BEFORE THE DEPARTMENT OF THE TREASURY
ALCOHOL AND TOBACCO TAX AND TRADE BUREAU
WASHINGTON, D.C.

In re: BELLION SPIRITS, LLC'S PETITION FOR HEALTH CLAIMS UNDER 27 C.F.R. §§ 70.701, 7.54(e)	)	**TTB DOCKET No. _____** **PETITION FOR HEALTH CLAIMS**

PETITION FOR HEALTH CLAIMS UNDER 27 C.F.R. § 70.701 & 7.54(e)

Bellion Spirits, LLC and Chigurupati Technologies (collectively "Bellion" or "Petitioners"), by counsel and pursuant to 27 CFR §§ 70.701 and 7.54(e), hereby submit this petition for health-related statements concerning the effect of NTX® in distilled spirits or beverages. NTX® is a proprietary blend of glycyrrhizin,[1] mannitol,[2] and potassium sorbate.[3] As discussed below and in the materials attached, when NTX® is infused into alcoholic beverages, it renders them safer, i.e., less toxic, than counterparts that do not contain NTX®. NTX® reduces the adverse effects of alcohol on the liver and on DNA. Thus, NTX® lessens certain deleterious effects caused from consumption of alcohol. Accordingly, Petitioners hereby request that the Alcohol and Tobacco Tax and Trade Bureau ("TTB") declare, via rulemaking or through the exercise of enforcement discretion, that the use of the Petitioners' proposed health-related statements concerning the hepatoprotective and DNA-protective effects of NTX® in the labeling and advertising of wines, distilled spirits, and malt beverages is permissible. The health claims

[1] Glycyrrhizin is an extract of licorice root.
[2] Mannitol is a sugar alcohol.
[3] Potassium sorbate is commonly used as a food preservative.

requested through this petition facilitate informed decisions concerning consumer health when purchasing and/or consuming alcoholic beverages. The health claims identified in this petition are truthful and non-misleading, supported by peer-reviewed scientific literature and human clinical studies. By giving consumers an ability to distinguish between alcoholic beverages that are less toxic than traditional counterparts, consumers may choose the healthier alcoholic beverage and, as a result, experience greater health and wellness than would otherwise be the case.

Petitioners' proposed health-related statements appear in section III below. Attached hereto, and incorporated in this petition, are the following supportive materials: (1) Expert Report of Dr. Sidney Stohs, Ph.D., FACN, CNS, ATS, FAPhA; (2) Expert Report of Dr. Harry G. Preuss, MD, MACN, CNS; (3) Stohs Curriculum Vitae; (4) Preuss Curriculum Vitae; and (5) scientific literature and studies supportive of the NTX technology.[4]

TABLE OF CONTENTS

[4] Bellion submits its confidential and proprietary scientific data "Confidential" under 5 U.S.C. § 552(B)(4).

I. Interests of the Petitioners

Bellion Spirits, LLC is the independent bottler and distributor of Bellion brand Vodka. Its principal place of business is Bellion Spirits LLC, One Harmon Plaza, Suite 805, Secaucus,

NJ 07094. Bellion Spirits was founded to pioneer innovation in the alcohol beverage industry, through the introduction of functional alcoholic beverages, which generally retain the customary characteristics of alcoholic beverages, while reducing or mitigating the unwanted negative effects, like damage to the liver, genetic injuries, and oxidative stress. Bellion's scientific and commercial goals focus on the education of consumers concerning the detrimental effects of alcohol consumption, along with the advantages of functional beverages or spirits that aid consumers in making smarter, safer, and healthier choices. Bellion's objectives include the distribution of products and technologies that help to educate consumers about the chemical interactions of alcohol in the body (both positive and negative). Bellion has coined the phrase "Functional Spirits" as a description of the benefits or protective properties conveyed through its technology, as described more fully below in this petition.

Chigurupati Technologies Private Ltd. is solely a Research & Development institution founded with the objective to "aid in the evolution of mankind." Its principal place of business is Plot No. 512/m/1. Road No. 31 Jubilee Hills, Hyderabad, 500033, Andhra Pradesh, India. By focusing on research and development, Chigurupati Technologies develops and leverages innovations that later provide beneficial health products for consumers globally, thus encouraging a physical and philosophical consumer evolution. Chigurupati Technologies depends on its ability to convey scientific literature and facts to consumers and businesses in association with its technologies.

Chigurupati Technologies Private Ltd. developed and owns a proprietary blend of three generally recognized as safe ingredients combined through a proprietary process and sold under the name "NTX." Those three ingredients are Glycyrrhizin Acid, D-Mannitol, and Potassium Sorbate. "NTX" is thus an ingredient in Bellion Vodka that Bellion purchases from Chigurupati

Technologies. Studies commissioned by Chigurupati Technologies reveal that "NTX," when infused into liquor, yields a protective effect on the human liver during alcohol consumption, lessening the adverse effect of alcohol on the liver. Heretofore that information has not appeared on the label, in the labeling, or in the advertising for alcohol containing products sold in the United States. NTX® also adds flavor and smoothness to the vodka drink. Bellion Vodka has not promoted the use of NTX for health purposes, but seeks to convey the liver and DNA protective effects of alcohol containing NTX® through this instant petition.

II. Procedural Background

In 2014, Bellion petitioned the TTB through the COLA process and requested approval for the use of Bellion's "NTX" designation on labeling. That designation reveals the presence of Bellion's proprietary blend in vodkas. In a letter dated December 17, 2014, the TTB informed Bellion it would send two pending COLAs[5] for Bellion Vodka back to the applicant, Frank-Lin Distillers Products, Ltd. ("Frank-Lin"), with a "Needs Correction" notification because the labels' referenced "NTX." According to the TTB, the acronym "NTX" was a misleading health claim.

At the time, Bellion's labeling included the following descriptive phrase as the statement of composition: "Vodka Infused With Natural Flavors." The label included the following statements:

For over 600 years, vodka has been made the same way.
No longer. Infused with NTX, Bellion changes vodka forever.

• Vodka Evolved • Infused With Natural Flavors • Created With NTX •

[5] The labels' TTB ID numbers are 1425001000403 and 14227001000056.

Bellion's labels did not explain or define the acronym "NTX." Both of Bellion's label applications, however, identified "NTX" as a fanciful name. One COLA submission provided the following explanation:

> NTX = NEW TECHNIX (OR NEW TECHNIQUES). THIS IS A BREAKTHROUGH COMBINATION OF INGREDIENTS AND PROPRIETARY TECHNIQUES THAT REMOVES THE UNDESIRABLE ATTRIBUTES [TASTES] COMMON IN MANY VODKAS AND PROMOTES SMOOTHNESS AND ROUNDNESS AND MOUTHFEEL BEYOND WHAT NORMAL FILTRATION AND MULTIPLE DISTILLATIONS CAN DO. WORLDWIDE PATENT AND TRADEMARK PROTECTION IS IN PROCESS. BRAND NAME, LOGO AND GRAPHICS MAY REPEAT; LOT NUMBER(S) MAY APPEAR/CHANGE.

According to the TTB, reference to "NTX" was an implied specific health claim in violation of 27 C.F.R. § 5.42(b)(8). The TTB determined that extraneous information not present on the Bellion label (e.g., in scientific publications elsewhere) touted the health benefits of "NTX." TTB asserted that "NTX" was an implied claim relating to Naltrexone, a prescription drug that was approved for the treatment of alcohol dependence, and "[t]he use of the drug NTX to treat alcohol dependency, as well as publicly available studies claiming that NTX has beneficial effects for patients with elevated liver enzymes, would be likely to reinforce the health claim . . . and cause further confusion about the term 'NTX.'" The TTB produced no consumer perception or survey data in support of its COLA denials.

On January 15, 2015, Bellion Spirits, L.L.C. appealed the TTB COLA denial through 27 CFR § 13.25. That appeal asserted that the TTB's censorship of the "NTX" labeling information violated the First Amendment of the U.S. Constitution because, inter alia, the TTB had no factual or legal bases to censor basic information related to product composition and ingredients. The

parties informally resolved the COLA disputes and, in April 2015, the TTB approved separate COLA applications for Bellion Vodka labels that referenced "NTX."[6]

Now, through this instant submission, Bellion petitions the TTB for use of nine (9) specific health claims under 27 CFR § 7.54(e) related to the NTX® ingredients contained in Bellion Vodka.

III. Requested Action

Petitioners hereby request that the TTB rule that alcoholic spirit beverages that contain NTX® may bear one or more the following health-related statements in its labeling, advertising, or promotional speech:

- NTX® provides antioxidant and anti-inflammatory support;
- NTX® helps protect against, i.e., reduces, alcohol-induced oxidative damage to the liver;
- NTX® helps maintain normal liver enzyme production and function;
- NTX® supports normal liver defenses and regenerative mechanisms;
- NTX® reduces the risk of alcohol-induced liver diseases, including fibrosis and cirrhosis;
- NTX® helps maintain normal liver functions;
- NTX® helps protect DNA from alcohol-induced damage; and
- NTX® reduces alcohol-induced DNA damage.

[6] The labels' TTB ID numbers are 15091001000076 (approved) and 15091001000077 (expired). Previously, the TTB had approved a label that was identical to the labels at issue in the appeal (TTB ID number 13294001000173). Frank-Lin voluntarily surrendered that label on July 31, 2014, however, at the request of the TTB. Two other labels (TTB ID numbers 13052001000468 and 13051001000241) were surrendered in 2014 because the applicant, Covington Spirits, L.L.C., no longer bottled Bellion Vodka.

Those health-related statements are accompanied by the following, prominently placed disclaimer:

> NTX® does not protect against all health risks associated with moderate and heavy levels of alcohol consumption, including, but not limited to, motor vehicle accidents, high blood pressure, stroke, cancer, birth defects, psychological problems, and alcohol dependency. Do not consume alcohol if: you are younger than the legal drinking age; you are pregnant or may become pregnant; you are taking medicine that can interact with alcohol; you have a medical condition for which alcohol is contraindicated; you plan to drive; or you cannot restrict your drinking to moderate levels. If you consume alcohol, only consume it in moderation. "Moderation" means up to one drink per day for women and up to two drinks per day for men.

The above-listed health-related statements are truthful and non-misleading. The disclaimer language is reasonable and adequate to properly identify the remaining risks inherent in alcohol consumption. As discussed below, the above-listed health claims are protected speech under the First Amendment to the United States Constitution.

IV. Legal and Statutory Framework

The provision of truthful and non-misleading health information is indispensable to informed consumer choice. The need for accurate health information at the point of sale is no less important in the alcoholic beverage context as it is in the food and dietary supplement context. *See, e.g., Pearson v. Shalala*, 164 F.3d 650 (D.C. Cir. 1999). Unlike the Federal Food and Drug Administration, the TTB has yet to adopt a cogent and workable regulatory regime for the evaluation of health-related statements. *C.f.* 21 CFR § 101.70 (articulating procedure and elements required for health claim petition). To determine whether a health claim satisfies Section 27 CFR 7.54, petitioners must have a full and fair opportunity to present all supportive science. The adequacy of that science must be evaluated in context with the benefit sought to be achieved.

Bellion submits this instant petition for agency action in lieu of (or in conjunction with) the COLA process, in part, because the TTB's existing regulatory framework concerning health claim petitions is inadequate and insufficient to provide constitutionally required protection for truthful and non-misleading health information on the label, in the labeling, and in the advertising of alcoholic beverages.

A. TTB's Extant Framework Involving Health-Related Statements

The Federal Alcohol Administration Act authorizes the Secretary of the Treasury to promulgate regulations concerning the labeling and advertising of alcoholic beverages, including regulations that are intended to prevent consumer deception and prohibit the use of misleading statements, irrespective of falsity. *See* 27 U.S.C. § 205(e)-(f).

In 2003, the TTB issued regulations for the use of health claims and health-related statements in the labeling and advertising of wines, distilled spirits, and malt beverages. *See* 68 Fed. Reg. 10076 (Mar. 3, 2003) (Health Claims and Other Health-Related Statements in the Labeling and Advertising of Alcohol Beverages); 27 C.F.R. § 4.39(i) (labeling of wine); *id*. at § 4.64(i) (advertising of wine); *id*. at § 5.42(b)(8) (labeling of distilled spirits); *id*. at § 5.65(d) (advertising of distilled spirits); *id*. at § 7.29(e) (labeling of malt beverages); and *id*. at § 7.54(e) (advertising of malt beverages). Those regulations attempted to balance the speaker's First Amendment right to label and advertise products through truthful and non-misleading scientific information and the public's right to be informed of the significant health risks associated with alcohol consumption.[7]

[7] The agency was motivated apparently by the well-established scientific link between moderate alcohol consumption (notably red wine consumption) and a reduced risk of coronary heart disease. *See, e.g.*, 68 Fed. Reg. 10076, 10082-83 (Mar. 3, 2003). The TTB had refused to permit health claims associated with red wine, despite the general agreement in support of that claim by the scientific and medical fields.

Under TTB's health claim regulations, alcoholic beverage labels and advertisements may not contain any health-related statement that is untrue in any particular or tends to create a misleading impression as to the effects on health of alcohol consumption. *See id.* at §§ 4.39(h)(2), 4.64(i)(2), 5.42(b)(8)(ii), 5.65(d)(2), 7.29(e)(2), 7.54(e)(2). For purposes of the TTB's regulations, a "health-related statement," means:

> [A]ny statement related to health . . . and includes statements of a curative or therapeutic nature that, expressly or by implication, suggest a relationship between the consumption of alcohol, distilled spirits, or any substance found within the distilled spirits, and health benefits or effects on health. The term includes both specific health claims and general references to alleged health benefits or effects on health associated with the consumption of alcohol, distilled spirits, or any substance found within the distilled spirits, as well as health-related directional statements. The term also includes statements and claims that imply that a physical or psychological sensation results from consuming the distilled spirits, as well as statements and claims of nutritional value (*e.g.,* statements of vitamin content). Statements concerning caloric, carbohydrate, protein, and fat content do not constitute nutritional claims about the product.

Id. at §§ 4.39(h)(1)(i), 4.64(i)(1)(i), 5.42(b)(8)(i)(A), 5.65(d)(1)(i), 7.29(e)(1)(i), 7.54(e)(1)(i). Additionally, a "specific health claim," is "a type of health-related statement that expressly or by implication, characterizes the relationship of the distilled spirits, alcohol, or any substance found within the distilled spirits, to a disease or health-related condition." *Id*. at §§ 4.39(h)(1)(ii), 4.64(i)(1)(ii), 5.42(b)(8)(i)(B), 5.65(d)(1)(ii), 7.29(e)(1)(ii), 7.54(e)(1)(ii). Examples of implied specific health claims include the following: "statements, symbols, vignettes, or other forms of communication that suggest, within the context in which they are presented, that a relationship exists between distilled spirits, alcohol, or any substance found within the distilled spirits, and a disease or health-related condition." *Id*.

Thus, the TTB has extended its definition of "health claims" to encompass nearly any statement that suggests a positive benefit for the human body from consumption of alcohol. The

TTB's definition of "health claim" is more expansive than similar interpretations by sister agencies like the FDA. *See* 21 U.S.C. §343(r)(1)(B) (defining a health claim, for purposes of the FDCA, to mean a claim that "characterizes the relationship of any nutrient . . . to a disease or a health-related condition . . .").

If the health-related statement conveys a misleading impression, the TTB may require a prominent disclaimer or other qualifying statement for the purpose of dispelling the misleading impression. *See, e.g.*, 27 C.F.R. § 5.42(b)(8)(ii)(A). Under the TTB's regulations, a specific health claim is misleading unless it: (1) is truthful and adequately substantiated by scientific or medical evidence; (2) is sufficiently detailed and qualified with respect to the categories of individuals to whom the claim applies; (3) adequately discloses the health risks associated with both moderate and heavier levels of alcohol consumption; and (4) outlines the categories of individuals for whom any alcohol consumption poses risks. *See, e.g., id*. at § 5.42(b)(8)(ii)(B)(2). That information must appear as part of the specific health claim on a label, and, in advertising, as prominent as the specific health claim. *See id*. The TTB has never produced a consumer survey or impact study that determined consumers' level of understanding concerning health-related claims and alcohol consumption.

B. The TTB Must Implement a Constitutional Regulatory Framework for Evaluation of Health Claim Petitions

Unlike the Federal Food and Drug Administration, the TTB has yet to issue workable regulatory procedures and criteria for the evaluation of health-related statements. That regulatory deficiency creates hardship for petitioners, like Bellion, by denying them a well-defined regulatory avenue within TTB jurisdiction that will ensure allowance of constitutionally protected speech on the labels, in the labeling, and in advertising of alcoholic beverages. The limited scope and applicability of extant TTB labeling and advertising regulations precludes the

sponsorship of health claim petitions that could satisfy the elements of the TTB's regulations in, for example, Section 5.42(b)(8). As discussed below the process available to advertisers and labelers fails under the First Amendment.

In prior meetings between Bellion and TTB staff members in July 2014, the TTB conceded that its health claim procedures were inadequate and regulations concerning same were dead letters. Rather than perform its administrative review, the TTB expressed its intent to transfer health claim petitions to the FDA for review—a procedure that would violate administrative procedure. "Agency action taken without statutory authorization, or which frustrates the congressional policy which underlies a statute, is invalid." *See Yankton Sioux Tribe v. Kempthorne*, 442 F.Supp.2d 774, 784 (D.S.D. 2006). The FDA lacks jurisdiction or authority to interpret the TTB's regulations. *See Am. Library Assn. v. FCC*, 406 F.3d 689, 702 (D.C. Cir. 2005) (an agency does not possess plenary authority to act within a given area simply because Congress has endowed it with some authority to act in that area); *see also* 5 U.S.C. § 706(2)(C) (under the APA, agency action must be set aside and deemed unlawful where the agency acts "in excess of statutory jurisdiction, authority, or limitations, or short of statutory right").

Congress tasked the TTB with the regulation of spirit products, which includes exclusive authority to regulate their labeling and advertising. *See* 27 U.S.C. §§ 201, *et seq.* The FDA lacks statutory authority to regulate alcoholic spirits that are within the TTB's purview. *See Brown-Forman Distillers Corp. v. Mathews*, 435 F. Supp. 5, 8 (W.D. Ky. 1976) ("it was Congress' intention to place exclusive jurisdiction in BATF with respect to regulating labeling of alcoholic beverages."). Nothing in the FAA Act grants the TTB authority to delegate its

administrative functions or responsibilities to the FDA, and the FDA similarly lacks primary authority to regulate the labels and advertising of alcoholic spirits. *Id.* at 12.

i. TTB's Mandatory Label Reviews (COLAs) Are Inadequate

Regardless of whether a label bears a health-related statement, the TTB must generally preapprove wine, distilled spirit, and malt beverage labels.[8] *See* 27 C.F.R. §§ 4.50-4.52 (wine); *id*. at §§ 5.55-5.56 (distilled spirits); *id*. at §§ 7.40-7.42 (malt beverages). To obtain the TTB's approval, alcohol producers may apply for a Certification/Exemption of Label/Bottle Approval ("COLA"). The TTB will reject the application if the label fails to comply with applicable regulations or is otherwise deficient. For instance, the TTB will decline to approve an application if the label bears a misleading health-related statement in violation of the regulations discussed above. The TTB evaluates health-related statements on a case-by-case basis during its label reviews. *See id*.

Although the TTB will examine health-related statements that appear on labels of alcoholic beverages during its standard review, the "COLAs Online" process is not equipped to receive information necessary to demonstrate that a particular-health related claim is substantiated (to wit, that the claim is truthful and non-misleading, and supported by reliable scientific evidence). For instance, the only type of label attachments included within a filed COLA application include JPEG and TIFF file formats that use only RGB color mode. *See* TTB, COLAs and Formulas Online FAQs.[9] Moreover, the paper COLA application, TTB F 5100.31,

[8] Certain changes to labels that the TTB has previously approved may be made without obtaining a new certification/exemption of label/bottle approval ("COLA") from the TTB. *See*, TTB, *List of Allowable Changes to Approved Labels*, *available at* http://www.ttb.gov/labeling/allowable_revisions.shtml#completeList (last accessed Mar. 31, 2016).

[9] *Available at* http://www.ttb.gov/faqs/colasonline.shtml (last accessed April 5, 2015).

which can be submitted in lieu of an electronic application, includes no exceptions or provisions that allow submission of detailed scientific information related to health statements on labels or labeling.

Even if the COLA process permitted the submission of information regarding a health-related statement that appears on the label of an alcoholic beverage, the TTB could, theoretically, leave applicants buried within "corrective" processes inherent to the COLA review process. The system of administrative appeals is not designed (nor efficient enough) to prevent significant constitutional injury or alleviate the burdens of the TTB's prior restraint of health claim language. As explained below, a deprivation of First Amendment rights, even for minimal periods of time, constitutes an irreparable constitutional injury. *Elrod v. Burns*, 427 U.S. 347, 373–74 (1976). To the extent Bellion is precluded from communicating its truthful scientific information, it suffers an irreparable constitutional injury.

The COLA process is also too narrow to achieve the proper review of claims that would be used beyond labels or labeling. The COLA process pertains solely and exclusively to content appearing on spirit labels or labeling. That approval process has no relationship to advertising statements, or promotional content that may appear beyond the "label" of an alcoholic product. Thus, the COLA process would provide inadequate and inefficient relief for Bellion here. Petitioners are not requesting the use of specific health-related statements on a specific label, but, rather, they seek permission to include the proposed health-related statements throughout their labels, advertising, and promotional content. Moreover, Bellion would choose from among the various health claims subject to approval when designing or implementing labeling changes. Bellion should not be required to independently clear health claims through the COLA process

when such approval can most efficiently and pragmatically proceed through one health claim petition, the instant petition.

ii. TTB Advanced Review of Advertisements Is Inadequate

Although the TTB must preapprove alcoholic beverage labels, its regulations do not require preapproval of alcoholic beverage advertisements, including those that contain health-related statements.[10] The TTB, however, may review advertisements that appear in various media (e.g., online, print, or broadcast) on a case-by-case basis, either on its own accord or in response to complaints it receives about specific advertisements. Like labels, the TTB reviews alcoholic beverage advertisements on a case-by-case basis. *See id*. at § 7.54(a), (e)(2)(i) (malt beverages); *id*. at § 5.65(a)(1), (d)(2)(i) (distilled spirits); and *id*. at §4.64(a)(1), (i)(2)(i) (wine).

An alcoholic beverage advertiser faces serious risk when it includes a health-related statement in advertisements. For instance, several years prior, the TTB targeted an advertisement for alcoholic beverages that contained ingredients associated with non-alcohol energy drinks on the grounds that they implied that ingestion of the extra ingredients would result in a stimulating or energizing effect or enable consumers to drink more of a product without feeling the effects of alcohol in violation of the TTB's advertising regulations. Rather than have an advertiser bear the risk that the TTB will initiate enforcement action for use of a health-related statement in an advertisement, the TTB must provide a clear pathway for premarket claims approval.

[10] Alcoholic beverage industry member may, voluntarily, pre-clear advertising with the TTB.

iii. Bellion Has the Right to Petition the TTB for Health Claims

The First Amendment guarantees the right to petition government for redress. *Am. Bus. Ass'n v. Rogoff*, 649 F.3d 734, 738 (D.C. Cir. 2011) ("The right to petition is cut from the same cloth as the other guarantees of [the First] Amendment, and is an assurance of a particular freedom of expression"). The right "extends to [petitioning] all departments of the Government, including administrative agencies and courts." *Id.* (quoting *Cal. Motor Transport Co. v. Trucking Unlimited*, 404 U.S. 508, 510 (1972)). That pathway is particularly important here, where Bellion's commercial speech might otherwise subject it to liability absent TTB's preapproval of health claim language.

Administrative agencies are obliged by the Administrative Procedures Act (APA) to respond to petitions of the kind here presented. *See* 5 U.S.C. § 555(e) (requiring a response to a petition along with a "statement of the grounds for denial").

Consistent with the constitutional requirement for prompt relief in matters trenching upon free speech, federal courts have routinely excused the exhaustion doctrine in the administrative context where, as here: (1) the unexhausted remedy would plainly be inadequate; (2) a constitutional claim remains at issue even after theoretical exhaustion occurred; (3) the relief requested cannot be granted by the agency (e.g., through a facial challenge to agency regulations); (4) exhaustion would be futile; and (5) the petitioner would otherwise suffer irreparable harm if unable to secure immediate judicial review. *See, e.g., Dawson Farms, LLC v. Farm Serv. Agency*, 504 F.3d 592, 606 (5th Cir. 2007); *Ace Prop. & Cas. Ins. Co. v. Fed. Crop Ins. Corp.*, 440 F.3d 992, 999–1000 (8th Cir. 2006); *McBride Cotton & Cattle Corp. v. Veneman*, 290 F.3d 973, 980 (9th Cir. 2002). In other words, to the extent the TTB refuses to promptly and timely respond to this petition, relief in the district courts will be appropriate to prevent Bellion's

imminent loss of First Amendment rights through TTB's prior restraint on truthful health claim language. It is the law that TTB prohibits health statements on the labels, in the labeling, and in the advertising of alcohol containing products, unless it acts to allow or approve of the statements. Hence, there is an immediate and all-encompassing prior restraint on precisely the claims Bellion seeks in this petition, which restraint can either be lifted promptly by TTB or judicial review is warranted under the common exceptions to the exhaustion doctrine.

The TTB's health claim regulations and review process described in Sections 7.54(e) and 5.42(b) have erected a total ban on health related claims in the marketplace, in part, because the TTB has yet to implement the administrative process or structure needed to consider a properly noticed health claim petition. The TTB conceded that point in prior rulemaking: "TTB agrees that the regulations make it difficult to present a substantive health claim (for example, one involving cardiovascular benefits associated with moderate alcohol consumption)…" *See* Final Rule, *Healht Claims and Other Health-Related Statements in the Labeling and Advertising of Alcohol Beverages*, 68 Fed. Reg. 10076 (Mar. 3, 2003). The lack of meaningful review procedures effects an unconstitutional prior restraint of health claims in the marketplace, and renders action on this instant petition necessary. TTB may not shirk its constitutional duty under the First Amendment to ensure that truthful health statements are not suppressed; consequently, in the face of the present petition, it must review it and it must do so promptly.

C. Bellion's Proposed Health Claims Are Truthful, Non-Misleading, and Satisfy the TTB's Health Claim Regulations

Bellion has proposed the use of eight (8) health claims that accurately reflect the scientific evidence concerning NTX® technology. Those statements are truthful, substantiated by scientific and medical evidence; are sufficiently detailed and qualified with respect to the

categories of individuals to whom the claim applies; adequately disclose the health risks associated with both moderate and heavier levels of alcohol consumption; and explain the categories of individuals for whom any alcohol consumption poses risks.

The claims sufficiently describe the categories of individuals to whom the claims apply. Bellion's functional spirit infused with NTX® is designed for use by all lawful consumers of alcoholic beverages. The ill-effects of alcohol use are common knowledge and sufficiently known by the TTB and consumers. According to the World Health Organization's most recent data, the harmful use of alcohol is among the top five risk factors for disease, disability, and death. *See* Vladimir Poznyak & Dag Rekve, *Global Status Report on Alcohol and Health 2014*, World Health Org., p. 2.[11] The long-term effects of alcohol consumption can damage most organs of the human body. *Caan, Woody; Belleroche, Jackie de, eds. (11 April 2002). Drink, Drugs and Dependence: From Science to Clinical Practice (1st ed.). Routledge. pp. 19–20.*

Liver diseases are the predominant health concern associated with alcohol consumption because over time heavy drinking causes the multifaceted disease known as Alcoholic Liver Disease ("ALD"). ALD is characterized by hepatic steatosis or increased fat deposits in the liver. It can cause alcoholic hepatitis, also known as inflammation of the liver, and cellular stress from oxidation of the liver. *See* Aditya Ambade & Pranoti Mandrekar, Oxidative Stress and Inflammation: Essential Partners in Alcoholic Liver Disease, International Journal of Hepatology, Volume 2012 (2012), Art. ID 853175.[12] After prolonged alcohol consumption, ALD culminates in irreversible destruction and scarring (fibrosis) of the liver tissue called

[11] *Available at* http://apps.who.int/iris/bitstream/10665/112736/1/9789240692763_eng.pdf?ua=1 (last accessed April 4, 2016).

[12] *Available at* http://www.hindawi.com/journals/ijh/2012/853175/ (last accessed April 4, 2016).

cirrhosis, which ultimately leads to liver failure. *See Mayo Clinic*, Alcohol Use Disorder: Complications, (July 25, 2015).[13] While it is axiomatic in the ALD context that the more heavily one consumes alcohol—and the greater frequency of drinking—the more likely one is to develop cirrhosis, it should be noted that alcohol tolerance varies from person to person, and for some people one drink a day is sufficient to leave permanent scars on the liver. *See* Jennifer, Robinson, M.D., *Understanding Cirrhosis of the Liver*, WebMD (February 1, 2015).[14] Thus, hepatoprotective effects that limit liver injury are cumulative and likely to benefit moderate drinkers over their adulthood.

The CDC reported 36,427 deaths in 2013 from chronic liver diseases, making it the 12th leading cause of death in the United States. *See* Jiaquan Xu, M.D., Sherry Murphy, B.S., Kenneth Kochanek, M.A., & Brigham Bastian, *Deaths: Final Data for 2013*, Nat'l Vital Statistics Reports Vol. 62 No. 2, 45 (February 16, 2016).[15] That category is broken down into two sections: (1) ALD, accounted for 18,146 deaths; and (2) other chronic liver diseases and cirrhosis, accounted for 18,281 deaths in the United States in 2013. *Id.* The annual inpatient cost for alcohol-related cirrhosis in 2014 was $850 million, and this figure does not account for the unknown millions more spent on outpatient treatments. *See* Lauren Beste, M.D., *Alcoholic Liver Disease*, Gastroenterology & Hepatology, Vol. 12, Issue 1, (January 2016).[16]

Aside from ALD, recent research is demonstrating that alcohol abuse causes the dual-

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[13] A*vailable at* http://www.mayoclinic.org/diseases-conditions/alcohol-usedisorder/basics/complications/con-20020866 (last accessed April 4, 2016).

[14] A*vailable at* http://www.webmd.com/digestive-disorders/understanding-cirrhosis-basic-information?page=2 (last accessed April 4, 2016).

[15] *Available at* http://www.cdc.gov/nchs/data/nvsr/nvsr64/nvsr64_02.pdf (last accessed April 4, 2016).

[16] A*vailable at* http://www.gastroenterologyandhepatology.net/index.php/archives/january-2016/alcoholic-liver-disease/ (last accessed April 4, 2016).

harms of accumulated DNA damage and alcohol-induced dysfunction to DNA repair, which coalesce into the well-known negative effects of alcohol on the brain, i.e. brain damage. *See* Inna Kruman, George Henderson, and Susan Bergeson, *DNA Damage and Nuerotoxicity of Chronic Alcohol Abuse*, 237 Exp Biol Med 7, 740-47 (July, 24, 2012).[17] Those twin effects of chronic alcohol consumption result in genomic instability and the death of neurons. *Id*. Those destructive effects to the very building blocks of the nervous system are likely the reason that brain damage, including central nervous system degeneration, are associated with chronic alcohol consumption. *Id*.

The ill-effects of alcohol abuse are well-documented. The problems associated with lower levels of alcohol consumption are also documented. *See, e.g.*, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, *State of the Science Report on the Effects of Moderate Drinking* (Dec. 19, 2003).[18] Studies suggest that even moderate drinking may contribute to genetic and organ damage. *See, e.g.*, Castelli, E., et al., *Indicators of genetic damage in alcoholics: reversibility after alcohol abstinence*, Hepatogastroenterology, 1999 May-Jun; Vol. 46(27):1664-8; Honor Whiteman, *Young adults "damage DNA" with weekend alcohol consumption*, MedicalNewsToday.com (Jan. 2, 2014);[19] Rendón-Ramirez, R., et al., *Oxidative damage in young alcohol drinkers: A preliminary study*, Alcohol Intn'l Biomed. Journal, 2013 November; Vol. 47(7):501-504. Given the frequency and volume of alcohol consumption in the United States, any product that affords liver and genetic protection during ethanol metabolism is

[17] A*vailable at* www.ncbi.nlm.nih.gov/pmc/articles/PMC3685494/ (last accessed April 4, 2016).

[18] Available at http://pubs.niaaa.nih.gov/publications/ModerateDrinking-03.htm (last accessed April 11, 2016).

[19] *Available at* http://www.medicalnewstoday.com/articles/270735.php (last accessed April 11, 2016).

of considerable health value to consumers nationwide—even if that protective effect merely mitigates, as opposed to eliminates, the damage.

D. Bellion's Health Claims Concerning NTX® Hepatoprotective Effects and DNA Protection Are Truthful and Adequately Substantiated by Scientific and Medical Evidence

The hepatoprotective effect of NTX® is supported by the attached exhibits 1, 3, and 5, and through the more than 100 peer-reviewed scientific publications attached. Those reports, studies, and datasets are herein incorporated into this petition by reference, including the expert reports of Dr. Sidney Stohs and Dr. Harry Preuss. Bellion's experts explain that the requested health claims are supported by the scientific evidence, including product-specific testing, peer-reviewed literature, and animal and in vitro test data.

As discussed above, the harms of alcohol consumption are well documented, and the most common chronic alcohol issue is ALD, which is defined by lesions on the liver ranging from hepatic steatosis to cirrhosis and eventually liver failure. The cascading negative effects of alcohol on the liver are attributable to the mechanisms of alcohol toxicity, which can be summarized in five steps: (1) the metabolism of alcohol to highly toxic acetaldehyde and free radical species; (2) the production of reactive oxygen (ROS) and nitrogen species with resulting oxidative stress; (3) the inflammatory release of cytokines as tumor necrosis factor-alpha and interleukin-6; (4) abnormal lipid metabolism, oxidative DNA damage, formation of protein, and DNA adducts with metabolites of alcohol and acetaldehyde; and (5) ultimately induction of apoptosis or necrosis with subsequent multi-system organ failure. *See* Stohs Rep. at 2.

Biomarkers are biological indicators of the health of an organ. Just as blood sugar elevation is associated with diabetes, common biomarkers can be used to track the progression of

ALD. Elevations of these biomarkers indicate liver disease, while decreases occur as the liver heals. *See id*. at 2-3.

Liver health is generally measured or evaluated through several generally-accepted biomarkers. *See* Preuss Rep. at 3. Those markers indicate disruption of the hepatic function. Scientists evaluate measurements related to the blood serum levels of enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), and alkaline phosophatase (ALP). *See id*. Elevated circulating levels of those enzymes indicate liver injury from excess alcohol intake. *See id*. Moreover, a reduction of those serum levels occur as the liver begins to repair itself. *See id*.

Scientists may look to other biomarkers to evaluate liver function. The hepatic metabolism of alcohol causes excess production of tissue-damaging free radicals and reactive oxygen species (ROS). Markers of oxidative stress and oxidative tissue damage include the production of ROS, the lipid malondialdehyde (MDA), protein carbonyl formation, and DNA damage. Moreover, reductions in glutathione (GSH) levels could indicate intercellular antioxidant and chemoprotectant characteristics in human tissues, in part, because reductions in GSH levels are associated with changes to oxidative stressors. *See id*. at 3-4. Therefore, GSH content is considered an important marker for ROS and free radicals. *See id*. at 4.

The two primary approaches for successful prevention and reversal of liver damage are abstinence from alcohol and appropriate nutritional support. *See id*. Because the vast majority of alcohol users will not abstain, the need to develop safer means of alcohol consumption to reduce the associated morbidity and mortality, as well as the costs to society, are pressing. *See id*. To prevent and treat alcohol-induced liver toxicity the mechanism must disrupt and either inhibit or reverse the five steps of toxicity described above. *See id*. Studies in humans and

animals have demonstrated the hepatic protective effects of NTX® as well as its individual components during acute and chronic alcohol consumption. *See id*.

The two primary ingredients in NTX® are glycyrrhizin and mannitol, which work synergistically to protect the liver from the harmful health effects of alcohol. *See* Stohs Rep. at 4. A randomized, double-blind, placebo-controlled cross-over clinical trial involving 12 human subjects found that NTX® produced a significant decrease in the biomarkers for liver toxicity. *See id*. Another similar test with 31 human subjects showed a significant reduction in biomarkers of liver disease and oxidative liver effects, and the authors indicated that NTX® may be effective at reducing the negative effects of alcohol consumption. *Id*. Furthermore, the synergistic effects of mannitol and glycyrrhizin were demonstrated in a 28 day study in rats where the substances restored the biomarkers of liver function to as high as 80% of normal. *See id*. at 5. In all, over 70 studies show the key components of NTX® produce liver protecting, antioxidant, and anti-inflammatory effects. *See id*. at 7.

Antioxidants and Anti-Inflammation:

The health-related statements regarding the antioxidant, anti-inflammatory effects of NTX® that serve to protect the liver against oxidative damage have been thoroughly substantiated. Human and animal studies alike demonstrate the positive biomarker effects of glycyrrhizin by inhibiting the lipid accumulation in the liver and decreasing inflammatory cytokines, which reduction protects liver function. *See id*. at 8. Several studies have confirmed the tissue protective effects of mannitol, as it is already used as an antioxidant in conjunction with heart bypass surgeries and as an anti-inflammatory in tissue baths following kidney transplants. *See id*. at 9. Fifty human studies and a number of animal studies confirm NTX®'s primary ingredients protect against oxidative liver damage. *See id*. at 12.

A series of animal studies have therefore shown that licorice extract and mannitol favorably decrease reactive oxygen species (ROS) and similar metabolites, lipid peroxidation (MDA formation) as well as carbonyl protein formation secondary to alcohol-induced production. *See* Preuss Rep. at 4. Those studies also show a positive increase in the levels of endogenous antioxidant reduced glutathione (GSH). *See id*. Those key findings concerning glycyrrhizin and mannitol have been corroborated in clinical studies. *See id*. NTX® was shown to decrease alcohol-induced DNA damage in human peripheral lymphocytes. *See id*. at 5. NTX® was also shown to enhance the levels of the enzymes catalase, GPX, and SOD that are responsible for decomposing ROS. *See id*. Thus, NTX® had an ability to protect hepatocytes from the damaging influences of ROS and oxidative stressors. *See id*. Those findings were supported by over thirty published animal, in vitro, and human clinical studies confirming the antioxidant and anti-inflammatory properties of the components in NTX®.

Helps Maintain Normal Liver Function:

The published scientific evidence strongly supports NTX®'s ability to maintain normal or healthy liver function during and after alcohol consumption. Several published human, animal, and cell culture studies revealed that NTX® and its components foster normalization or near-normalization of many liver enzymes, including ALT, AST, GGT, ALP, SOD, GPX, and catalase. *See id*. at 6. NTX®'s proprietary formula (and its components) ameliorate alcohol-induced inflammation. Glycyrrhizin itself has been shown to reduce serum ALT and AST levels in patients with chronic hepatitis by at least thirty-five percent (35%). *See id*.

NTX®'s beneficial effects were documented in a randomized, doubled-blind, placebo-controlled clinical trial involving twelve human subjects through a cross-over model. NTX® provided significant decreases and normalization in the examined biomarkers correlating with

liver toxicity, to wit, ALT, AST, GGT, and ALP. *See id*. Another randomized, double-blind, placebo-controlled cross-over clinical trial involving thirty one (31) human subjects revealed that consumption of alcohol with NTX® significantly reduced the levels of ROS and facilitated development of normal liver enzyme circulating concentrations. *See id*.

The enzymes that are normalized were biomarkers of liver health. When those enzymatic levels decrease, as several randomized double-blind human studies have documented, the ability of NTX® to preclude alcohol toxicity and help maintain normal liver function is substantiated. *See* Stohs Rep. at 13. Several animal studies have shown the defensive and regenerative properties of NTX®, but one in particular on rats actually produced a marked reduction in alcohol-induced liver lesions. *See id*. at 17.

Supports the Liver's Regenerative Mechanism:

The health-related statements regarding reduction of risk for and amelioration of liver diseases including fibrosis and cirrhosis have also been scientifically substantiated. A published meta-analysis summarized the effects of a glycyrrhizin product in 838 patients through 12 randomized trials confirmed that the substance significantly decreased serum levels and improved liver function in alcoholic liver disease cases. *See id*. at 20. NTX® co-administered with alcohol has demonstrated a significant decrease in liver toxicity biomarkers, thereby facilitating increased liver function and regeneration, which reduces the risk of liver diseases like fibrosis and cirrhosis. *See id*. at 22.

Almost fifty published human clinical studies and a substantial number of animal studies have demonstrated that NTX and its specific components (e.g., glycyrrhizin and mannitol) support the liver's normal defense mechanisms against alcohol and other hepatoxins, thus promoting general liver health in those that might drink moderately. A meta-analysis of twelve

randomized human clinical studies evaluated data collected from 838 patients with alcoholic liver disease and concluded that glycyrrhizin significantly decreased serum ALT and AST levels. *See* Preuss Rep. at 8. Another study determined that glycyrrhizin reduced the incidence of liver fibrosis in rats co-administered glycyrrhizin. *See id.* Another double-blind, comparative, cross-over clinical trial examined the effects of alcohol on blood levels of ROS in the absence and presence of co-administered NTX®. *See id*. The results demonstrated that NTX® statistically and significantly reduced the alcohol-induced generation of ROS and ROS metabolites, and further decreased serum lipid peroxidation product MDA, while increasing serum levels of the endogenous antioxidant GSH. *See id*. at 8-9. NTX® also significantly decreased serum protein carbonyl levels. *See id*. at 9. That data demonstrated that NTX® co-administered with alcohol decreased the oxidative tissue damage that would otherwise have been observed during alcohol consumption and metabolism.

Reduces Alcohol-Induced DNA Damage:

NTX® aids in protection of DNA by reducing alcohol-induced damage. Health claims related to that effect are scientifically substantiated. A randomized, double-blind human study revealed that alcohol consumption is damaging to DNA because it damages peripheral lymphocytes, but NTX® significantly reduces the level of damage caused, revealing that it protects DNA. *See* Stohs Rep. at 30. One of the key ways NTX® protects DNA is via its anti-oxidant and suppression of oxidative damage effects. *See id*. Glycyrrhizin has been show to prevent DNA fragmentation and modulated programmed cell death. *See id*. One mechanism of DNA protection was shown in an in vitro model where glycyrrhizin directly bonded to the major and minor grooves and phosphate backbone of DNA, thereby protecting it from damage. *See id*. at 32.

Excess alcohol consumption generally contributes to the production of oxidative damage to DNA and the epigenome. *See* Preuss Rep. at 13. Reactive oxygen species activate or repress epigenetic elements like chromatin remodeling, micro-RNAs, DNA (de)methylation and histone modification. *See id*. at 13-14. Epigenetic changes, even slight alterations, may affect gene expression and could ultimately result in liver disorders. *See id*. at 14. After ingestion, alcohol can form adducts with DNA, inhibiting the formation of various proteins essential for healthy hepatic function. *See id*.. NTX® has been shown through scientific studies and laboratory work to inhibit those negative effects, in part, by restraining the formation of alcohol-induced DNA damage.

In one study involving thirty one (31) subjects in a randomized, double-blind, placebo-controlled cross-over clinical trial, patients challenged with alcohol presented apparent DNA damage in peripheral lymphocytes as measured by single cell electrophoresis comet assay, the cytokinesis-block micronucleus assay, and 8-hydroxy-2-deoxyguanosine formation. When similarly challenged with NTX-infused alcohol, patients presented with a significant reduction in DNA damage. *See id*. In addition, various other studies using human and animal tissues have shown that individually glycyrrhizin and mannitol prevent oxidative DNA damage. *See id*.

Based on the wealth of peer-reviewed literature, animal studies, human clinical test data, and product-specific testing, NTX® and its major components have been shown to reduce DNA damage from DNA single and double strand breaks induced by alcohol and other ROS generating systems in the liver.

Totality of the Scientific Evidence:

The well-documented scientific reports of Drs. Stohs and Preuss present balanced and objective evidence concerning the efficacy of NTX®. The more than 100 studies attached to this

Petition in Exhibit 5 substantiate the health claims requested in section III of this Petition. Significantly, in performing its review of the available scientific evidence under this Petition, TTB must in conformity with First Amendment standards consider all scientific evidence supportive of the requested claims. The TTB must acknowledge that human clinical testing in areas concerning alcohol exposure carries significant health and liability risks that render repeat (or expansive) testing impractical, costly, and perhaps unethical (e.g., long-term studies). The universe of scientific data is therefore limited by those practical considerations. Animal and in vitro models must be considered where supportive of the mechanism of action, or where such studies explain the biophysical or biochemical responses. Animal and in vitro models are often essential to develop or prove causal connections between the test components and a statistically significant effect later observed in human models.

E. NTX® Is Safe and Lawful when Added to Alcoholic Beverages

The TTB has already acknowledged that NTX® may safely and lawfully be added to alcoholic beverages because it has approved the use of NTX® in at least one alcoholic beverage, Bellion branded vodka. Plus, the TTB has authorized the use of mannitol, glycyrrhizin, and potassium sorbate, which are the three ingredients that comprise NTX®, in alcoholic beverages generally, subject to limitations. *See* TTB, *Limited Ingredients: Flavoring Substances and Adjuvants Subject to Limitation or Restriction* (explaining that mannitol, glycyrrhizin, and potassium sorbate may be added so long as they do not exceed 2.5%, 0.1%, and 0.1%, respectively).[20]

[20] *Available at* http://www.ttb.gov/ssd/limited_ingredients.shtml (last accessed April 5, 2016).

Similarly, the federal Food and Drug Administration ("FDA") allows mannitol, glycyrrhizin, and potassium sorbate to be added to foods, which also establishes that NTX® is safe. *See* 21 C.F.R. § 180.25 (approving the use of mannitol as a food additive, and noting that the ingredient is used as an anticaking agent and free flow agent); *id.* at §182.3640 (affirming that potassium sorbate, a chemical preservative, is generally recognized as safe ("GRAS") when it is used in accordance with good manufacturing practice); *id*. at §184.1408 (affirming that licorice and licorice derivatives, including glycyrrhizin, are GRAS when used as flavor enhancers in various foods, including alcoholic beverages).

To further attest to the safety of NTX®, Dr. Stohs' expert report identifies various studies that have evaluated the safety of NTX® and its constituents, and explains that during such studies, no serious adverse events were observed. *See* Stohs Rep. at 33-34.

F. The Proposed Claim Adequately Explains the Health Risks Associated with Both Moderate and Heavier Levels of Alcohol Consumption and Explains the Categories for Whom Any Alcohol Consumption Poses Risks

Consistent with section III *supra*, all Bellion advertising, labeling, and promotional material will feature the following disclaimer that corresponds with the proposed health claims:

> NTX® does not protect against all health risks associated with moderate and heavy levels of alcohol consumption, including, but not limited to, motor vehicle accidents, high blood pressure, stroke, cancer, birth defects, psychological problems, and alcohol dependency. Do not consume alcohol if: you are younger than the legal drinking age; you are pregnant or may become pregnant; you are taking medicine that can interact with alcohol; you have a medical condition for which alcohol is contraindicated; you plan to drive; or you cannot restrict your drinking to moderate levels. If you consume alcohol, only consume it in moderation. "Moderation" means up to one drink per day for women and up to two drinks per day for men.

That disclaimer adequately and succinctly identifies the range of individuals who should not consumer alcohol, including, e.g., those with medical conditions or pregnant women. The claim

identifies the risks of moderate and heavy alcohol consumption that are not addressed through the NTX® technology.

Read in conjunction with the requested health claims, the speech conveyed to consumers precludes any suggestion or impression that alcohol is unequivocally safe to consume. Rather, the claim conveys the truthful and non-misleading impression that alcohol infused with NTX® is healthier than conventional alcoholic beverages, but not a "healthy" product in general terms.

G. The Proposed Claims Do Not Render NTX® a "Drug" Under Federal Law

The TTB has primary jurisdiction over the labeling and advertising of alcoholic beverages that are subject to the federal Alcohol Administration Act ("FAA Act"). *See* 27 U.S.C. § 205(e), (f); 27 C.F.R. Parts 4, 5, 7. The FAA Act applies to distilled spirits and certain wines and malt beverages. *See* 27 U.S.C. § 211(a)(5) (defining "distilled spirit" to mean "ethyl alcohol, hydrated oxide of ethyl, spirits of wine, whiskey, rum, brandy, gin, and other distilled spirits, including all dilutions and mixtures thereof, for non-industrial use"); *id*. at § (a)(6) (defining "wine" as, *inter alia*, containing 7 percent or more alcohol by volume); *id*. at § 211(a)(7) (defining "malt beverage" as "a beverage made by the alcoholic fermentation of an infusion or decoction, or combination of both, in potable brewing water, of malted barley with hops, or their parts, or their products, and with or without other malted cereals, and with or without the addition of unmalted or prepared cereals, other carbohydrates or products prepared therefrom, and with or without the addition of carbon dioxide, and with or without other wholesome products suitable for human food consumption").

The FDA has also noted "that certain curative, therapeutic, or disease-prevention claims for an alcoholic beverage might place the product in the category of a drug under the Federal

Food, Drug, and Cosmetic Act (FFDC Act), 21 U.S.C. 321(g)(1)(B). *See* 68 Fed. Reg. 10076, 10078 (Mar. 3, 2003). The TTB agreed with the FDA, but also acknowledged that the TTB's system of "health claims" regulation is a separate regulatory model that mirrors the FDA's health claim model for foods and dietary supplements. In the preamble to its final rule for health claims and other health-related statements, the TTB explained:

> After giving careful consideration to these comments, and consulting with FDA, TTB does not agree that its health claim regulations should be identical to those of FDA. FDA regulations were promulgated pursuant to a very specific grant of authority by Congress under the NLEA. Because of the differences in statutory authority, as well as the differences in the products regulated under these two statutes, TTB's regulatory scheme for health claim labeling will differ from FDA's regulatory scheme.
>
> However, TTB agrees with the FDA comment in several respects. Most importantly, we agree that it is important to ensure that alcohol beverage producers do not violate the new drug provisions of the FFDC Act when seeking to use specific health claims on alcohol beverage labels. It would be where the use of that claim would render the product subject to FDA's jurisdiction over drugs. Furthermore, FDA's authority over new drugs has significant public health and safety consequences. TTB does not wish to create any confusion on the part of industry members regarding their obligations to comply with FDA's requirements over drug claims.
>
> In the past, ATF merely advised industry members that they should be aware of the fact that the use of a health claim on an alcohol beverage label may subject the product to FDA's jurisdiction. However, after reviewing the comments on this issue, we met with FDA to discuss a process whereby TTB and FDA could consult on the use of specific health claims on alcohol beverage labels. In this way, FDA would have an opportunity to object to the use of a specific health claim, based on its jurisdiction over drugs, prior to any TTB action.
>
> Accordingly, the final rule now provides that TTB will consult with FDA, as needed, on the use of specific health claims on labels. If FDA determines that a specific health claim is a drug claim that is not in compliance with the requirements of the FFDC Act, TTB will not approve the use of such statement on a label. There is no similar provision in the advertising regulations, since advertisers are not required to obtain prior approval from TTB. We will of course consult with FDA, as appropriate, if the question arises as to whether an advertisement is in violation of the FFDC Act.

Id. at 10098.

While the TTB has enacted regulations governing the use and dissemination of "specific health claims," and has accepted jurisdiction by "evaluat[ing] such statements on a case-by-case basis…,"[21] the TTB also lacks authority to impose FDA regulatory requirements on spirit products. Neither the FAA Act nor the interpretive case law confers authority on the TTB to look beyond the TTB's enabling regulations in, for instance, 27 C.F.R. § 7.29(e) to determine whether a product claim is permissible under regulations enforced by other sister agencies without jurisdiction.

The ATF (TTB's predecessor) has acknowledged that "there are differences between ATF's statutory mandate to prevent misleading statements on labels and in advertising of alcoholic beverages under the FAA Act, and the more specific authority given to FDA in regulating health claims on food labels pursuant to the NLEA." *See Health Claims in the Labeling and Advertising of Alcoholic Beverages*, ATF IC 9308, 1993 WL 719948 (ATF Aug. 2, 1993).

Those points notwithstanding, the TTB health claim regulations are clearly designed to exempt certain "health claims" from the federal "drug" model when used for spirit beverages and subject to TTB preapproval. The TTB regulations unambiguously provide for TTB approval of "health claims" and "specific health claims" that "characterize[] the relationship of the malt beverage, alcohol, or any substance found within the malt beverage, to a disease or health-related condition." *See* 27 CFR § 7.54(e)(ii). That language mirrors the FDA's "health claim" exemptions under the NLEA, wherein the FDA approves claims that also "characterize the relationship between the substance in a food to a disease or health-related condition…" *See, e.g.,*

[21] *See, e.g.,* 27 C.F.R. § 7.54(e).

21 C.F.R. § 101.70(f); 21 U.S.C. § 343(r)(1)(B) (defining a health claim to mean a claim that "characterizes the relationship of any nutrient . . . to a disease or a health-related condition . . .").. Through the passage of 27 C.F.R. §§ 7.29, 7.54, and similar regulations, the TTB unambiguously established a pathway for health claim approval that is specific to TTB's jurisdiction and independent of the FDA's statutory authority and regulatory models.

Nonetheless, the health claims requested by this petition are not treatment or drug claims but, rather, claims that describe the role of the NTX® dietary ingredients that is intended to affect the structure or function of humans through well-documented mechanisms showing that NTX® maintains, protects, or supports those structure (to wit, hepatic systems and DNA). The FDA expressly excludes such claims from the definition of "drug" under Section 403(r)(6) of the federal Food, Drug, and Cosmetic Act. *See* 21 U.S.C. § 343(r)(6). Certain of the claims pertain to the reduction in the risk of disease effected by NTX® but they are, in that respect, indistinguishable from claims FDA and the federal courts regard as health claims. The FDA expressly excludes such "health claims" from the definition of "drug." *See id*. at § 343(r)(1)(B), (3)-(5). Although the FDA's regulatory model regarding health claims is not applicable here in the TTB context, the FDA's system—from which the TTB's regulation is apparently modeled—expressly excludes health claims from the "drug" context. *See id*. at § 343(r)(1)(B) (defining health claim as one that characterizes a relationship between a substance and a disease or health-related condition).

H. The Proposed Health-Related Statements Constitute Protected Commercial Speech under *Central Hudson* and Its Progeny

The proposed health-related statements are protected commercial speech under *Central Hudson* and its progeny. *See generally Central Hudson Gas & Elec. Corp. v. Pub. Serv.*

Comm'n of N.Y., 447 U.S. 557 (1980); *see also Rubin v. Coors Brewing Co.*, 514 U.S. 476 (1995) (applying *Central Hudson*'s test to strike regulations prohibiting beer producers from truthfully disclosing the alcohol content of beers). In *Central Hudson*, the U.S. Supreme Court acknowledged that the First Amendment protects commercial speech from unwarranted government intrusion, albeit less so than other constitutionally guaranteed expression. *See* 447 U.S. at 562. "The protection available for particular commercial expression turns on the nature both of the expression and of the governmental interests served by its regulation." *Id*. at 563. If a commercial "communication is neither misleading nor related to unlawful activity, the government's power is more circumscribed." In that instance, courts evaluate four elements in determining whether government censorship of commercial speech violates the First Amendment: (1) whether the speech concerns lawful activity and is not misleading; (2) whether the government's interest in prohibiting the speech is "substantial;" (3) whether the prohibition at issue "directly advances the governmental interest asserted;" and (4) whether the prohibition is "more extensive than is necessary to serve that interest." *Rubin*, 514 U.S. at 482 (citing *Central Hudson*, 447 U.S. 557 (1980)).

"The party seeking to uphold a restriction on commercial speech carries the burden of justifying it." *Bolger v. Youngs Drug Products Corp.*, 463 U.S. 60, 71, n. 20 (1983). "This burden is not satisfied by mere speculation or conjecture; rather, a governmental body seeking to sustain a restriction on commercial speech must demonstrate that the harms it recites are real and that its restriction will in fact alleviate them to a material degree." *Edenfield v. Fane*, 507 U.S. 761, 771 (1993) (citations omitted). To ban the proposed health-related statements, the TTB must show either that the language is not protected speech or that TTB's interest in government censorship is substantial and the method of censorship advances those interests in a direct and

material way and that there are no obvious, less speech-restrictive alternatives (such as claim qualifications). *Id.* at 767.

i. The Health-Related Statements Concern Lawful Activity and are Not Misleading

As explained in section IV(C), *infra*, and through the supporting evidence attached hereto, the available scientific record substantiates the truthfulness of the proposed health-related statements. The sale of Bellion Vodka and/or other alcoholic beverages containing NTX through the use of truthful and non-misleading health claims constitutes constitutionally protected activity. Moreover, no evidence demonstrates that the proposed health-related statements create a misleading impression concerning the effects on health of alcohol. No evidence suggests that Bellion's health claims would promote an increase in alcohol consumption. Where TTB imposes an outright ban on a claim based on the alleged existence of implied claims, the TTB's decision must be supported by empirical evidence demonstrating that consumers actually understand the label to convey the implied claims and are misled by them.[22] The TTB's regulations acknowledge that claims should be remedied through disclaimers. Here, Bellion's claims are supported by reliable scientific evidence. Thus, at worst, the proposed health-related statements are only potentially misleading. That distinction is critical because potentially misleading speech

[22] Where TTB imposes an outright ban on a health-related statements, the TTB's decision must be supported by empirical evidence demonstrating that consumers actually understand the statement to convey the misleading claims, are misled by those claims, and that the proposed qualifying language would not otherwise remedy that misleadingness. *See Fleminger, Inc. v. U.S. Dep't of Health and Human Servs.*, 854 F. Supp. 2d 192, 216 (D. Conn. 2012) (explaining that, based on established precedent, agencies must have "empirical evidence in connection with the government's outright ban on the proposed health claim"); *see also Whitaker v. Thompson*, 248 F. Supp. 2d 1, 11 (D.D.C. 2002) (citing *Pearson v. Shalala*, 164 F.3d 650, 659–60 (D.C. Cir. 1999) (explaining that a complete ban of a claim would be approved only under narrow circumstances, i.e., when there was almost no qualitative evidence in support of the claim and where the government provided empirical evidence proving that the public would still be deceived even if the claim was qualified by a disclaimer").

is entitled to constitutional protection under the First Amendment and may not be banned outright by this or any other federal agency. *See Pearson v. Shalala*, 164 F.3d 650 (D.C. Cir. 1999) ("*Pearson I*").

To establish that the proposed health-related statements are potentially misleading (as opposed to truthful and non-misleading), the TTB must have factual evidence showing that they are misleading before it may censor them because the TTB—not Bellion—has the burden to justify restrictions on speech. *See Pearson I*, 164 F.3d at 659 (explaining, in part, that the required use of disclaimers is a burden on speech). The TTB cannot declare a statement misleading, and bar that statement outright, by uttering the word "misleading" or resorting to categorical labels. To prove any of the proposed health-related statements is deceptive, the TTB "must offer consumer data or other extrinsic evidence to show that the audience to which the advertisement is directed is in fact misled [or capable of being misled] by the advertisement." *Stokely-Van Camp, Inc. v. Coca-Cola Co.*, 646 F. Supp. 2d 510, 525 (S.D.N.Y. 2009) (citing *Time Warner Cable, Inc. v. DIRECTV, Inc.*, 497 F.3d 144, 158 (2d Cir. 2007)); *see also Scotts Co. v. United Indus. Corp.*, 315 F.3d 264, 273 (4th Cir. 2002) (explaining that if a plaintiff challenges "a claim of implied falsehood, a plaintiff must demonstrate, by extrinsic evidence, that the challenged [advertisements] tend to mislead or confuse consumers"); *Pizza Hut, Inc. v. Papa John's Int'l, Inc.*, 117 F.3d 489, 497 (5th Cir. 2000) ("if the statements at issue are either ambiguous or true but misleading, the plaintiff must present evidence of actual deception") (citations omitted).

The TTB cannot show that any of Bellion's proposed health-related statements is misleading, because the TTB cannot show that (1) the health-related statement misleads consumers into thinking that alcoholic beverages containing NTX® are healthy; or (2) that

consumers will consume more alcoholic beverages than they otherwise would without the health-related statement.

Consumers remain aware that alcohol is unhealthy when consumed in excess or on a regular basis. *See* RESTATEMENT (SECOND) OF TORTS § 402A cmt. j (1965) (exempting alcohol sellers from liability for failing to warn of alcohol dangers, which includes dangers from consumption "in excessive quantit[ies]" and consumption "over a long period of time," because those dangers are "generally known and recognized" by the public). Consumer impact surveys in the mid-1990s concerning health claims on wine products found that participants exposed to those health-related statements did not exhibit a diminished understanding of the risks of drinking. *See Lieberman*, *supra*, 58 Food & Drug L.J. at 515. Moreover, the proposed claim qualification, and indeed any comparable and reasonable qualification—which would be acceptable to Bellion—disabuses consumers of any notion that alcohol consumption is safe or that drinking to excess is advisable.

To the extent that the TTB would view the proposed health-related statements as specific health claims, the qualification or disclaimer proposed herein that will appear as part of them ensures that any potential misleadingness as to the effects on health of alcohol consumption will be dispelled. The proposed disclaimer: (1) is sufficiently detailed and qualified with respect to explaining the categories of individuals to whom the claim applies; (2) adequately discloses the health risks associated with both moderate and heavier levels of alcohol consumption; and (3) outlines the categories of individuals for whom any levels of alcohol consumption may cause health risks.

In sum, there is no basis to conclude that the proposed health-related statements, with the accompanying claim qualification or disclaimer, are potentially misleading or misleading in any respect.

ii. Outright Censorship of the Truthful and Non-Misleading Health-Related Statements Does not Advance any Governmental Interest and Contradicts the TTB's Stated Objectives

Under *Central Hudson*, the government must assert a "substantial interest" in restricting speech. *Coors*, 514 U.S. at 483. Assertion of a substantial interest is not a trifling or insignificant burden on government action. Identifying the asserted interest is critical to an assessment under *Central Hudson*.

The TTB has previously explained that health-related statements must be regulated to prevent alcohol abuse by those consumers who might mistake alcohol for a healthy (or at least less harmful) product. In other words, the TTB has historically argued that it has an interest in prohibiting truthful statements which, by implication, "tend to create a misleading impression" that might encourage consumers to consume more alcohol to their detriment. If the TTB claims a state interest in protecting consumers from truthful information that could lead to abusive behaviors, it should be mindful of the fact that courts have already rejected that approach. *See 44 Liquormart*, 517 U.S. at 503 (explaining that courts express skepticism over government interests that "rest on the offensive assumption that the public will respond 'irrationally' to the truth").

Health-related statements for alcoholic beverages do not materially influence consumer drinking patterns, which undercuts the TTB's theory that protection of consumers from health claims is a significant state interest. *See* Lieberman, *The Power of Positive Drinking: Are Alcoholic Beverage Health Claims Constitutionally Protected?* 58 Food & Drug L.J. 511, 515

(2003). The ATF, the TTB's predecessor, considered this issue in 1998 when wine sellers asked for authority to promote the consumption of wine in moderation given evidence that consumption produced beneficial health effects. *Id.* The ATF commissioned a consumer impact study that found health claims on wine labels did not "induce wine drinkers to alter their drinking pattern, quantitatively or otherwise." *Id.* That study also found that "nothing in the proposed labels appeared to diminish focus group participant perceptions about the risk of drinking." *Id.* In other words, the health-related statements did not sway consumers into suddenly believing that the risks of alcohol consumption had dissipated.

Although the TTB might claim a substantial interest in protecting United States consumers from the ills of alcohol abuse, that interest is legally and factually distinct from a state interest in protecting consumers from truthful labeling content. Protecting consumers from truthful, verifiable, and non-misleading ingredient disclosures is a practice at odds with the TTB's mission and purpose, and lacks a "substantial" undergirding state interest. Perhaps for that reason, all nine Justices on the United States Supreme Court agreed that the ATF's prior label restrictions were unconstitutional in *Coors*, and at least one Justice would have applied "full First Amendment protection" to similar label disclosures involving alcohol content. *See Coors Brewing*, 514 U .S. at 491-92 (Stevens, J., concurring).

iii. Censoring the Health-Related Statements Will Not Advance an Interest in Protecting Consumers from the Ills of Alcohol Abuse

Even if the proposed health-related statements are banned, there is "no evidence to suggest that [the TTB's] speech prohibition will *significantly* reduce [or effect] marketwide consumption." *See 44 Liquormart*, 517 U.S. at 506. Under the third element of *Central Hudson*, a restriction on commercial speech is only valid if it directly advances the asserted governmental interest. *Coors*, 514 U.S. at 486. The government bears the burden "of showing that the

challenged regulation advances the Government's interest 'in a direct and material way.'" *Id.* at 486–87 (quoting *Edenfield v, Fane*, 507 U.S. 761, 767 (1993)). "That burden 'is not satisfied by mere speculation or conjecture; rather, a governmental body seeking to sustain a restriction on commercial speech must demonstrate that the harms it recites are real and that its restriction will in fact alleviate them to a material degree.'" *Id.* at 487 (quoting *Edenfield*, 507 U.S. at 770–71).

To meet its burden and show that regulation advances the governmental interest in a "direct and material way," the TTB must produce actual evidence that allowing the speech would harm the interest it seeks to further through the prevention of that speech. *See Pearson v. Edgar*, 153 F.3d 397, 404 (7th Cir. 1998) (overturning regulation prohibiting real estate brokers from soliciting where government "produced no evidence in this case that real estate solicitation harms or threatens to harm residential privacy"). The courts have made clear the TTB cannot meet that burden "by mere speculation or conjecture; rather a governmental body seeking to sustain a restriction on commercial speech must demonstrate that the harms it recites are real and that its restriction will in fact alleviate them to a material degree." *Fla. Bar v. Went For It, Inc.*, 515 U.S. 618, 625–26 (1995). "Thus, the government must come forward with some quantum of evidence, beyond its own belief in the necessity for regulation, that the harms it seeks to remedy are concrete and that its regulatory regime advances the stated goals." *Pagan v. Fruchey*, 492 F.3d 766, 771 (6th Cir. 2007) (citing *Edenfield*, 507 U.S. at 770–72).

In *Pagan*, for instance, the Sixth Circuit concluded that an affidavit submitted by the government was insufficient to prove that the regulation at issue, the posting of "for sale" signs on cars, directly and materially advanced the state's interests in traffic safety and aesthetics. *Id.* at 772–73. While the government argued that "it would be difficult, expensive, and time-consuming to conduct studies and provide empirical evidence in support of [the regulation]," the

court held that government must nevertheless offer some "actual evidence" that the regulation "will directly advance the government's asserted interest." *Id.* at 773–74; *cf. Anderson v. Treadwell*, 284 F.3d 453, 462 (2d Cir. 2002).

The TTB must have "credible evidence" that allowing the speech at issue would harm the interest asserted. *Id.* at 489; s*ee also 44 Liquormart*, 517 U.S. at 503 (requiring the government to provide evidence that prohibiting price advertising would actually advance the governmental interest in temperance). For example, in *Coors* the government asserted that allowing breweries to disclose the amount of alcohol on their labels would promote "strength wars."[23] *Id.* However, the Court recognized that prohibition of alcohol content on beer labels failed to suppress strength wars to any degree. *Id*. at 490–91 (noting that "the Government did not offer any convincing evidence that the labeling ban has inhibited strength wars").

Here the TTB has no credible evidence that the proposed health-related statements would harm any state interest or that consumers would be misled by them, especially when those health claims are accompanied by the proposed disclaimer. Consumers decide how to drink alcoholic beverages (quantitatively and qualitatively) based on a variety of reasons that may include sociological and psychological factors. Convenience and circumstance likely factor more in the decision than health related-statements. For instance, the quantity of alcohol consumed may depend on the social environment, the availability of transportation, the age of the drinker, etc. Accordingly, the concept that prohibiting a health-related statement will influence the rate, amount, of frequency of alcohol consumption is sheer speculation.

iv. Prohibiting the Proposed Health-Related Statements Is Not Sufficiently Tailored to Any Governmental Goal

[23] A strength war, in that context, is a competition between breweries who "seek to compete for customers on the basis of alcohol content." *Coors*, 514 U.S. at 483.

To pass constitutional muster under *Central Hudson*, the final analysis requires the TTB to prove that a speech restriction is sufficiently tailored to its articulated goal. *Coors,* 514 U.S. at 490. Assuming *arguendo* that the TTB has a defined and substantial interest in regulating the proposed health-related statements, that interest is not directly advanced by the TTB's censorship of the claims here in isssue.

Even assuming that the TTB could demonstrate an important state interest, and that unqualified health-related statement have the potential to mislead, the TTB must then *prove* that there are no less speech-restrictive alternatives, such as the disclaimers required under its own regulations for specific health claims. *Pearson I,* 164 F.3d at 658-59 (requiring reliance on disclaimers as a less speech restrictive alternative to outright suppression); *Alliance for Natural Health U.S. v. Sebelius*, 714 F. Supp. 2d 48, 71 (D.D.C. 2010) ("*ANH I*") (invalidating FDA disclaimer language and holding that FDA acted unconstitutionally by requiring a more onerous disclaimer without considering whether a short and succinct disclaimer would be a less restrictive means); *see also Pearson v. Shalala*, 130 F. Supp. 2d 105, 120 (D.D.C. 2001) ("*Pearson II*").

Here, as discussed above, the Petitioners propose a disclaimer to accompany the health-related statements that is sufficient to cure any potential for misleadingness regarding the effects of alcohol on health. The Petitioners proposed that disclaimer despite TTB having conceded in prior agency statements that burdensome disclaimers for specific health claims would be "extremely unlikely [to] fit on a normal alcoholic beverage label." *See* Health Claims in the Labeling and Advertising of Alcoholic Beverages, ATF IC 93-8, 1993 WL 719948 (ATF Aug. 2, 1993). Because "no label has [ever] met," the standard clearly reveals that the burdens of the TTB regulations have, to this point, effected a de facto market ban. *See, e.g.*, Erik Bierbauer,

Liquid Honesty: The First Amendment right to Market the Health Benefits of Moderate Alcohol Consumption, 74 N.Y.U. L. Rev. 1057, 1068 (1999) (explaining that "[t]he only health statement that ATF said it would accept was a four-page report with thirty-four footnotes, which the agency said presented a balanced view of drinking's good and ill effects.").

To the extent that the TTB will require a more onerous, lengthy, and burdensome disclaimer than the one proposed, the TTB would violate the First Amendment's requirement for reasonable claim qualification, which is to mandate "short, succinct, and accurate" qualifications. *Pearson II*, 130 F. Supp. 2d at 120. Moreover, there is no proof that onerous and lengthy disclaimers are the only ones capable of effectuating a legitimate government interest such that there are no "less restrictive means" through short, succinct and accurate disclaimers, such as the one proposed in this petition. *See Pearson I*, 164 F.3d at 658. Absent extrinsic evidence that such onerous disclaimers are required to cure any alleged misleadingness caused by the proposed health-related statements, requiring onerous disclaimers is unconstitutional. Indeed, the federal courts have explained repeatedly that the government acts unconstitutionally unless it can "demonstrate with empirical evidence that [much shorter] disclaimers … would bewilder consumers and fail to correct for deceptiveness." *Whitaker v. Thompson*, 248 F. Supp. 2d 1, 5 (D.D.C. 2002); *Pearson I*, 164 F.3d at 659-60; *ANH I*, 714 F. Supp. 2d at 63 (invalidating FDA censorship because the "[a]gency has not provided any empirical evidence, such as 'studies' or 'anecdotal evidence,' that consumers would be misled").

V. Conclusion

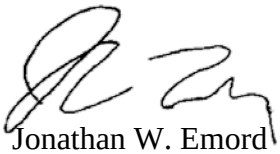
For the foregoing reasons, the Petitioners request that TTB authorize the proposed health-related statements concerning NTX®. Those claims include the following health claims in Bellion labels, labeling, advertising, and promotional statements:

- NTX® provides antioxidant and anti-inflammatory support;
- NTX® helps protect against alcohol-induced oxidative damage to the liver;
- NTX® helps maintain normal liver enzyme production and function;
- NTX® supports normal liver defenses and regenerative mechanisms;
- NTX® reduces the risk of alcohol-induced liver diseases, including fibrosis and cirrhosis;
- NTX® helps maintain normal liver functions;
- NTX® helps protect DNA from alcohol-induced damage; and
- NTX® reduces alcohol-induced DNA damage

Any questions concerning this Petition may be directed to Jonathan W. Emord, Esq. Emord & Associates, P.C., 11808 Wolf Run Lane, Clifton, VA 20124, (202) 466-6937. The undersigned certify on behalf of the Petitioners that to the best of their knowledge and belief, the Petition includes all information and views on which the Petitioners rely and is a representative and balanced submission that includes unfavorable information as well as favorable information known by the Petitioner to be pertinent to evaluation of the proposed health claims.

DATED: April 12, 2016.

Sincerely,



Jonathan W. Emord
Peter A. Arhangelsky
Bethany R. Kennedy
Counsel to Bellion Spirits, LLC
And Chigurupati Technologies
Private Ltd.

CERTIFICATE OF SERVICE

I HEREBY CERTIFY that on this April 11, 2016, copies of the foregoing Health Claim Petition and all supporting exhibits were mailed via UPS Next Day Air (in hardcopy and electronic format) to:

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April 12, 2016

VIA FEDEX OVERNIGHT:
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Teresa G. Knapp
Gail H. Davis
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Washington, D.C. 20005

Re: Petition for Health Related Statements for Bellion Vodka with NTX®

Dear Mr. Manfreda and Mss. Knapp and Davis:

Please find enclosed two hardcopies of Bellion Sprits, L.L.C.'s and Chigurupati Technologies Private Ltd.'s petition for health-related statements concerning the effect of NTX® in distilled spirits and/or other alcoholic beverages. We have enclosed compact discs containing electronic versions of the petitions in PDF Format. Those CDs contain two versions of Exhibit 5: a public redacted version and a confidential version containing confidential and proprietary data subject to protection under 5 U.S.C. § 552(b)(4).

Please do not hesitate to contact us if you have any questions.

Sincerely,

Jonathan W. Emord
Peter A. Arhangelsky
Bethany R. Kennedy

RESEARCH SUPPORTING THE HEALTH CLAIMS FOR NTX, A PRODUCT COMPOSED OF GLYCYRRHIZIN AND MANNITOL, IN CONJUNCTION WITH ALCOHOL CONSUMPTION

SIDNEY J. STOHS, Ph.D., FACN, CNS, ATS, FAPhA

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ABSTRACT

The detrimental effects of chronic and heavy alcohol consumption are well documented, and alcohol is a risk factor or cause of numerous diseases. The most common problem associated with chronic alcohol consumption is alcoholic liver disease which can range from hepatic steatosis to more advanced stages as hepatitis, cirrhosis, hepatocellular carcinoma and ultimately liver failure. NTX is a patented product composed of the primary ingredients glycyrrhizin and mannitol that act synergistically. Numerous studies in humans and animals have demonstrated the liver protective effects of NTX as well as its individual components during alcohol consumption. Furthermore, the safety of the ingredients in NTX is well-established. Therefore, health claims regarding NTX have been set forth based on the totality of the scientific literature, and each health claim is substantiated and supported by the scientific literature.

INTRODUCTION

The detrimental effects of chronic and/or heavy alcohol consumption are well established, and alcohol is considered a risk factor or cause of numerous diseases. More than 50 % of Americans consume alcohol. According to a 2014 report of the Center for Disease Control (CDC), the cost for excessive drinking in the United

States was $223.5 billion in 2006 in terms of health care costs and lost productivity[1], a number that undoubtedly has increased in subsequent years. Furthermore, the CDC estimates that there are approximately 88,000 alcohol related deaths annually[2].

The most common problem associated with chronic alcohol consumption is alcoholic liver disease which is defined by histological lesions on the liver that range from hepatic steatosis to more advanced stages as alcoholic steatohepatitis, cirrhosis, hepatocellular carcinoma and liver failure due to necrosis or apoptosis (programmed cell death)[3,4]. Over 14,000 deaths occur annually in the United States due to alcoholic liver cirrhosis[4].

The mechanisms associated with alcohol toxicity and the induction of alcoholic liver disease can be summarized as follows: metabolism of alcohol to highly toxic acetaldehyde and free radical species, production of reactive oxygen (ROS) and nitrogen species with resulting oxidative stress, release of inflammatory cytokines as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), abnormal lipid metabolism, oxidative DNA damage, formation of protein and DNA adducts with metabolites of alcohol and acetaldehyde, and ultimately induction of apoptosis or necrosis with subsequent multi-system organ failure[3-9].

Biomarkers are biological indicators of the state of health of an organ or tissue. Just as blood sugar (glucose) elevation is indicative of and associated with diabetes, various common biomarkers are useful in assessing the existence, progression and pathogenesis of alcoholic liver disease. These biomarkers include measurements of the blood serum levels of the enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT) and alkaline phosphatase (ALP). Elevations of the serum levels of these enzymes occur when the liver is damaged as is the case with alcoholic liver disease. Conversely, decreases in the serum levels of these enzymes occur as the liver begins to heal[8-12].

Other biomarkers that are commonly used to assess the stages of alcoholic liver disease include total bilirubin, lipid profiles (cholesterol and triglycerides), hemoglobin or serum ferritin, albumin, platelets, prothrombin time and

international normalized ratio (INR). For example, elevations in blood levels of cholesterol and triglycerides are associated with faulty liver metabolism of these lipids which occurs in conjunction with alcoholic liver disease. Similarly, elevated bilirubin occurs as a result of faulty liver metabolism.

As previously noted, the hepatic metabolism of alcohol results in the production of free radicals and reactive oxygen species (ROS) leading to oxidative stress which produces tissue damaging effects. Markers of oxidative stress and oxidative tissue damage include reactive oxygen species (ROS) production, the lipid metabolite malondialdehyde (MDA), protein carbonyl formation, and DNA damage. Increases in these biomarkers serve as indices and measures of alcoholic liver damage. Reduced glutathione (GSH) content is one of the primary intracellular antioxidants and chemoprotectants in human tissues, and a decrease in GSH content occurs as it neutralizes ROS and free radicals. Therefore, a decrease in GSH is associated with an increase in oxidative stress as occurs in the case of alcoholic liver disease[8-12].

Blood and tissue levels of the enzymes catalase, glutathione peroxidase (GPX) and superoxide peroxidase (SOD) are also used to obtain information regarding alcoholic liver disease[10-12]. These enzymes play important roles in the decomposition of ROS as hydrogen peroxide. Increases in the levels of these enzymes are indicative of hepatoprotection.

In individuals with alcohol-associated steatosis, studies indicate that approximately 20% will develop cirrhosis within 10 years[6], and it is estimated that 20% of heavy drinkers will develop acute alcoholic hepatitis[13]. The two primary approaches for successful prevention and reversal of liver damage are abstinence from alcohol and appropriate nutritional support. Realistically, one cannot expect the vast majority of alcohol consumers to abstain. Therefore, there is a pressing need to develop safer strategies for alcohol consumption and treatment in order to reduce alcohol-associated morbidity and mortality, and the high cost to society.

The high morbidity and mortality rate of alcoholic liver disease is attributed to the cascading sequence of events described above which can ultimately culminate in multisystem organ failure and death[3-9,13]. Systems designed to prevent and treat alcohol-induced liver toxicity and alcoholic liver disease must therefore focus on disrupting, inhibiting and/or reversing these pathological pathways. As a consequence, a new category of alcoholic beverages called Functional Spirits has been created and a product has been patented[14] that prevents and/or ameliorates the hepatotoxic effects of alcohol while retaining it desired characteristics.

NTX is a patented product composed of the primary ingredients glycyrrhizin glycyrrhizic acid) and mannitol[14]. It also contains potassium sorbate as an antioxidant, preservative and antimicrobial. The potassium may also provide benefits with respect to electrolyte balance. Recent studies in humans and animals have demonstrated the hepato-protective effects of NTX during alcohol consumption[14-19].

In a randomized, double-blind, placebo-controlled cross-over clinical trial involving human 12 subjects, NTX afforded significant liver protection during 12 days of alcohol consumption as evidenced by significant decreases in the serum levels of the enzyme biomarkers of liver toxicity ALT, AST, GGT and ALP[15]. No adverse effects were observed by any of the subjects in the presence or absence of the NTX.

A second randomized, double-blind, placebo-controlled cross-over clinical trial involving human 31 subjects was conducted whereby all participants received 1.5 g alcohol/kg with and without NTX. A seven day washout was provided between treatments[16]. Consumption of alcohol alone caused an increase in serum ROS and ROS metabolites which persisted for up to 4 hours. In addition, alcohol consumption resulted in a decrease in serum GSH, an increase in MDA (lipid peroxidation), an increase in protein carbonyl, and an increase in oxidative DNA damage in peripheral lymphocytes as determined by the single cell electrophoresis comet assay, the cytokinesis-block micronucleus assay, and 8-

hydroxy-2-deoxyguanosine formation. Consumption of alcohol with NTX significantly reduced the levels of ROS and all the oxidative markers. According to the authors, the results indicated that NTX may "be effective in reducing alcohol-induced pathophysiological changes"[16].

A double blind, comparative, cross-over clinical trial was conducted which examined the effects of alcohol on blood levels of ROS in the absence and presence of co-administered NTX[17]. Twelve normal healthy subjects were used. The results demonstrated that NTX significantly dampened the alcohol-induced generation of ROS and ROS metabolites. NTX also decreased serum MDA, a product of alcohol-induced lipid peroxidation, while increasing serum levels of the endogenous antioxidant and chemoprotectant GSH. Furthermore, NTX administration with alcohol significantly decreased serum protein carbonyl levels which are another marker of alcohol-induced oxidative stress[17].

A 28 day experiment was conducted in rats that daily received 2.5 g/kg/day with or without various doses of mannitol, glycyrrhizin or both[14]. The results demonstrated that mannitol and glycyrrhizin alone afforded significant hepato-protection while the combination of these two substances (NTX) was synergistic, affording protection that was greater than the sum of the individual ingredients. The combination restored biomarkers of liver function to as much as 80 % of normal. The combination of mannitol and glycyrrhizin (NTX) provided significant hepato-protection from alcohol toxicity as evidenced by decreases in serum ALT levels, decreases in the levels of the inflammatory cytokine TNF-α, and decreases in the lipid peroxidation product MDA.

This combination (NTX) product synergistically decreased alcohol induced production of reactive oxygen species (ROS) by enhancing the levels of the enzymes catalase, GPX and SOD that decompose tissue damaging ROS[14]. Reduced glutathione (GSH) is the primary antioxidant within cells that protects against ROS. Alcohol consumption results in a depletion of GSH due to alcohol-induced production of ROS. Concurrent consumption of the combination of mannitol and

glycyrrhizin (NTX) provided significant restoration of endogenous GSH levels in the liver[14], indicative of a more normal healthy functioning liver.

A study was conducted in rats that evaluated the effects of NTX on oxidative stress induced by a single dose of alcohol[18]. The alcohol alone resulted in an increase in the production of ROS, a decrease in GSH and an increase in MDA, all indicators of alcohol-induced toxicity. Co-administration of NTX with the alcohol resulted in a significant modulation of these alcohol-induced effects, indicating a hepato-protective effect of the NTX.

Another study in rats has evaluated the hepatoprotective effects of NTX against alcohol induced hepatotoxicity[19]. Rats were given orally 1, 2 or 3 grams of ethanol per kg daily for 28 days with and without NTX. Alcohol treatment caused the elevation of serum enzymes ALT, AST, GGT and ALP, denoting hepatic toxicity. In addition, the lipid peroxidation product MDA was elevated while the endogenous antioxidant GSH was decreased. Co-administration of NTX with the alcohol resulted in 20-40 % decreases in the levels of ALT, AST, GGT and ALP, demonstrating the hepatoprotective effects of the NTX. Furthermore, 26-47 % decreases occurred in lipid peroxidation (MDA) with 24-28 % increases in the intracellular antioxidant and hepatoprotectant GSH.

The published scientific literature has been reviewed regarding glycyrrhizin and mannitol as well as potassium sorbate. The author used PubMed as well as Google Scholar. PubMed is the search engine which accesses primarily the MEDLINE database of references and abstracts on biomedical topics and life sciences. The PubMed database is maintained by the U.S. National Library of Medicine at the National Institutes of Health. Google Scholar also covers articles in journals that are less well known as well as a greater number of foreign language journals than PubMed. As a consequence, the author located a number of articles via Google Scholar that were not detected by PubMed.

This review addresses not only human studies associated with alcoholic liver disease, NTX and its components glycyrrhizin and mannitol, but also relevant

animal and in vitro studies. Animal and in vitro studies provide valuable information regarding mechanisms of action, safety and efficacy that can be readily extrapolated to humans, and that cannot be conducted in humans because of ethical and potential safety considerations. Furthermore, animal studies corroborate and can greatly extend information derived from human studies.

In summary, the above human and animal studies demonstrate that co-consumption of NTX with alcohol can ameliorate the hepatotoxic effects of alcohol and thereby improve the health of alcohol consumers as well as decrease the alcohol-related burden on society in a cost-effective manner.

HEALTH CLAIMS

Animal and human studies have been conducted with NTX in combination with alcohol[14-19], and described above. In addition, over 70 human, animal and in vitro studies have been conducted and published on the hepatoprotective, antioxidant and anti-inflammatory effects of glycyrrhizin and mannitol, the key components of NTX which have been shown to work synergisitcally[14]. Therefore, the following health claims regarding NTX have been set forth. Each health claim is substantiated and supported by the available scientific literature.

- **Provides antioxidant and anti-inflammatory support**
- **Helps protect against oxidative damage to the liver**
- **Helps maintain normal liver enzyme production and function**
- **Supports normal liver defenses and regenerative mechanisms**
- **Reduces the risk of liver diseases including fibrosis and cirrhosis**
- **Ameliorates the symptoms associated with liver disease including fibrosis and cirrhosis**
- **Helps maintain normal liver functions**
- **Helps protect DNA from alcohol-induced damage**
- **Reduces alcohol-induced DNA damage**

PROVIDES ANTIOXIDANT AND ANTI-INFLAMMATORY SUPPORT

Human and animal studies have shown that NTX decreases alcohol-induced production of reactive oxygen species (ROS) and ROS metabolites, lipid peroxidation (MDA formation) and carbonyl protein formation, while increasing the levels of the endogenous antioxidant reduced glutathione (GSH)[14, 16-19]. Furthermore, NTX decreases the alcohol-induced increase in DNA damage in human peripheral lymphocytes as determined by the single cell electrophoresis comet assay, the cytokinesis-block micronucleus assay, and 8-hydroxy-2-deoxyguanosine formation[16]. NTX also enhances the levels of the enzymes catalase, GPX and SOD that decompose ROS[14], thereby affording protection from the tissue damaging effects of ROS.

In an animal study, a licorice extract predominantly containing glycyrrhizin has been shown to effectively inhibit alcohol induced increases in TNF-α, lipid accumulation in liver (steatosis) and decreases in the antioxidant GSH[20]. The hepatoprotective effect was confirmed histologically. In animals, glycyrrhizin also inhibits alcohol-induced increases in the inflammatory cytokine NF-κB[21, 22], and increases in MDA as well as serum lipids[23], while preventing liver injury and cirrhosis.

Various in vitro studies have demonstrated the antioxidant and free radical scavenging ability of glycyrrhizin[24-29]. In cell culture experiments, glycyrrhizin has been shown to inhibit inflammatory TNF-α secretion[30,31] and MDA production (lipid peroxidation)[32-34], while enhancing the antioxidant enzyme SOD and the antioxidant and tissue protectant GSH[32,33,35].

In a number of animal experiments, glycyrrhizin has been shown to decrease the production of the inflammatory cytokines TNF-α, IL-1 and IL-6[27, 36-38], decrease lipid peroxidation (MDA)[36, 38-40], increase hepatic GSH content[40,41], and increase levels of the reactive species-eliminating enzymes SOD and GPx[42].

Glycyrrhizin also provides protection against nephrotoxic drugs[43] as well as experimental acute pancreatitis in rats[44]. In the latter case, glycyrrhizin decreased

concentrations of the inflammatory cytokines TNF-α, IL-1 and IL-6 and also decreased MDA production. In this study, the protective effects of glycyrrhizin were corroborated histologically[44].

Glycyrrhizin has been shown to ameliorate liver damage associated with high fructose intake in rats[38] which is characterized by oxidative tissue damage and inflammation. Glycyrrhizin administration significantly deceased levels of AST, ALT, ALP, ROS, carbonyl protein, TNF-α, lipid peroxidation (MDA) and apoptosis, all characteristic of liver damage. The antioxidant, anti-inflammatory and hepatoprotective effects of the glycyrrhizin were affirmed histologically[38].

Mannitol is the second of the two primary components of NTX. Several human studies have demonstrated the antioxidant and tissue protective effects of mannitol. Mannitol has been shown to exhibit tissue protective effects in acute ischemic-reperfusion injuries[45], and antioxidant and free radical scavenging properties in conjunction with cardiopulmonary bypass[46]. Mannitol is used as an anti-inflammatory and antioxidant in tissue baths associated with kidney transplants[47].

In a study in rats, mannitol was demonstrated to provide protection against alcohol-induced gastric mucosal damage due to its antioxidant and anti-inflammatory properties[48]. Mannitol has also been shown to exhibit anti-inflammatory and antioxidant activity in rats after traumatic brain injury[49]. Mannitol decreased production of MDA and normalized the levels of the antioxidant enzymes catalase and GPx.

In summary, over 30 human, animal and in vitro published studies have confirmed the antioxidant and anti-inflammatory properties of NTX and its primary components glycyrrhizin and mannitol. It should be noted that glycyrrhizin and mannitol afford tissue and organ protection against oxidative stress and ROS regardless of their source, including chronic alcohol consumption.

HELPS PROTECT AGAINST OXIDATIVE DAMAGE TO THE LIVER

As noted above, common biomarkers used in assessing the existence and progression of alcoholic liver damage and disease include the measurement of serum levels of the enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT) and alkaline phosphatase (ALP)[50-52]. Other biomarkers that are also commonly used to determine the degree of tissue damage include total bilirubin, lipid profiles, hemoglobin or serum ferritin, albumin, and platelets.

A randomized, double-blind, placebo-controlled clinical trials involving 12 human subjects in each study have shown that NTX provides significant liver protection during 12 days of alcohol consumption as evidenced by decreases in the biomarkers of liver toxicity ALT, AST, GGT and ALP[15].

A second randomized, double-blind, placebo-controlled cross-over clinical trial involving human 31 subjects was conducted whereby all participants received alcohol with and without NTX. A seven day cross-over washout was provided between treatments[16]. Consumption of alcohol alone caused an increase in serum ROS and ROS metabolites, a decrease in serum GSH, an increase in MDA, an increase in protein carbonyl, and an increase in DNA damage in peripheral lymphocytes as determined by the single cell electrophoresis comet assay, the cytokinesis-block micronucleus assay, and 8-hydroxy-2-deoxyguanosine formation. Co-consumption of alcohol with NTX significantly reduced the levels of ROS and all the oxidative markers, thus demonstrating the tissue protective effects of NTX.

A meta-analysis of 12 randomized controlled trials (RCTs) has been published that summarizes the effects of a glycyrrhizin product on liver function in 838 patients with alcoholic liver disease[53]. The study results showed that glycyrrhizin significantly decreased serum ALT and AST levels and the authors concluded that the glycyrrhizin product significantly improved liver function in alcoholic liver disease[53].

Several animal studies have demonstrated that NTX provides significant protection against alcohol-induced oxidative damage to the liver as evidenced by decreases in serum levels of ALT, AST, GGT and ALP[14,19]. Increases in hepatic MDA are an indicator of oxidative liver damage, and NTX significantly reduced the alcohol-induced production of MDA[14,19]. The hepatoprotective effects of NTX in rats were confirmed by histopathological examination of livers that revealed a marked reduction in alcohol-induced lesions with an absence of necrotic cells[19]

In a number of animal experiments, glycyrrhizin has been shown to decrease the production of MDA (lipid peroxidation)[36, 38-40], increase hepatic content of the antioxidant and free radical scavenger GSH[40,41], and increase levels of the reactive species-eliminating enzymes SOD and GPx[42]. Glycyrrhizin also provides protection against nephrotoxic drugs[43] as well as experimental acute pancreatitis in rats[44]. In the latter case, glycyrrhizin decreased MDA production, am indicator of liver oxidative damage, and the protective effects of glycyrrhizin were demonstrated histologically[44].

Glycyrrhizin has been shown to ameliorate oxidative liver damage associated with high fructose intake in rats[38]. Glycyrrhizin administration significantly deceased levels of AST, ALT, ALP, ROS, carbonyl protein, TNF-α, lipid peroxidation (MDA) and apoptosis, all characteristic of oxidative liver damage. The antioxidant and hepatoprotective effects of the glycyrrhizin were affirmed histologically[38].

In cell cultures, glycyrrhizin has been shown to inhibit TNF-α secretion[30,31] and MDA production[32-34], while enhancing the antioxidant enzyme SOD and the endogenous antioxidant GSH[32,33,35].

Glycyrrhizin, one of the active constituents in NTX, has been shown to significantly reduce serum ALT and AST in patients with chronic hepatitis[54-67], with the reduction in these enzymes being an indication of the extent of liver damage. The frequency of ALT normalization was dependent upon duration of glycyrrhizin administration, with the highest normalization reported being approximately 35 %[54]. The improved health of the liver was confirmed histologically in one study where glycyrrhizin treatment resulted in a 45 % improvement in the necrosis

score[63]. Glycyrrhizin administration has also been shown to decrease the incidence of hepatocarcinoma in individuals with hepatitis by as much as 25 %[54, 59, 65-67].

A meta-analysis of 9 randomized controlled trials involving 687 patients with chronic hepatitis treated with diammonium glycyrrhizinate, a salt of glycyrrhizin, has been published[68]. The study concluded that the diammonium glycyrrhizinate significantly decreased serum ALT and AST levels as well as provided significant improvements in total bilirubin and albumin, thus demonstrating improved liver function.

Another meta-analysis reviewed 24 randomized control trials involving 3201 patients with chronic hepatitis who were treated with diammonium glycyrrhizinate[69]. The authors concluded that the diammonium glycyrrhizinate significantly reduced serum ALT and total bilirubin and ameliorated fibrosis.

In summary, approximately 50 published human studies as well as a number of animal studies have shown that NTX as well as its primary active constituents glycyrrhizin and mannitol afford protection against oxidative damage to the liver, keeping in mind that the mechanism of alcohol toxicity and liver disease involves oxidative stress and oxidative damage.

HELPS MAINTAIN NORMAL LIVER ENZYME PRODUCTION AND FUNCTION

Animal and cell culture studies have shown that NTX and its active constituents glycyrrhizin and mannitol help maintain a wide range of liver enzymes at normal levels including ALT, AST, GGT and ALP, affirming the lack of alcohol-induced inflammation and tissue damage. Furthermore, antioxidant enzymes as SOD, GPX and catalase are supported so that they can preclude formation of products of oxidative tissue damage including ROS, carbonyl protein and MDA. These results demonstrate that glycyrrhizin and mannitol protect the liver, and support and facilitate normal liver function.

A randomized, double-blind, placebo-controlled clinical trial involving 12 human subjects received alcohol daily for 12 days with or without NTX. NTX co-administration with alcohol provided significant decreases and normalization in the enzyme biomarkers of liver toxicity ALT, AST, GGT and ALP[15,16]. The results demonstrated that NTX precluded alcohol toxicity and helped maintain normal liver function.

A second randomized, double-blind, placebo-controlled cross-over clinical trial involving human 31 subjects was conducted whereby all participants received a single dose of alcohol with and without NTX. A seven day washout was provided between treatments[16]. Consumption of alcohol alone caused an increase in serum ROS and ROS metabolites, a decrease in serum GSH, an increase in MDA, an increase in protein carbonyl, and an increase in DNA damage in peripheral lymphocytes as determined by the single cell electrophoresis comet assay, the cytokinesis-block micronucleus assay, and 8-hydroxy-2-deoxyguanosine formation. Co-consumption of alcohol with NTX significantly reduced the levels of ROS and all the oxidative markers[16], thus helping to maintain normal liver enzyme levels and functions.

Glycyrrhizin, one of the active constituents in NTX, has been shown in a number of clinical studies to significantly reduce and normalize serum ALT and AST in patients with chronic hepatitis[54-67]. The frequency of ALT normalization was dependent upon duration of glycyrrhizin administration, with the highest normalization reported being approximately 35 %[54].

Several animal studies have demonstrated that NTX provides significant protection against alcohol-induced oxidative damage to the liver as evidenced by decreases and normalization of the serum levels of ALT, AST, GGT and ALP[14,19]. Rats were given ethanol daily for 28 days with and without NTX[19]. Alcohol treatment produced an elevation of serum enzymes ALT, AST, GGT and ALP, denoting hepatic toxicity. In addition, the endogenous antioxidant GSH was decreased. Co-administration of NTX with the alcohol resulted in 20-40 %

decreases in the levels of ALT, AST, GGT and ALP with significant increases in the antioxidant GSH, demonstrating the ability of NTX to decrease the hepatotoxic effects of alcohol, and help maintain normal liver enzyme levels. The ability of NTX to provide protection against alcohol-induced toxicity was confirmed by histopathological examination of the livers[19].

The combination of glycyrrhizin and mannitol, the active ingredients in NTX, synergistically decreased alcohol induced production of reactive oxygen species (ROS) by enhancing the levels of the enzymes catalase, GPX and SOD that decompose tissue damaging ROS[14], thus contributing to the maintenance of normal liver function. In addition, consumption of mannitol and glycyrrhizin (NTX) prevented alcohol-induced depletion of endogenous GSH levels in the liver[14], indicative of hepatoprotection by NTX and a normal healthy functioning liver.

Glycyrrhizin ameliorates oxidative liver damage associated with high fructose intake in rats[38]. Glycyrrhizin administration significantly normalized levels of AST, ALT, ALP, ROS, carbonyl protein, lipid peroxidation (MDA) and apoptosis, all characteristic of oxidative liver damage. The tissue effects of the glycyrrhizin and normalization of liver function were affirmed histologically[38].

In cell culture experiments, glycyrrhizin has been shown to inhibit TNF-α secretion[30,31] and MDA production[32-34], while enhancing the antioxidant enzyme SOD and the antioxidant GSH[32,33,35].

In summary, numerous published human, animal and cell culture studies have shown that NTX and its primary active constituents glycyrrhizin and mannitol normalize a wide range of liver enzymes including ALT, AST, GGT, ALP, SOD, GPX and catalase while inhibiting alcohol-induced inflammation and formation of products of oxidative tissue damage including ROS, carbonyl protein and MDA. These results demonstrate that glycyrrhizin and mannitol protect the liver, and support and facilitate normal liver function.

SUPPORTS NORMAL LIVER DEFENSES AND REGENERATIVE MECHANISMS

Almost 50 published human clinical studies and a wide variety on animal studies have demonstrated that NTX and its individual components glycyrrhizin and mannitol support normal defense mechanisms against alcohol and other hepatotoxins, allowing normal function and regeneration of the liver.

A randomized, double-blind, placebo-controlled cross-over clinical trial has demonstrated that NTX provides significant liver protection during 12 days of alcohol consumption as compared to receiving alcohol alone as evidenced by decreases and normalization of the hepatic enzyme biomarkers of liver toxicity ALT, AST, GGT and ALP[15]. A four week washout period was provided between the cross-over treatments.

A second randomized, double-blind, placebo-controlled cross-over clinical trial involving human 31 subjects was conducted whereby all participants received a single dose alcohol with and without NTX. A seven day washout was provided between treatments[16]. Consumption of alcohol alone caused an increase in serum ROS and ROS metabolites, a decrease in serum GSH, an increase in MDA, an increase in protein carbonyl, and an increase in DNA damage in peripheral lymphocytes as determined by the single cell electrophoresis comet assay, the cytokinesis-block micronucleus assay, and 8-hydroxy-2-deoxyguanosine formation. Consumption of alcohol with NTX significantly reduced the levels of ROS and all the oxidative markers and increased serum GSH. The results indicated that NTX may "be effective in reducing alcohol-induced pathophysiological changes"[16].

A double blind, comparative, cross-over clinical trial was conducted which examined the effects of alcohol on blood levels of ROS in the absence and presence of co-administered NTX[17]. The results demonstrated that NTX significantly decreased the alcohol-induced generation of ROS and ROS metabolites, and decreased serum lipid peroxidation product MDA, while increasing serum levels of the endogenous antioxidant GSH. NTX administration

with alcohol also significantly decreased serum protein carbonyl levels which are another marker of alcohol-induced oxidative stress[17]. Thus, NTX co-administration with alcohol decreased oxidative tissue damage and supported normal liver function.

A 28 day experiment was conducted in rats that daily received alcohol with or without various doses of mannitol, glycyrrhizin or both[14]. The results demonstrated that individually mannitol and glycyrrhizin provided significant hepato-protection while the combination of these two substances (NTX) was synergistic, affording protection that was greater than the sum of the individual ingredients. The combination of glycyrrhizin and mannitol restored biomarkers of liver function to as much as 80 % of normal. The combination (NTX) also provided significant hepatoprotection from alcohol toxicity as evidenced by decreases in serum ALT levels as well as the inflammatory cytokine TNF-α and the lipid peroxidation product MDA.

Glycyrrhizin and mannitol synergistically decreased alcohol-induced production of reactive oxygen species (ROS) by enhancing the levels of the enzymes that decompose tissue damaging ROS, namely catalase, GPX and SOD[14]. Reduced glutathione (GSH) is the primary antioxidant within cells that protects against ROS. Alcohol consumption results in a depletion of GSH as a result of the alcohol-induced production of ROS. Concurrent consumption of the combination of mannitol and glycyrrhizin (NTX) provided significant restoration of endogenous GSH levels in the liver[14], indicative of a more normal healthy functioning liver.

A study was conducted in rats that evaluated the effects of NTX on oxidative stress induced by a single dose of alcohol[18]. The alcohol alone increased hepatic production of ROS, decreased GSH and increased MDA, all indicators of alcohol-induced toxicity. Co-administration of NTX with the alcohol significantly modulated these alcohol-induced effects indicating a hepatoprotective effect of the NTX.

Another study in rats evaluated the hepatoprotective effects of NTX against alcohol induced hepatotoxicity[19]. Rats were given ethanol daily for 28 days with and without NTX. Alcohol treatment resulted in an elevation of the serum enzymes ALT, AST, GGT and ALP, denoting hepatic toxicity. In addition, the lipid peroxidation product MDA was elevated while the endogenous antioxidant GSH was decreased. Co-administration of NTX with the alcohol resulted in 20-40 % decreases in the levels of ALT, AST, GGT and ALP, demonstrating the hepatoprotective effects of the NTX. Furthermore, significant decreases occurred in alcohol-induced lipid peroxidation (MDA) with significant increases in the antioxidant and tissue protectant GSH, demonstrating the liver protective effects of NTX. The hepatoprotective effects of NTX in this rat study were confirmed by histopathological examination that showed a marked reduction in alcohol-induced lesions with an absence of necrotic cells[19].

A study has examined the beneficial effects of a glycyrrhizin product on alcohol-induced fibrosis in rats[21]. Pathological liver fibrosis was observed when rats were treated daily with alcohol for 16 weeks. Fibrosis was assessed and demonstrated by measuring serum levels of hyaluronic acid, laminin, procollagen III and collagen type IV as well as histopathology. All indices of fibrosis were significantly decreased in rats co-administered the glycyrrhizin product, thus demonstrating that the glycyrrhizin exhibits tissue protective effects and supports normal liver function[21].

A study in rats has examined the inhibitory effects of glycyrrhizin on alcohol plus carbon tetrachloride-induced liver cirrhosis[22]. The alcohol plus carbon tetrachloride significantly increased serum ALT and the inflammatory marker NF-κB, and histologically liver steatosis and fibrosis were severe. Co-treatment with a glycyrrhizin product markedly improved the steatosis and fibrosis, significantly reduced the serum ALT levels and returned the NF-κB levels to near normal. The authors concluded that the glycyrrhizin protected the liver from hepatotoxin-induced liver injury and cirrhosis[22].

A published meta-analysis of 12 randomized controlled trials (RCTs) has summarized the effects of a glycyrrhizin product on liver function in 838 patients with alcoholic liver disease[53]. The analysis showed that glycyrrhizin significantly decreased serum ALT and AST levels, and the authors concluded that the glycyrrhizin product significantly improved liver function in alcoholic liver disease[53].

A published meta-analysis of 9 randomized controlled trials involving 687 patients with chronic hepatitis treated with diammonium glycyrrhizinate, a salt of glycyrrhizin, concluded that the diammonium glychrryizinate significantly decreased serum ALT and AST levels[68]. Furthermore, the treatment provided significant improvements in total bilirubin and albumin, thus demonstrating improved liver function.

Another meta-analysis reviewed 24 randomized control trials involving 3201 patients with chronic hepatitis who were treated with diammonium glycyrrhizinate[69]. The authors concluded that the diammonium glycyrrhizinate significantly reduced serum ALT and total bilirubin and ameliorated fibrosis, therefore supporting normal liver function.

REDUCES THE RISK OF LIVER DISEASES INDLUDING FIBROSIS AND CIRRHOSIS

Alcoholic liver disease exhibits a histopathological progression that ranges from steatosis to more advanced stages as steatohepatitis, cirrhosis, hepatocellular carcinoma and liver failure due to necrosis or apoptosis (programmed cell death)[3,4]. Common biomarkers used to assess the progression of alcoholic liver damage and disease included the measurement of serum levels of the enzymes ALT, AST, GGT and ALP[50-52]. Other biomarkers that are also used to determine the degree of tissue damage and impairment of liver function include total bilirubin, lipid profiles, hemoglobin or serum ferritin, albumin, and platelets.

Numerous human and animal studies have demonstrated that NTX and its individual components glycyrrhizin and mannitol support normal defense mechanisms and preclude alcohol liver diseases including fibrosis and cirrhosis, thereby allowing normal function and regeneration of the liver.

A randomized, double-blind, placebo-controlled cross-over clinical trial has demonstrated that NTX provides significant liver protection during 12 days of alcohol consumption as compared to receiving alcohol alone as evidenced by decreases and normalization of the hepatic enzyme biomarkers of liver toxicity ALT, AST, GGT and ALP[15]. A four week washout period was provided between the cross-over treatments.

Another randomized, double-blind, placebo-controlled cross-over clinical trial involving human 31 subjects was conducted whereby all participants received a single dose alcohol with and without NTX. A seven day washout was provided between cross-over treatments[16]. Alcohol alone resulted in an increase in serum ROS and ROS metabolites, a decrease in serum GSH, an increase in MDA, an increase in protein carbonyl, and an increase in DNA damage in peripheral lymphocytes as determined by the single cell electrophoresis comet assay, the cytokinesis-block micronucleus assay, and 8-hydroxy-2-deoxyguanosine formation. Consumption of alcohol with NTX significantly reduced the levels of ROS and all the oxidative markers and increased serum GSH. The results indicated that NTX may "be effective in reducing alcohol-induced pathophysiological changes"[16].

Glycyrrhizin, one of the two active constituents in NTX, has been shown in a number of clinical studies to significantly reduce and normalize serum ALT and AST in patients with chronic hepatitis[54-67]. The frequency of ALT normalization was dependent upon duration of glycyrrhizin administration, with the highest normalization being approximately 35 %[54].

Animal studies have demonstrated that NTX provides significant protection against alcohol-induced oxidative damage to the liver as evidenced by decreases

and normalization of the serum levels of ALT, AST, GGT and ALP[14,19]. The combination of glycyrrhizin and mannitol, the active ingredients in NTX, synergistically decreased alcohol induced production of reactive oxygen species (ROS) by enhancing the levels of the enzymes catalase, GPX and SOD that decompose tissue damaging ROS[14], thus contributing to the maintenance of normal liver function. In addition, consumption of mannitol and glycyrrhizin (NTX) prevented alcohol-induced depletion of endogenous GSH levels in the liver[14], indicative of hepatoprotection by NTX and a normal healthy functioning liver.

The ability of NTX to protect against alcohol induced hepatotoxicity was evaluated in rats[19]. Rats were given ethanol daily for 28 days with and without NTX. Alcohol treatment produced an elevation of serum enzymes ALT, AST, GGT and ALP, while the endogenous antioxidant GSH was decreased, denoting hepatic toxicity. Co-administration of NTX with the alcohol resulted in 20-40 % decreases in the levels of ALT, AST, GGT and ALP with significant increases in the intracellular antioxidant and tissue protectant GSH, demonstrating the ability of NTX to significantly decrease the hepatotoxic effects of alcohol. Histopathological examination confirmed that NTX decreased alcohol-induced damage to the liver and resulted in a lack of alcohol-induced necrotic cells[19].

A published meta-analysis has summarized the effects of a glycyrrhizin product on liver function in 838 patients with alcoholic liver disease involving 12 randomized controlled trials (RCTs)[53]. Glycyrrhizin administration significantly decreased serum ALT and AST levels, and the authors concluded that the glycyrrhizin product significantly improved liver function in alcoholic liver disease[53].

A published meta-analysis of 9 randomized controlled trials involving 687 patients with chronic hepatitis treated with diammonium glycyrrhizinate, a salt of glycyrrhizin, demonstrated that the diammonium glychrryizinate significantly decreased serum ALT and AST levels[68]. Furthermore, the treatment provided significant improvements in total bilirubin and albumin, thus demonstrating a reduction in the effects of the hepatitis and an improvement in liver function.

A meta-analysis reviewed 24 randomized control trials involving 3201 patients with chronic hepatitis who were treated with diammonium glycyrrhizinate[69]. The diammonium glycyrrhizinate significantly reduced serum ALT and total bilirubin and ameliorated fibrosis, therefore supporting normal liver function.

A study in rats has evaluated the hepato-protective effects of NTX against alcohol induced hepatotoxicity[19.]. Rats were given ethanol daily for 28 days with and without NTX. Alcohol treatment resulted in an elevation of the serum enzymes ALT, AST, GGT and ALP, denoting liver toxicity. In addition, the lipid peroxidation product MDA was significantly elevated while the endogenous antioxidant GSH was decreased as a result of the alcohol treatment. Co-administration of NTX with the alcohol resulted in 30-48 % decreases in the levels of ALT, AST, GGT and ALP, demonstrating the hepatoprotective effects of the NTX. Furthermore, a 61 % decrease occurred in lipid peroxidation (MDA) with over a 50 % increase in GSH, demonstrating the liver protective effects of NTX.

The beneficial effects of a glycyrrhizin product on alcohol-induced fibrosis in rats have been studied[21]. Pathological liver fibrosis occurred when rats were treated daily with alcohol for 16 weeks. Fibrosis was assessed and demonstrated by measuring serum levels of hyaluronic acid, laminin, procollagen III and collagen type IV as well as histopathology. All indices of fibrosis were significantly decreased in rats co-administered the glycyrrhizin product, demonstrating that glycyrrhizin exhibits tissue protective effects and supports normal liver function[21].

Glycyrrhizin has been shown to inhibit alcohol plus carbon tetrachloride-induced liver cirrhosis in rats[22]. Alcohol plus carbon tetrachloride significantly increased serum ALT and the inflammatory marker NF-κB, while histologically liver steatosis and fibrosis were observed to be severe. Co-treatment with a glycyrrhizin product markedly improved the steatosis and fibrosis, significantly reduced the serum ALT levels and returned the NF-κB levels to near normal. The authors noted that the glycyrrhizin “protects liver from hepatotoxin-induced liver injury and cirrhosis”[22].

Magnesium isoglycyrrhizinate, a magnesium salt form of glycyrrhizin, improves liver function in humans with alcoholic liver[70], disease chronic hepatitis[71,72], cirrhosis[73] as demonstrated by decreases in the serum levels of ALT, AST and total bilirubin. These results have been confirmed in rats with non-alcoholic steatohepatitis[74] and mice with acute hepatic injury[75] treated with magnesium isoglycyrrhizinate based on serum measurements of AST, ALT, MDA, lipid profiles and TNF-α as well as liver histopathology.

AMELIORATES THE SYMPTOMS ASSOCIATED WITH LIVER DISEASE INCLUDING FIBROSIS AND CIRRHOSIS

The histopathological progression of alcoholic liver disease ranges from steatosis to more advanced stages as steatohepatitis, cirrhosis, hepatocellular carcinoma and liver failure due to necrosis or apoptosis (programmed cell death)[3,4]. Biomarkers are commonly used to assess the progression of alcoholic liver damage and disease that include measurement of serum levels of the enzymes ALT, AST, GGT and ALP[50-52]. Biomarkers that are also used to determine the degree of tissue damage as hepatitis, fibrosis and cirrhosis include total bilirubin, lipid profiles, hemoglobin or serum ferritin, albumin, and platelets.

Numerous human and animal studies have demonstrated that NTX and its individual components glycyrrhizin and mannitol support normal defense mechanisms and ameliorate liver diseases including fibrosis and cirrhosis, thereby facilitating normal function and regeneration of the liver.

NTX co-administration with alcohol has been shown to provide significant decreases and normalization in the enzyme biomarkers of liver toxicity ALT, AST, GGT and ALP[15]. A randomized, double-blind, placebo-controlled cross-over clinical trial involving 12 human subjects received alcohol daily for 12 days or alcohol in combination with NTX. A four week washout cross-over period was provided. The results demonstrated that NTX suppressed alcohol toxicity and helped maintain normal liver function.

A second randomized, double-blind, placebo-controlled cross-over clinical trial involving human 31 subjects was conducted whereby all participants received 1.5 g alcohol/kg with and without NTX. A seven day washout was provided between treatments[16]. Alcohol alone caused an increase in serum ROS and ROS metabolites, a decrease in serum GSH, an increase in MDA, an increase in protein carbonyl, and an increase in DNA damage in peripheral lymphocytes as determined by the single cell electrophoresis comet assay, the cytokinesis-block micronucleus assay, and 8-hydroxy-2-deoxyguanosine formation. Consumption of alcohol in combination NTX significantly reduced the levels of ROS and all the oxidative markers. The results indicated that NTX may "be effective in reducing alcohol-induced pathophysiological changes"[16].

Glycyrrhizin, one of the two active constituents in NTX, has been shown in a number of clinical studies to significantly reduce and normalize serum ALT and AST in patients with chronic hepatitis[54-67]. The frequency of ALT normalization was dependent upon duration of glycyrrhizin administration, with the highest normalization being approximately 35 %[54].

A meta-analysis has summarized the effects of a glycyrrhizin product on liver function in 838 patients with alcoholic liver disease involving 12 randomized controlled trials (RCTs)[53]. Glycyrrhizin administration significantly decreased serum ALT and AST levels. The authors concluded that the glycyrrhizin product significantly improved liver function in alcoholic liver disease, thus ameliorating the symptoms associated with the disease[53].

Animal studies have demonstrated that NTX provides significant protection against alcohol-induced oxidative damage to the liver as evidenced by decreases in the serum levels of ALT, AST, GGT and ALP[14,19]. Oxidative liver damage ultimately leads to hepatitis, fibrosis and cirrhosis. The combination of glycyrrhizin and mannitol, the active ingredients in NTX, synergistically decreased alcohol induced production of reactive oxygen species (ROS) by enhancing the levels of the enzymes catalase, GPX and SOD that decompose tissue damaging ROS[14], thus

contributing to the maintenance of normal liver function. In addition, consumption of mannitol and glycyrrhizin (NTX) prevented alcohol-induced depletion of endogenous GSH levels in the liver[14], indicative of hepato-protection by NTX and contributing to a normal healthy functioning liver.

A study in rats evaluated the ability of NTX to protect against alcohol induced hepatotoxicity[19.]. Rats were given ethanol daily for 28 days with and without NTX. Alcohol treatment elevated serum enzymes ALT, AST, GGT and ALP, denoting liver toxicity. In addition, serum levels of the endogenous antioxidant GSH decreased. Co-administration of NTX with the alcohol resulted in 20-40 % decreases in the levels of ALT, AST, GGT and ALP with significant increases in GSH, demonstrating the ability of NTX to significantly decrease the hepatotoxic effects of alcohol. . Histopathological examination confirmed that NTX decreased alcohol-induced damage to the livers of these rats and resulted in a lack of alcohol-induced necrotic cells[19].

A published meta-analysis of 9 randomized controlled trials involving 687 patients with chronic hepatitis treated with diammonium glycyrrhizinate, a salt of glycyrrhizin, demonstrated that the diammonium glychrryizinate significantly decreased serum ALT and AST levels[68]. Furthermore, the treatment provided significant improvements in total bilirubin and albumin, thus demonstrating a significant amelioration of the hepatitis in terms of liver function.

A meta-analysis reviewed 24 randomized control trials involving 3201 patients with chronic hepatitis who were treated with diammonium glycyrrhizinate[69]. The diammonium glycyrrhizinate reduced significantly serum ALT and total bilirubin, and ameliorated fibrosis, therefore supporting normal liver function.

The beneficial effects of a glycyrrhizin product on alcohol-induced fibrosis in rats have been studied[21]. Pathological liver fibrosis occurred when rats were treated daily with alcohol for 16 weeks. Fibrosis was assessed and demonstrated by measuring serum levels of hyaluronic acid, laminin, procollagen III and collagen type IV as well as liver histopathology. All indices of fibrosis were significantly

decreased in rats co-administered the glycyrrhizin product, demonstrating that glycyrrhizin exhibits tissue protective effects and ameliorates alcohol-induced liver fibrosis[21].

Glycyrrhizin has been shown to inhibit alcohol plus carbon tetrachloride-induced liver cirrhosis in rats[22]. Alcohol plus carbon tetrachloride significantly increased serum ALT and the inflammatory marker NF-κB, while histologically liver steatosis and fibrosis were observed to be severe. Co-treatment with a glycyrrhizin product markedly ameliorated the steatosis and fibrosis, significantly reducing the serum ALT levels and returning the NF-κB levels to near normal. The authors noted that the glycyrrhizin "protects liver from hepatotoxin-induced liver injury and cirrhosis"[22].

Studies have shown that magnesium isoglycyrrhizinate, a magnesium salt form of glycyrrhizin, improves liver function in humans with alcoholic liver[70], disease chronic hepatitis[71,72], cirrhosis[73] as demonstrated by decreases in the serum levels of ALT, AST and total bilirubin. These results have been confirmed in rats with non-alcoholic steatohepatitis[74] and mice with acute hepatic injury[75] treated with magnesium isoglycyrrhizinate based on serum measurements of AST, ALT, MDA, lipid profiles and TNF-α as well as liver histopathology.

HELPS MAINTAIN NORMAL LIVER FUNCTIONS

Common biomarkers used to assess liver function and disease include the measurement of blood levels of the enzymes ALT, AST, GGT and ALP[50-52]. Other biomarkers that are used to determine the degree of tissue damage and impairment of liver function include total bilirubin, lipid profiles, hemoglobin or serum ferritin, albumin, and platelets.

Numerous human and animal studies have demonstrated that NTX and its individual components glycyrrhizin and mannitol support normal defense

mechanisms and preclude oxidative tissue damage and inflammation, thereby allowing normal function of the liver.

The co-administration of NTX with alcohol significantly decreases and normalizes the enzyme biomarkers of liver toxicity ALT, AST, GGT and ALP[15], documenting the hepatoprotective effects of the NTX. A randomized, double-blind, placebo-controlled cross-over clinical trial involving 12 human subjects received alcohol daily for 12 days with or without NTX. A four week washout cross-over period was provided between treatments. NTX decreased the biomarkers of toxicity and demonstrated that NTX suppressed alcohol toxicity and helped maintain normal liver function.

A second randomized, double-blind, placebo-controlled cross-over clinical trial involving human 31 subjects was conducted whereby all participants received a single dose of alcohol with and without NTX. A seven day washout was provided between treatments[16]. Alcohol alone increased serum ROS and ROS metabolites, decreased serum GSH, increased MDA, increased protein carbonyl, and increased DNA damage in peripheral lymphocytes. Consumption of alcohol in combination NTX significantly reduced the levels of ROS and all the oxidative markers. The results indicated that NTX may "be effective in reducing alcohol-induced pathophysiological changes"[16], helping to maintain normal liver function.

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Animal studies have demonstrated that NTX provides significant protection against alcohol-induced oxidative damage to the liver as evidenced by normalization of the serum levels of ALT, AST, GGT and ALP[14,19]. Rats were given ethanol daily for 28 days with and without NTX[19]. Alcohol treatment produced an elevation of serum enzymes ALT, AST, GGT and ALP, while the endogenous antioxidant GSH was decreased, denoting liver toxicity. Co-administration of NTX with the alcohol resulted in 20-40 % decreases in the levels of ALT, AST, GGT and ALP with 24-28 % increases in the levels of the antioxidant and tissue protectant GSH, demonstrating the ability of NTX to significantly decrease the hepatotoxic effects of alcohol. Histopathological examination confirmed that NTX decreased alcohol-induced damage to the liver and resulted in a lack of alcohol-induced necrotic cells[19].

Oxidative liver damage ultimately leads to hepatitis, fibrosis and cirrhosis. The combination of glycyrrhizin and mannitol, the active ingredients in NTX, synergistically decreased alcohol induced production of reactive oxygen species (ROS) by enhancing the levels of the enzymes catalase, GPX and SOD that decompose tissue damaging ROS[14], thus contributing to the maintenance of normal liver function. In addition, consumption of mannitol and glycyrrhizin (NTX) prevented alcohol-induced depletion of endogenous GSH levels in the liver[14], indicative of hepato-protection by NTX and contributing to a normal healthy functioning liver.

A published meta-analysis of 9 randomized controlled trials involving 687 patients with chronic hepatitis treated with diammonium glycyrrhizinate, a salt of glycyrrhizin, demonstrated that the diammonium glychrryizinate significantly decreased serum ALT and AST levels[68]. Furthermore, the treatment provided significant improvements in total bilirubin and albumin, thus demonstrating an amelioration of the symptoms of the hepatitis and an improvement in liver function.

A meta-analysis reviewed 24 randomized control trials involving 3201 patients with chronic hepatitis who were treated with diammonium glycyrrhizinate[69]. The diammonium glycyrrhizinate reduced significantly serum ALT and total bilirubin, and ameliorated fibrosis, therefore supporting normal liver function.

A licorice extract predominantly containing glycyrrhizin has been shown to effectively inhibit alcohol induced fatty liver disease as exemplified by increases increases in ALT and AST, increases in TNF-α, lipid accumulation in liver (steatosis) and decreases in the antioxidant GSH[20]. The hepatoprotective effect of the glycyrrhizin preparation was confirmed histologically.

The effects of a glycyrrhizin product on alcohol-induced liver fibrosis in rats have been studied[21]. Pathological liver fibrosis occurred when rats were treated daily with alcohol for 16 weeks. Fibrosis was assessed and demonstrated by measuring serum levels of hyaluronic acid, laminin, procollagen III and collagen type IV as well as histopathology. All indices of fibrosis were significantly decreased in rats co-administered the glycyrrhizin product, demonstrating that glycyrrhizin exhibits tissue protective effects and helps normalize liver function[21].

Glycyrrhizin has been shown to inhibit alcohol plus carbon tetrachloride-induced liver cirrhosis in rats[22]. Alcohol plus carbon tetrachloride significantly increased serum ALT and the inflammatory marker NF-κB, while histologically liver steatosis and fibrosis were observed to be severe. Co-treatment with a glycyrrhizin product markedly ameliorated the steatosis and fibrosis, significantly reducing the serum ALT levels and returning the NF-κB levels to near normal. The authors noted that the glycyrrhizin "protects liver from hepatotoxin-induced liver injury and cirrhosis"[22].

Glycyrrhizin ameliorates oxidative liver damage associated with high fructose intake in rats[38]. Glycyrrhizin administration significantly normalized levels of AST, ALT, ALP, ROS, carbonyl protein, lipid peroxidation (MDA) and apoptosis, all characteristic of oxidative liver damage. The tissue protective effects of the glycyrrhizin and normalization of liver function were affirmed histologically[38].

In cell culture experiments, glycyrrhizin has been shown to inhibit TNF-α secretion[30,31] and MDA production[32-34], while enhancing the antioxidant enzyme SOD and the antioxidant GSH[32,33,35]. These results provide supporting evidence of the ability of glycyrrhizin to promote normal liver function.

Mannitol is the second of the two primary components of NTX. Several human studies have demonstrated the antioxidant and tissue protective effects of mannitol. Mannitol has been shown to exhibit tissue protective effects in acute ischemic-reperfusion injuries[45], and antioxidant and free radical scavenging properties in conjunction with cardiopulmonary bypass[46]. Mannitol is used as an anti-inflammatory and antioxidant in tissue baths associated with kidney transplants[47].

In a study in rats, mannitol was demonstrated to provide protection against alcohol-induced gastric mucosal damage due to its antioxidant and anti-inflammatory properties[48]. Mannitol has also been shown to exhibit anti-inflammatory and antioxidant activity in rats after traumatic brain injury[49]. Mannitol decreased production of MDA and normalized the levels of the antioxidant enzymes catalase and GPx.

HELPS PROTECT DNA FROM ALCOHOL-INDUCED DAMAGE

The metabolism of alcohol in the liver results in the production of reactive oxygen species (ROS) that produce DNA damage including strand breaks and fragmentation as well as damage to other tissue components[76, 77]. ROS can also activate or repress epigenetic elements as chromatin remodeling, micro-RNAs, DNA (de)methylation and histone modification that affect gene expression, thereby leading to various liver disorders[77,78]. Furthermore, ethanol is metabolically activated to free radical species that form adducts with DNA, precluding the formation of various proteins essential for healthy liver function[79,80]. Various human, animal and in vitro studies have shown that NTX and it components glycyrrhizin and mannitol can protect against the formation of DNA damage induced by alcohol and other agents.

A randomized, double-blind, placebo-controlled cross-over clinical trial involving human 31 subjects was conducted whereby all participants received alcohol with and without NTX. A seven day cross-over washout was provided between treatments[16]. Consumption of alcohol alone caused an increase in DNA damage in peripheral lymphocytes as determined by the single cell electrophoresis comet assay, the cytokinesis-block micronucleus assay, and 8-hydroxy-2-deoxyguanosine formation. Co-consumption of alcohol with NTX significantly reduced the levels of DNA damage by protecting the DNA and preventing damage from occurring

Various studies using human and animal tissues have shown that glycyrrhizin can prevent oxidative damage to DNA. Incubation of human lymphocytes with oxidative mutagens as hydrogen peroxide prevented the formation of DNA damage by as much as 70 %[81]. Similarly, glycyrrhizin significantly decreases ROS-induced DNA damage in human epithelial cells as determined by a single cell gel electrophoresis assay[82]. Glycyrrhizin has also been shown to protect cellular DNA from radiation induced double strand breaks in human peripheral blood leukocytes and in peripheral blood leukocytes and bone marrow of mice by scavenging free radicals and reactive oxygen species[83].

In rat primary hepatocyte cultures, glycyrrhizin prevented peroxide-induced DNA fragmentation and modulated programmed cell death (apoptosis)[33]. Several studies in rats have demonstrated that glycyrrhizin ameliorates oxidative hepatic DNA damage associated with metabolic syndrome, thus demonstrating its therapeutic potential against hepatocellular damage[38,84]. In a rat study, glycyrrhizin has been referred to as a potent chemopreventive, preventing oxidative tissue damage and normalizing DNA synthesis and metabolism[85]. In mice, glycyrrhizin was shown to prevent oxidative stress-induced DNA fragmentation and micronucleus formation as well as maintaining normal tissue histology[86].

Mannitol, the other component in NTX in addition to glycyrrhizin, has also been shown to prevent DNA damage in cell culture and cell free systems. Mannitol has been demonstrated to prevent oxidative DNA damage in vitro systems involving

normal human keratinocytes[87,88]. Mannitol was an effective scavenger of ROS and an effective inhibitor of oxidative DNA damage. Mannitol in Chinese hamster ovary (CHO) cells in culture stabilizes DNA and prevents DNA single and double strand breaks[88-90]. Mannitol provided significant inhibition of copper and iron induced DNA damage in isolated rat hepatic nuclei[91]. Mannitol has also been shown to prevent DNA damage associated with various ROS generating agents in in vitro cell-free systems[92-94].

The above studies show that NTX as well as its primary components glycyrrhizin and mannitol can prevent DNA damage as DNA single and double strand breaks induced by alcohol and other ROS generating systems in liver and other tissues. The animal and in vitro studies corroborate the human studies with NTX, and provide information regarding the mechanism of action its primary components.

REDUCES ALCOHOL-INDUCED DNA DAMAGE

Hepatic metabolism of alcohol results in the production of reactive oxygen species (ROS) that induce DNA damage including strand breaks and fragmentation as well as damage to other tissue components[76, 77]. ROS can also modulate epigenetic elements as chromatin remodeling, micro-RNAs, DNA (de)methylation and histone modification that affect gene expression, thereby leading to various liver disorders[77,78]. Ethanol is also metabolically activated to free radical species that form adducts with DNA, thereby inhibiting DNA and normal healthy liver function[79,80]. Human, animal and in vitro studies have shown that NTX and it components glycyrrhizin and mannitol can reduce the formation of DNA damage induced by alcohol and other ROS-generating agents, thus affording tissue and organ protection.

A randomized, double-blind, placebo-controlled cross-over clinical trial involving human 31 subjects was conducted whereby all participants received alcohol with and without NTX. A seven day cross-over washout was provided between treatments[16]. Consumption of alcohol alone caused an increase in DNA damage in peripheral lymphocytes as determined by the single cell electrophoresis comet

assay, the cytokinesis-block micronucleus assay, and 8-hydroxy-2-deoxyguanosine formation. Consumption of alcohol with NTX significantly reduced the levels of DNA damage.

Human and animal tissue studies have shown that glycyrrhizin, a primary component in NTX, can reduce oxidative damage to DNA. Incubation of human lymphocytes with oxidative mutagens as hydrogen peroxide reduced ROS formation of DNA damage by as much as 70 %[81]. In another study, glycyrrhizin significantly reduced ROS-induced DNA damage in human epithelial cells as determined by a single cell gel electrophoresis assay[82]. Glycyrrhizin reduces cellular DNA from radiation induced double strand breaks by scavenging free radicals and reactive oxygen species[83]. This ability of glycyrrhizin was demonstrated in peripheral blood leukocytes derived from humans and mice as well as bone marrow cells of mice[83].

In rat primary hepatocyte cultures, glycyrrhizin reduced peroxide (ROS)-induced DNA fragmentation and programmed cell death (apoptosis)[33]. Studies in rats have demonstrated that glycyrrhizin reduces ROS-induced hepatic DNA damage associated with metabolic syndrome, demonstrating its therapeutic potential against hepatocellular damage[38,84]. Glycyrrhizin also accelerates liver regeneration following partial hepatectomy and decreases elevated serum levels of ALT and AST, thus demonstrating ability of glycyrrhizin to enhance regeneration of liver mass and function[95]. One mechanism whereby glycyrrhizin reduces and inhibits oxidative DNA damage in tissues may be by the direct binding of glycyrrhizin to the major and minor grooves and phosphate backbone of DNA as has been shown in an in vitro model[96].

Mannitol, the other component in NTX in addition to glycyrrhizin, has also been shown to prevent DNA damage in cell culture and cell free systems. Mannitol has been demonstrated to reduce oxidative DNA damage in vitro systems involving normal human keratinocytes[87,88]. Mannitol was an effective scavenger of ROS and an effective inhibitor of oxidative DNA damage. Mannitol in Chinese hamster ovary (CHO) cells in culture stabilizes DNA and reduces DNA single and double

strand breaks[88-90]. Mannitol provided significant inhibition of DNA damage associated with copper and iron initiated oxidative stress in isolated rat hepatic nuclei[91]. Mannitol has also been shown to reduce DNA damage associated with various ROS generating agents in in vitro cell-free systems[92-94].

In summary, NTX and its primary components glycyrrhizin and mannitol can reduce DNA damage as DNA single and double strand breaks induced by alcohol and other ROS generating systems in liver and other tissues. In addition, a study in rats has demonstrated the ability of glycyrrhizin to restore function and enhance regeneration of the liver, a benefit that can be readily applied to chronic alcohol consumption and alcoholic liver disease.

SAFETY

Various studies have assessed the safety of NTX and its constituents, glycyrrhizin and mannitol. A randomized, double-blind, placebo-controlled cross-over clinical trial involving 12 human subjects received alcohol daily for 12 days or alcohol in combination with NTX. A four week washout cross-over period was provided. NTX co-administration with alcohol has been shown to provide significant decreases and normalization in the enzyme biomarkers of liver toxicity ALT, AST, GGT and ALP[15]. NTX had no effect on heart rate, blood pressures or respiratory rate, and no adverse effects were reported in these human subjects[15]. The results demonstrated that NTX suppressed alcohol toxicity while exhibiting no adverse effects under the experimental conditions employed in humans.

A 28 day NTX detailed safety study was conducted in rats[19]. Rats were orally administered 0, 0.5 gm/kg, 0.75 gm/kg, and 1.0 gm/kg NTX daily for 28 days. The rats were observed daily for visible signs of reaction to the NTX such as eye and mucus membrane changes, respiration, central nervous system, circulatory, somatomotor activity and behavioral patterns. No untoward changes were observed. Furthermore, no changes were observed with respect to body weights or food consumption.

Clinical pathology assessments were conducted on blood samples drawn prior to treatment at two weeks of treatment and at 28 days of treatment[19]. As compared to the untreated (vehicle only) controls, at no time points did NTX treatment at any of the three doses result in significant changes with respect to leukocyte count, erythrocyte count, lymphocyte count, monocyte count, eosinophil count, basophil count, hemoglobin, differential count, blood clotting time, packed cell volume, serum sodium and potassium, plasma AST, ALT, ALP, blood urea nitrogen (BUN), creatinine or total protein. Microscopic examination of the urine sediment of control and NTX treated groups did not reveal the presence of any epithelial casts, bacterial cells, erythrocytes or any other abnormalities at any of the time points over the 28 days of treatment[19].

Upon post-mortem examination at the end of the 28 days of the study, the NTX treated animals did not reveal any significant lesions with respect to vital organs including livers, lungs, hearts, kidneys, spleens and urinary bladders. In summary, the results of this detailed safety study in rats clearly demonstrate the safety of NTX over a range of doses when given daily for up to 28 days[19]. No adverse effects of any kind were observed.

The safety of glycyrrhizin and various salts and derivatives thereof has been extensively reviewed[97]. The oral LD50 for glycyrrhetinic acid, the aglycone of glycyrrhizin, was 610 mg/kg or higher in rats and mice. Based on the differences in molecular weights, an equivalent amount of glycyrrhizin would be over 1.1 g/kg in rats which would be over 14.2 grams for an 80 kg human. For the sake of comparison, the LD50 for caffeine is believed to be in the range of about 10 grams in humans. In an 8 week study involving 39 healthy volunteers, the no effect level was conservatively determined to be 2 mg/kg/day or 160 mg for an 80 kg individual. Glycyrrhizin was shown to be non-phototoxic, Glycyrrhizate salts were non-carcinogenic in mice, and exhibited no reproductive or developmental toxicities in rats, mice, hamsters or rabbits[97].This detailed report concluded that glycyrrhizin and its salts exhibited little acute, short-term, subchronic or chronic toxicity[97].

Ingestion of glycyrrhizin at high doses can have physiological effects including sodium and water retention, potassium loss, weight gain and increased blood pressure. However, studies have been conducted where subjects have been given 300-700 mg glycyrrhizin per week for up to 16 years without significant adverse effects[54,98]. Under the conditions of use of NTX, the consumption of glycyrrhizin would be below the levels required to produce these adverse events.

A review has summarized the use of glycyrrhizin in the treatment of liver diseases[99]. Ten clinical studies are reviewed using doses up to 200 mg per day, primarily given intravenously. Six of the 10 studies reported no adverse effects, including a study that averaged approximately 10 years. Hypertension with skin rash was reported in one study that lasted for up to 14.5 years and involved daily intravenous injections which results in much higher blood and tissue levels than achieved by oral administration due to the gastro-intestinal metabolism of glycyrrhizin. Two studies reported headache and three studies reported an increase in blood pressure[99], which cannot be correlated with similar doses given orally.

Numerous plants and fungi produce mannitol, and as a consequence, mannitol is a common sugar alcohol present in the diet of human[100]. The subchronic oral toxicity of mannitol was studied in rats that were given approximately 12 grams of mannitol per kg per day for 90 days[101]. This dose would be equivalent to an 80 kg individual consuming about 155 grams of mannitol per day. The authors concluded that mannitol did not produce signs of toxicity.

The patent for NTX notes that glycyrrhizin is used at a concentration of 0.05-0.3 % while mannitol is used in the range of 0.5-3.0 %[14]. For someone consuming 60 ml of an alcoholic beverage (4 shots) containing NTX, they would consume 30-180 mg of glycyrrhizin and 300 mg-1.80 grams of mannitol, amounts that are clearly within the safety ranges for both ingredients.

CONCLUSIONS

The adverse health effects and societal costs of chronic and heavy alcohol consumption are well documented, and alcohol is a risk factor or cause of numerous diseases with alcoholic liver disease being the most common problem associated with chronic alcohol consumption. As a consequence, there is a pressing need to develop strategies for safer alcohol consumption and treatment in order to reduce alcohol-associated morbidity and mortality, and the high cost to society.

NTX is a patented product composed of the primary ingredients glycyrrhizin and mannitol that act synergistically. Based on the totality of the scientific information and evidence, NTX and its active ingredients glycyrrhizin and mannitol are safe and exhibit beneficial properties with respect to the potentially detrimental effects of alcohol. Therefore, health claims regarding NTX have been set forth, and each health claim is substantiated and extensively supported by the scientific literature.

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89. Hall AH Jr, Earnes RZ, Waymack PP Jr, Patterson RM. 1988. Acute effects of a superoxide radical-generating system on DNA double-strand stability in Chinese

hamster ovary cells. Determination by a modified fluorometric procedure. Mutat Res 198: 161-168.
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91. Sahu SC, Washington MC. 1991. Effects of antioxidants on quercetin-induced nuclear DNA damage and lipid peroxidation. Cancer let 60: 259-264.
92. Kim NH, Kang JH. 2006. Oxidative damage to DNA induced by the cytochrome C and hydrogen peroxide system. J Biochem Mol Biol 39: 452-456.
93. Sarker AH, Watanabe S, Seki S, Akiyama T, Okada S. 1995. Oxygen radical-induced single-strand DNA breaks and repair of the damage in a cell-free system. Mutat Res 337: 85-95.
94. Tsou TC, Lai HU, Yang JL. 1999. Effects of mannitol or catalase on the generation of reactive oxygen species leading to DNA damage by chromium (VI) reduction with ascorbate. Chem Res Toxicol 12: 1002-1009.
95. Kimura M, Moro T, Motegi H, Maruyama H, Sekine M, Okamoto H, Inoue H, Sato T, Ogihara M. 2008. In vivo glycyrrhizin accelerates liver regeneration and rapidly lowers serum transaminase activities in 70 % partially hepatectomized rats. Eur J Pharmacol 579: 357-364.
96. Nafisi S, bonsai M, Manouchahri F, Abdi K. 2012. Interaction of glycyrrhizin and glycyrrhetinic acid with DNA. DNA Cell Biol 31: 114-121.
97. Cosmetic Ingredient Review expert Panel. 2007. Final report on the safety assessment of glycyhrretinic acid, potassium glycyrrhetinate, disodium succinoyl glycyrrhetinate, glyceryl glycyrrhetinate, glycyrrhetinyl stearate, stearyl glycyrrhetinate, glycyrrhizic acid, ammonium glycyrrhizate, dipotassium glycyrrhetinate, disodium glycyrrhizate, trisodium glycyrrhizate, methyl glycyrrhizate, and potassium glycyrrhizate. Int J Toxicol 26: Suppl. 2: 79-112.
98. Ikeda K, Arase Y, Kobayashi M, Saitoh S, Someya T, Hosaka T, Sezaki H, Akuta N, Suzuki Y, Suzuki F, Kumada H. 2006. A long-term glycyrrhizin injection therapy reduces hepatocellular carcinogenesis rate in [patients with interferon-resistant active chronic hepatitis C: a cohort study of 1249 patients. Dig Dis Sci 51: 603-609.
99. Li JY, Cao HY, Liu P, Cheng CGH, Sun MY. 2014. Glycyrrhizic acid in the treatment of liver diseases: Literature review. BioMed Res Int 2014: Article ID 872139. doi: 10.1155/2014/872139.
100. Meena M, Prasad V, Zehra A, Gupta VK, Upadhyay RS. 2015. Mannitol metabolism during pathogenic fungal-host interaction under stressed conditions. Front Microbiol 6: 1019. doi: 10.3389/fmicb.2015.01019.

101. Til HP, Kuper CF, Falke HE Bar A. 1996. Subchronic oral toxicity studies with erythritol in mice and rats. Regul toxicol Pharmacol 24: S221-231.

04-09-16

EXHIBIT 2

CURRICULUM VITAE

S.J. STOHS, Ph.D.

Birth: Ludell, Kansas USA

EDUCATIONAL BACKGROUND:

Secondary School	- Scribner Public High School, Graduated 1957
B.S. in Pharmacy	- The University of Nebraska, Lincoln, 1962
M.S. in Natural Products and Biochemistry	- The University of Nebraska, Lincoln, 1964
Ph.D. in Biochemistry	- The University of Wisconsin, Madison, 1967
Post-Graduate Study	- Karolinska Institute, Stockholm, Sweden, 1975-76
	- National Institute of Environmental Health Sciences Research Triangle Park, NC 1987-88

PHARMACY LICENSE:

Nebraska

ACADEMIC APPOINTMENTS:

Assistant Professor, Department of Pharmacognosy, University of Nebraska, September 1967.

Assistant Professor and Acting Chairman, Department of Pharmacognosy, University of Nebraska, September 1968.

Member of the Graduate Faculty, University of Nebraska, June 1969.

Chairman, Department of Pharmacognosy, University of Nebraska, September 1970.

Associate Professor and Chairman (tenured), Department of Pharmacognosy, University of Nebraska, September 1971.

Associate Professor and Chairman, Department of Medicinal Chemistry and Pharmacognosy, University of Nebraska, September 1972.

Professor and Chairman, Department of Medicinal Chemistry and Pharmacognosy, University of Nebraska Medical Center, July 1974.

Leave of Absence from the University of Nebraska, and Visiting Research Associate, Department of Forensic Medicine, Karolinska Institute, Stockholm, Sweden, 1975 - 76.

Professor and Chairman, Department of Biomedicinal Chemistry, University of Nebraska Medical Center, August 1977.

Professor, Eppley Institute, February 1982.

Assistant Dean for Graduate Studies and Research, College of Pharmacy, Professor, Pharmaceutical Sciences and Eppley Institute, University of Nebraska Medical Center, November 1985.

Professor, Pharmaceutical Sciences and Eppley Institute, University of Nebraska Medical Center, July 1987 - January 1989.

Faculty Development Leave from University of Nebraska, and Visiting Professor, Systemic Toxicology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, September 1987 through June, 1988.

Assistant Dean for Research and Professor, School of Pharmacy and Allied Health Professions, and Professor of Pharmacology, School of Medicine, Creighton University, January, 1989.

Acting Dean, School of Pharmacy and Allied Health Professions, Creighton University, October, 1990.

Dean, School of Pharmacy and Health Professions, Creighton University, July, 1991 - June, 2003.

Dean Emeritus, School of Pharmacy and Health Professions, Creighton University, July, 2003.

INDUSTRIAL APPOINTMENTS:

Senior Vice President of Research and Development, AdvoCare International, Carrollton, TX, October 2003 – 2009(retired). Responsible for development of over 25 new products and reformulation of over 40 products. New product development resulted in submitting 3 patent applications with one patent awarded and two pending.. Also had overall responsibility for product quality control/quality assurance, adverse events reporting, structure/function claims submitted to FDA, product training, clinical pilot studies on new and reformulated products, contract research studies regarding AdvoCare products, and audits of manufacturing facilities. In addition, interfaced with all other departments/areas, assisted in negotiation of product and raw material prices, and assisted in resolution of products/supply chain issues. Answered numerous distributor-related questions regarding ingredients, products and product applications.

Vice President for Scientific Affairs, AdvoCare International May 2014—January 1, 2015.

Kitsto Consulting LLC, Frisco, TX, January 2009 to present. Founding partner in consulting company specializing in toxicology, nutrition and wellness, research design and organizational analysis.

ADMINISTRATIVE EXPERIENCE:

Vice for Scientific Affairs, ADVOCARE International, May 2014-January 1, 2015.

Sr. Vice President of Research & Development, ADVOCARE International, 2003-2009(retired).

Responsible for the development of a research and development program, including the hiring of staff, and establishing operational policies and procedures. Overall responsibility for product development, product review and reformulation, quality assurance/quality control, regulatory affairs, adverse events,

documentation and records and good manufacturing practices for manufacturing facilities. Established research protocols for selected ADVOCARE products and identified research facilities. Member of supply chain team and product launch team. Extensive interface with distributors through product training presentations, email, AdvoLink and face-to-face interactions. Responsible for product training and participated in over 25 Success Schools since 1998.

Dean, School of Pharmacy and Allied Health Professions, Creighton University, 1991 –2003.

Program had 23 faculty members and six staff when position was assumed. In 2003, the School has over 100 faculty and approximately 45 staff. Developed the Doctor of Pharmacy degree as the sole professional degree. Initiated distance-learning Doctor of Pharmacy pathway for practitioners. Initiated the first entry-level Doctor of Physical Therapy and Doctor of Occupational Therapy programs in the country. Developed a post-professional Doctor of Occupational Therapy pathway. Initiated distance-learning pathways for the Doctor of Occupational Therapy and the Doctor of Physical Therapy for practitioners. Initiated a M.S. in Pharmaceutical Sciences. Initiated a Master of Health Services Administration program. Developed the nation's first web-based entry-level Doctor of Pharmacy pathway. Developed a B.S. program in Health Sciences for students in the Doctors of Pharmacy, Physical Therapy and Occupational Therapy Programs.

Acting Dean, School of Pharmacy and Allied Health Professions, Creighton University, 1990 – 1991.

Assistant Dean for Research, Creighton University, 1989 - 1990.

Assistant Dean for Research and Development, University of Nebraska Medical Center, 1986 - 1987.

Assistant Dean for Research and Graduate Education, University of Nebraska Medical Center, 1985 - 1986.

Chaired various committees at the campus, state and national levels including: Departmental Graduate Committee; College Policy and Procedures Committee; Pharmaceutical Sciences Area Graduate Committee; State Commission on Drugs, Education Subcommittee; UNMC Marshalling Corps; Faculty Senate Grievance Committee; AACP Biological Sciences Resolutions Committee; Academy of Pharmaceutical Sciences Kilmer Prize Committee; Program Committee, American Society of Pharmacognosy; Biological Sciences Section of AACP; AACP Biological Sciences Nominating Committee; Special Projects Grants Committee of AACP; University of Nebraska Chapter of Sigma Xi; and University wide Strategic Planning Subcommittee, Search Committees for Deans of School of Dentistry, School of Law and College of Business Administration.

Directed the research of over 40 graduate students, and the research and budgets

of over 60 grants and contracts.

Chairman of Department, 1968-85. Responsible for the budgets and programs of the Department of Biomedicinal Chemistry. Responsible for promotion and tenure recommendations, departmental budget, recommendations for faculty salaries and teaching assignments. The Department had 9 tenure track faculty (3 through Eppley), 4 additional adjunct faculty, 18 graduate students, one full-time secretary and additional secretarial assistance.

SOCIETY MEMBERSHIPS:

Sigma Xi, Rho Chi Pharmacy Honor Society, American Association of Colleges of Pharmacy, Drug Metabolism Group of the American Society for Pharmacology and Experimental Therapeutics, International Union of Pharmacology Section on Toxicology, International Society for the Study of Xenobiotics, Society of Toxicology, International Association of Biomedical Gerontology, Phi Lambda Sigma Leadership Honor Society, Phi Beta Delta Honor Society for International Scholars, American Pharmaceutical Association, Association of Schools of Allied Health Professions, American College of Nutrition, American Nutraceutical Association, Society of Free Radical Biology and Medicine, International Federation of Sports Medicine.

AWARDS AND HONORS:

B.S. in Pharmacy with Distinction, 1962; NIH Predoctoral Fellowship, 1962-64; NIH Trainee Fellowship, 1964-67; elected Sigma Xi, 1966; selected to participate in the American Association of Colleges of Pharmacy Visiting Lecturer's Program, 1972-73; major professor of the Outstanding Graduate Student at the University of Nebraska for 1973-74 (Ronald Talcott), for 1976-77 (Lester Reinke), and for 1979-80 (Walid Al-Turk) selected by Sigma Xi chapter; selected as an Outstanding Educator of American, 1975; listed in American Men and Women of Science, 1968-present; elected to membership into the American Society for Pharmacology and Experimental Therapeutics, 1976; selected to Notable Americans of 1976-77; Sigma Xi Outstanding Scientist Award for 1977-78 at University of Nebraska; University of Nebraska Medical Center Outstanding Teacher Award, 1981; President, University of Nebraska Chapter of Sigma Xi, 1984-85; Visiting Professor, Tanta University, Tanta, Egypt, April 1985; Alumni Faculty Award for Outstanding Research, 1985; 1985 APhA Foundation/APhA Academy of Pharmaceutical Sciences Research Achievement Award in Pharmacodynamics; 1988 Burlington Northern Faculty Achievement Award, University of Nebraska; American Association of Colleges of Pharmacy Teacher of the Year Award, 1987-88; Native Son Award, Scribner, NE, 1992; Phi Beta Delta, Honor Society for International Scholars, 1992; Phi Lambda Sigma Excellence in Leadership Award, Creighton University, 1994-95; Creighton University Black Employee Network Service Award, 1995; Nebraska Pharmacists Association Cora Mae Briggs Outstanding Service to Nebraska Pharmacy Award, 1996; Elected into Fellowship in the Academy of Toxicological Sciences (Fellow ATS), 1998 (recertified in 2003);

Honorary Member of the Turkish Clinical Pharmacy Society, 1998; Elected into Fellowship in the American College of Nutrition (Fellow ACN), 1999; Elected into Fellowship in the Association of Schools of Allied Health Professions (Fellow ASAHP, 1999); Holder, Gilbert F. Taffe, Jr. Endowed Chair, 2000-2003; National Multiple Sclerosis Corporate Achievers Award for Outstanding Leadership and Community Involvement, 2000; John Doull Award in Toxicology, Central States Chapter, Society of Toxicology, 2001; Certified Nutrition Specialist (CNS) by the Certification Board of the American College of Nutrition, 2002; Dean Emeritus, School of Pharmacy and Health Professions, Creighton University, 2003; American College of Nutrition Industrial Recognition Award, 2007; University of Nebraska Medical Center 2010 Distinguished Pharmacy Alumni Award; Fellowship in Americal Pharmacists Association (Fellow APhA, 2014).Excellence in Nutrition Science Award, Emord & Associates, 2014; Excellence in Integrative Medicine Award, Emord & Associates, 2014; Pharmacy Education Recognition Award, Fourth International Meeting of Pharmacy and Pharmaceutical Sciences, Istanbul, turkey, 2014.

DISSERTATIONS AND THESES DIRECTED:

1. Incorporation of 14C-Acetate into Khellin and Visnagin in *Ammi visnaga* Plants and Suspension Cultures. 1969. Min-Hsiu Chen, M.S.

2. The Production of Diosgenin and the Metabolism of 14C-Cholesterol by *Dioscorea deltoidea* Wall Tissue Cultures. 1969. Balkrishena Kaul, Ph.D.

3. The In Vitro Metabolism of ^{3}H-Digoxigenin. 1972. Ronald E. Talcott, M.S.

4. Studies of pH Effects on the Therapy of Chronic Urinary Tract Infections In Vivo. 1973. Victor A. Padron, M.S.

5. Phytochemical Studies on Tissue Cultures of *Withania somnifera*. 1973. Poli Chen Yu, M.S.

6. Some Aspects of Cardenolide Metabolism with Particular Reference to the Hepatic Microsomal NADPH-Dependent Mixed-Function Oxidase System. 1973. Ronald E. Talcott, Ph.D.

7. A Phytochemical Investigation of *Corchorus olitorius* and *C. capsularis* Tissue Cultures. 1974. Cindy Tarng, M.S.

8. The In Vitro and In Vivo Metabolism of H^3-Digitoxin and H^3-Digitoxigenin in the Rabbit and Some of the Related Aspects. 1974. William H. Bulger, Ph.D.

9. The Influence of Selected Sedative-Hypnotics on Mixed-Function Oxidases. 1975. Lester A. Reinke, M.S.

10. The Microencapsulation and Properties of Selected Drugs. 1975. Kedar Naik, Ph.D.

11. A Rapid In Vivo Preliminary Screening Technique in Mice for Antiarrhythmic Drugs. 1976. Peter Nwangwu, M.S.

12. Some Affects of Streptozotocin-Induced Diabetes on Hepatic Mixed-Function Monooxygenase Enzymes in Rats. 1977. Lester A. Reinke, Ph.D.

13. Hepatic Glutathione Levels in Diabetic Rats. 1977. Jeanne M. Hassing, M.S.

14. An Evaluation of Several Methods for the Determination of Catecholamines in Plasma. 1978. Glen A. Scratchley, M.S.

15. In Vitro Production of Estrone and Estriol from Selected Steroid Precursors in the Rabbit. 1978. Victor A. Padron, Ph.D.

16. Effects of Lithium Chloride on Aconitine-Induced Arrthythmias, Atrial and Ventricular Tachycardias, and on the Distribution of Selected Electrolytes and ATPases in the Mouse Heart. 1978. Oladipo Oredipe, M.S.

17. The Antiarrythmic Activities, Acute Toxicity Profile and Hemodynamic Properties of Some New and Selected Quinidine Analogs. 1979. Peter U. Nwangwu, Ph.D.

18. Some Aspects of Sex Dependent Regulation of Drug Metabolizing Enzymes in Hepatic and Extrahepatic Tissues. 1979. Walid A. Al-Turk, Ph.D.

19. The Effects of Chronic Inorganic Lead Poisoning on Erythrocyte 5'-Nucleotides in the Rabbit. 1979. Mark S. Swanson, M.S.

20. The Effects of Various Xenobiotics on Aryl Hydrocarbon Hydroxylase Activity of Intestinal and Hepatic Microsomes from Male Rats. 1981. Lin-Jau Wu, M.S.

21. The Synthesis and Preliminary in vitro Biological Evaluation of 5,8 Endoethano-3-hydroxy-N-methylmorphinan and Its Immediate Precursors. 1981. Victoria F. Roche, Ph.D.

22. Studies on the Effect of Intragastrically Administered 3-Methylcholanthrene on Intestinal Mixed-Function Oxidase Activity and Protein Synthesis. 1982. Jeanne Marie Hassing, Ph.D.

23. Effect of Diphenylhydantoin on Hepatic and Extrahepatic Mixed Function Oxidases in the Female Rat. 1982. Ronald J. Heinicke, M.S.

24. Changes in Glutathione and Glutathione Metabolizing Enzymes in Erythrocytes and Lymphocytes of Humans and Mice as a Function of Age. 1982. Fatma H. El-Rashidy, M.S.

25. Studies on Polyamine Decarboxylases, DNA Synthesis and Ultrastructural Alterations in 17-Estradiol-Stimulated Rat Target Organs and Estriol Antagonism. 1982. Lynn A. Lavia, Ph.D.

26. Percutaneous Absorption of Iodochlorhydroxyquin. 1983. Fikrat Wahbi Ezzedeen, Ph.D.

27. Some Biochemical Characteristics of Pyrimidine 5'-Nucleotidase Deficient Erythrocytes. 1983. Mark S. Swanson, Ph.D.

28. A Possible Role of Lipid Peroxidation in TCDD Toxicity. 1984. Mohammad Q. Hassan, Ph.D.

29. Biochemical and Toxicological Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin Congeners in Female Rats. 1985. Mike A. Shara, M.S.

30. Lipid Peroxidation, Calcium, Iron and TCDD Toxicity in Rats. 1985. Zainab A.F. Al-Bayati, Ph.D.

31. Biochemical and Functional Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) on the Hearts of Female Rats. 1987. S.J. Hermansky, M.S.

32. Lipid Peroxidation Inducibility of Carbon Tetrachloride, Hexachlorocyclohexane, Dieldrin, Hexafluorobenzene and Hexachlorobenzene in Female Rats. 1987. M.R. Goel, M.S.

33. The Role of Divalent Cations in TCDD Induced Lipid Peroxidation and DNA Damage in Rats. 1988. Z.Z. Wahba, Ph.D.

34. Effects of TCDD on Lipid Peroxidation and Membrane Fluidity in the Mitochondria and Plasma Membranes of Rat Liver. 1988. N. Alsharif, M.S.

35. Effects of the Blue-Green Algal Hepatotoxin Microcystin-LR in Female Mice. 1990. S.J. Hermansky, Ph.D.

36. Evidence of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-Induced Oxidative Stress in Female Rats. 1990. M.A. Shara, Ph.D.

37. TCDD, Phagocytic Cells and Oxidative Stress. 1992. N.Z. Alsharif, Ph.D.

38. Mechanism of Ricin-Induced Oxidative Stress in Mice. 1995. Daniel F. Muldoon, Ph.D.

OTHER TRAINING ACTIVITIES:

Professional and undergraduate students who have received research training -
Naser Alsharif
Pat Casey
Phil Dickson
Julie Dopheide
Joseph Guzzo
Jeanette Haggerty
Joseph Hanna-Salem
Jeanne M. Hassing
Julia Kelly
Brian Knobel
Craig Kubitcek
Lon Lowry
Hamid Mohammadpour
Jeff Moser
Peter Nwangwu
John Obrist
Zeke Paster
Lester A. Reinke
Julie Sabatka
Sohel Sachek
Alan Smalljohn
Jorja Sturek
Philip Vuchetich
Christine Wagner
Bruce Weber
Vikki Miller
Kate Takacs

Post-doctoral fellows -
Dr. Paul Akubue
Dr. Hanan Al-Kaysi
Dr. Walid Al-Turk
Dr. Albert Awad
Dr. Debasis Bagchi
Dr. Manashi Bagchi
Dr. Laura Cook
Dr. Ezdihar Hassoun
Dr. Frank Luman
Dr. Rodney Markin
Dr. Zuhair Muhi-Eldeen
Dr. Gita Mukherjee

PATENTS:

U.S. patent 6,291,517 issued September 18, 2001 to D. Bagchi, M. Bagchi and S. J. Stohs on a “Method for preventing or reducing stress-induced gastric injury using grape seed proanthocyanidin extract.”

U.S. patent 7,645,742 issued January 12, 2010 to S. J. Stohs on “Composition for enhancing cellular energy” (Muscle fuel-AdvoCare International).

U.S. patent 7,989,009 issued August 2, 2011 to C. Kandaswami, S. J. Stohs and T. Coyle on “Composition and method for promoting weight loss”.

U.S. Patent 8.975,236 B2 issued March 10, 2015 to S. J. Stohs and H. Miller on “Dietary supplement composition of citrus derivatives”.

RESEARCH PUBLICATIONS (ABSTRACTS NOT INCLUDED):

1. Stohs, S.J. and Staba, E.J. Production of Cardiac Glycosides by Plant Tissue Cultures. IV. Biotransformation of Digitoxigenin and Related Substances. J. Pharm. Sci. 54, 56-58 (1965).

2. Stohs, S.J., Zull, J.E. and DeLuca, H.F. Vitamin D Stimulation of H^3-Orotic Acid Incorporation into Ribonucleic Acid of Rat Intestinal Mucosa. Biochemistry 6, 1304-1310 (1967).

3. Stohs, S.J. and DeLuca, H.F. Subcellular Location of Vitamin D and Its Metabolites in Intestinal Mucosa after a 10 IU Dose. Biochemistry 6, 3338-3349 (1967).

4. Stohs, S.J. The Metabolism of Progesterone by Plant Microsomes. Phytochemistry 8, 1215-1219 (1969).

5. Stohs, S.J. The Production of Scopolamine and Hyoscyamine by Datura stramonium L. Suspension Cultures. J. Pharm. Sci. 58, 703-705 (1969).

6. Chen, M., Stohs, S.J. and Staba, E.G. The Biosynthesis of Radioactive Khellin and Visnagin from ^{14}C-Acetate by Ammi visnaga Plants. Planta Medica 12, 319-327 (1969).

7. Stohs, S.J., Kaul, B. and Staba, E.J. Dioscorea Tissue Cultures II. The Metabolism of ^{14}C-Cholesterol by D. Deltoidea Suspension Cultures. Phytochemistry 8, 1679-1686 (1969).

8. Kaul, B., Stohs, S.J. and Staba, E.J. Dioscorea Tissue Cultures III. Influence of Various Factors on Diosgenin Production by Dioscorea deltoidea Callus and Suspension Cultures. Lloydia 32, 347-359 (1969).

9. Chen, M., Stohs, S.J. and Staba, E.J. The Biosynthesis of Visnagin from 2-^{14}C-Acetate by Ammi visnaga and A. majus. Lloydia 32, 347-359 (1969).

10. Stohs, S.J., Reinke, L.A. and El-Olemy, M.M. Metabolism in vitro of Digitoxigenin by Rat Liver Homogenates. Biochem. Pharmacol. 20, 437-446 (1971).

11. Stohs, S.J. and El-Olemy, M.M. Transformation of Cholesterol by Cheiranthus cheiri Leaf and Tissue Culture Homogenates. J. Steroid Biochem. 2, 293-298 (1971).

12. Stohs, S.J. and El-Olemy, M.M. 4-ß-Sitosten-3-one from ß-Sitosterol by Leaf Homogenates. Phytochemistry 10, 2987-2990 (1971).

13. Stohs, S.J. and El-Olemy, M.M. Pregnenolone and Progesterone from 20 -Hydroxycholesterol by Cheiranthus cheiri Leaf Homogenates. Phytochemistry 10, 3053-3056 (1971).

14. El-Olemy, M.M., Stohs, S.J. and Schwarting, A.E. Heimidine, A New Alkaloid from Heimia salicifolia. Lloydia 34, 439-441 (1971).

15. Talcott, R.E. and Stohs, S.J. Metabolism of Aniline and Hexobarbital by Liver Homogenates of Spironolactone-pretreated Male Rats. J. Pharm. Sci. 61, 296-297 (1972).

16. Stohs, S.J. and El-Olemy, M.M. Biotransformation of Progesterone by Dioscorea deltoidea Suspension Cultures. Phytochemistry 11, 1397-1400 (1972).

17. Stohs, S.J. and El-Olemy, M.M. 4-Androsten-3,17-dione Metabolism by Dioscorea deltoidea Suspension Cultures. Lloydia 35, 81-83 (1972).

18. Stohs, S.J. and El-Olemy, M.M. Progesterone and Pregnenolone Metabolism by Leaf Homogenates of Cardenolide Producing Plants. Phytochemistry 11, 2409-2413 (1972).

19. Talcott, R.E., Stohs, S.J. and El-Olemy, M.M. Metabolites and Some Characteristics of the Metabolism of 3H-Digoxigenin by Rat Liver Homogenates. Biochem. Pharmacol. 21, 2001-2006 (1972).

20. Bulger, W.H., Talcott, R.E. and Stohs, S.J. Separation of Hydroxylation Products of Digitoxigenin by TLC. J. Chromatog. 70, 187-189 (1972).

21. Talcott, R.E. and Stohs, S.J. The Effect of Cyproterone Acetate Pretreatment on the In Vitro Metabolism of Aniline, Hexobarbital, and ^{3}H-Digitoxigenin. J. Pharmacol. Exptl. Therap. 184, 419-423 (1973).

22. Talcott, R.E., Bulger, W.H. and Stohs, S.J. Metabolism of ^{3}H-Digitoxigenin by Rat Liver, Adrenal and Ovary Homogenates. Steroids 21, 87-94 (1973).

23. Bulger, W.H. and Stohs, S.J. 5ß-Hydroxydigitoxigenin: A Metabolite of Digitoxigenin by Rabbit Liver Homogenates. Biochem. Pharmacol. 22, 1745-1950 (1973).

24. Talcott, R.E. and Stohs, S.J. Effect of Phenobarbital and Spironolactone Pretreatment on Digitoxin Induced Mortality in Male and Female Rats. Arch. Int. Pharm. Therap. 204, 86-88 (1973).

25. Stohs, S.J. and Haggerty, J.S. Metabolism of 4-Cholesten-3-One to 5 -Cholestan-3-one by Leaf Homogenates. Phytochemistry 12, 2869-2872 (1973).

26. Talcott, R.E. and Stohs, S.J. Enzyme Induction with Pregnenolone-16-Carbonitrile: Onset of Action and Effect of Thioacetamide. Biochem. Pharmacol. 23, 1221-1224 (1974).

27. Bulger, W.H., Wheeler, D.M.S. and Stohs, S.J. 6ß-Hydroxy-3-Epidigitoxigenin: The Major Metabolite of Digitoxigenin by Rabbit Liver Homogenates. Biochem. Pharmacol. 23, 921-929 (1974).

28. El-Olemy, M.M., Sabatka, J.J. and Stohs, S.J. Sapogenins of Yucca glauca seeds. Phytochemistry 13, 489-492 (1974).

29. Rosenberg, H. and Stohs, S.J. Directed Biosynthesis of Unnatural Alkaloids in Dolichothele sphaerica. Phytochemistry 13, 823-827 (1974).

30. Stohs, S.J., Stoller, E.W., El-Olemy, M.M., Yu, P.C. and Tarng, C.S. Fatty Acid Composition of Lipids of Corchorus, Yucca, Dioscorea, Withania, and Rivea Tissue Cultures. Lloydia 37, 309-312 (1974).

31. Rosenberg, H. and Stohs, S.J. Aberrant Formation of N-Cinnamoylhistamine in Dolichothele sphaerica. Lloydia 37, 313-314 (1974).

32. Rosenberg, H. and Stohs, S.J. The Utilization of Tyrosine for Mescaline and Protein Biosynthesis in Lophophora williamsii. Phytochemistry 13, 1861-1863 (1974).

33. Stohs, S.J., Sabatka, J.J. and Rosenberg, H. Incorporation of 4-^{14}C-22,23-^{3}H-Sitosterol into Diosgenin by Dioscorea deltoidea Tissue Suspension Cultures. Phytochemistry 13, 2145-2148 (1974).

34. Stohs, S.J., Sabatka, J.J., Obrist, J.J. and Rosenberg, H. The Steroidal Sapogenins of Yucca glauca Tissues Cultures. Lloydia 37, 504-505 (1974).

35. Stohs, S.J., Rosenberg, H. and Billets, S. Sterols of Yucca glauca Tissue Cultures and Seeds. Planta Medica 27, 257-261 (1975).

36. Bulger, W.H. and Stohs, S.J. Effect of Phenobarbital on the Biotransformation of ^{3}H-Digitoxigenin to 6ß-Hydroxy-3-Epidigitoxigenin by Rabbit Liver Homogenates. Drug Metab. Disp. 2, 361-364 (1974).

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181. Protective role of Clarithromycin against Helicobacter pylori-induced oxidative stress and DNA damage in human gastric cells. T.R. McGinn, B. Buchanan, D. Bagchi, M. Hutzler and S.J. Stohs. Annual Meeting of the American College of Gastroenterology, 1997. Chicago, IL.

182. Protective effects of vitamins C and E, and a grape seed proanthocyanidin extract (GSPE) on smokeless tobacco-induced oxidative stress and apoptotic cell death in human oral keratinocytes. M. Bagchi, J. Balmoori, D. Bagchi, C. Kuszynski and S.J. Stohs. Annual Meeting of the Oxygen Society, 1997. San Francisco, CA.

183. Comparative in vitro and in vivo free radical scavenging abilities of a novel grape seed proanthocyanidin extract and selected antioxidants. S.J. Stohs, R.P. Scheckenbach, R.L. Krohn, J. Balmoori, M. Bagchi, C.A. Kuszynski, A. Garg and D. Bagchi. International Symposium on Stress Adaptation, Prophylaxis and Treatment, 1998. Calcutta, India.

184. Acute and chronic stress-induced oxidative gastrointestinal mucosal injury in rats, and protection by bismuth subsalicylate (BSS). D. Bagchi, C.B. Williams, M. Milnes, R.L. Krohn, M. Bagchi, J. Balmoori, X. Ye, M.X. Tran, S.J. Stohs, O.R. Carryl and S. Mitra. Digestive Disease Week, 1998. New Orleans, LA.

185. Mechanism of gastroprotection by bismuth subsalicylate against chemically-induced oxidative injury in human gastric mucosal cells. D. Bagchi, T.R. McGinn, X. Ye, J. Balmoori, M. Bagchi, S.J. Stohs, O.R. Carryl and S. Mitra. Digestive Disease Week, 1998. New Orleans, LA.

186. The cytotoxic effects of a novel grape seed proanthocyanidin extract on cultured human cancer cells. S.S. Joshi, X. Ye, W. Liu, M. Bagchi, H.G. Preuss, D. Bagchi and S.J. Stohs. American Association for Cancer Research, 1998. New Orleans, LA.

187. Cytotoxicity of a novel grape seed proanthocyanidin extract against selected human cancer cells. S.S. Joshi, X. Ye, W. Liu, M. Bagchi, H.G. Preuss, D. Bagchi and S.J. Stohs. Second International Conference on Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease, 1998. Helsinki, Finland.

188. In vitro and in vivo free radical scavenging abilities of a grape seed proanthocyanidin extract and selected antioxidants. D. Bagchi, R.L. Krohn, J. Balmoori, M. Bagchi, C.A. Kuszynski, A. Garg and S.J. Stohs. Second

International Conference on Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease, 1998. Helsinki, Finland.

189. Smokeless tobacco-induced oxidative stress, DNA damage and apoptotic cell death in human oral keratinocytes, and protection by antioxidants. D. Bagchi, M. Bagchi, S.S. Joshi, J. Balmoori, C. Kuszynski and S.J. Stohs. The Gordon Conference, 1998. Newport, RI.

190. Acute and chronic stress-induced gastrointestinal injury in rats, protection by a novel grape seed proanthocyanidin extract (GSPE). M. Bagchi, C.B. Williams, M. Milnes, J. Balmoori, X. Ye, D. Bagchi and S.J. Stohs. Annual Meeting of the Oxygen Society, 1998. Washington, DC.

191. Role of oxidative stress in the chronic toxicity of TCDD in C57BL/6J female mice. N.Z. Alsharif, L. Tang, E. Hassoun, T. El Metwally, C. Pedersen, M. Shara, and S.J. Stohs. Society of Toxicology, 1999. New Orleans, LA.

192. Role of p53 tumor suppressor gene in the toxicity of TCDD, endrin, naphthalene and chromium (VI) in the liver and brain tissues of mice. D. Bagchi, J. Balmoori, M. Bagchi, X. Ye, C.B. Williams, and S.J. Stohs. Society of Toxicology, 1999. New Orleans, LA

193. Smokeless Tobacco Induced Modulation of p53 Gene and Protective Effect of Antioxidants. M. Bagchi, S.S. Joshi, D. Bagchi, X. Ye, and S.J. Stohs. Society of Toxicology, 1999. New Orleans, LA.

194. Free Radicals and Grape Seed Proanthocyanidin Extract: Importance in Human Health and Disease. D. Bagchi, M. Bagchi, and S.J. Stohs. Conference on Emerging Potentials of Antioxidant Therapy, 1999. Goa, India.

195. Effective Integration of Laptop Computers in Classroom and Clerkship Sites and Creighton University. S.E. Ohia, G. G. Glynn, M. C. Makoid, M.S. Monaghan, and S.J. Stohs. A.A.C.P. Annual Meeting, 2000. San Diego, CA.

196. Protective effects of vitamins C and E and a novel grapeseed proanthocynadin extract on smokeless tobacco induced oxidative stress and modulation of Bcl-2, p53 and c-myc genes. M. Bagchi, S.S. Joshi, D. Bagchi, X. Ye, and S.J. Stohs. SFRR Europe Winter Meeting, 1999. Dinard, France.

197. Role of p53 tumor suppressor gene in chromium (VI)-induced oxidative stress and DNA damage. D.Bagchi, M. Bagchi, and S.J. Stohs. Conference on Molecular Mechanisms of Metal Toxicity and Carcinogenesis, National Institute of Occupational Safety and Health, 2000. Morgantown, WV.

198. Comparative toxicological evaluation of chromium polynicotinate and chromium picolinate. M. Bagchi, P. Lim, S.J. Stohs, H. Preuss and D. Bagchi. Society of Toxicology, 2001. San Francisco, CA.

199. Beneficial Effects of a Novel Niacin Bound Chromium in Athletes and Hypercholesterolemic Patients. M. Bagchi, H. Preuss, P. Lim, S.J. Stohs and D. Bagchi. International Congress of Toxicology, 2001. Brisbane, Australia.

200. Entry-Level Professional Doctoral Programs in Physical Therapy and Occupational Therapy at Creighton University. S. J. Stohs, A. J. Threlkeld, and R. Padilla. World Congress III of Health Professions, 2001. San Juan, Puerto Rico.

201. Transitional (Post-Professional) Web-Based Clinical Doctoral Programs in Physical Therapy and Occupational Therapy at Creighton University. S. J. Stohs, G. Jensen, B. Coppard, A. J. Threlkeld, and R. Padilla. World Congress III of Health Professions, 2001. San Juan, Puerto Rico.

202. Grape seed proanthocyanidins as novel dietary antioxidants. D. Bagchi, H.G. Preuss, M. Bagchi and S.J. Stohs. European Society for Free Radical Research, 2001. Rome, Italy.

203. Oxidative stress and toxicity. S.J. Stohs. Central States Society of Toxicology, 2001. Kansas City, MO.

204. Who owns intellectual property associated with web-based courses? S.J. Stohs and G.D. Jahn. American Assocation of Colleges of Pharmacy Annual Meeting, 2001. Toronto, Canada.

205. Entry-level and a post-professional clinical doctoral programs in occupational therapy (OTD). S.J. Stohs, R. Padilla, B. Coppard, and C. Royeen. World Congress of Occupational Therapists, 2002. Stockholm, Sweden.

206. Antimicrobial effects of natural antioxidants on Helicobacter pylori. A. Chatterjee, D. Bagchi, T. Yasmin and S.J. Stohs. Infectious Diseases Society of American Annual Meeting, 2002. Chicago, IL.

207. Entry level and post-professional doctoral programs in physical therapy (DPT) and occupational therapy (OTD) at Creighton University. S.J. Stohs, R. Padilla, and R. Sandstrom. Association of Schools of Allied Health Professions, 2002. San Diego, CA.

208. A 90-day chronic toxicity study of a novel natural (-) – Hydroxycitric acid from *Garcinia cambogia*. S.E. Ohia, S.J. Stohs, M. Shara, T. Hossain, A. Chatterjee, M. Bagchi and D. Bagchi. Society of Toxicology, 2003. Salt Lake City, UT.

209. Invivo exposure to an ephedra containing metabolic nutrition system does not alter serum biochemistry and histopathology of seven vital target organs of

B6C3F1 mice. S. Stohs, S. Gross, C. Patel, R. Hackman and S. Ray. Society of Toxicology, 2003. Salt Lake City, UT.

210. Prior exposure of Helicobacter pylori to bile salts and sodium chloride increases susceptibility to clarithromycin. A. Chatterjee, T. Yasmin, A. Hossain, D. Bagchi and S. Stohs. American Society of Microbilogy, 2003. Washington, DC.

211. Mechanisms of cell death after acetaminophen overdose: Apoptosis or oncotic necrosis? S.D. Ray, S. Gross, A. Chou, C. Brucculeri, D. Bagchi and S. Stohs. Experimental Biology Annual Meeting, 2003, San Diego, CA.

212. Entry-level and post-professional clinical doctoral programs in physical therapy (DPT): an administrative perspective. S.J. Stohs, G.M. Jensen, C. Goulet and R.W. Sandstrom. World Conference on Physical Therapy, 2003. Barcelona, Spain.

213. The effects of hydroxycitric acid extract and fluoxetine on rat brain cortex neurotransmitters after 30, 60 and 90 days treatment. M. Shara., S.J. Stohs, D. Bagchi, M. Bagchi and S. Ohia. Society of Toxicology, 2004. Baltimore, MD.

214. Exposure for one year to a metabolic nutrition system containing ephedra and caffeine does not alter serum chemistry profile or target organ histopathology of B6 C3 F1 mice. S.D. Ray, S. Stohs and R. Hackman. Society of Toxicology, 2004. Baltimore, MD.

215. Safety of a novel, natural extract of hydroxycitric acid, a supplement for weight management. D. Bagchi, M. Shara, S. E. Ohia, T. Yasmin, M. Bagchi, A. Chatterjee, and S.J. Stohs. Society of Toxicology, 2004. Baltimore, MD.

216. Citrus aurantium: Safe and effective in weight management? S.J. Stohs, D. Bagchi and H. Preuss. Society of Free Radicals Research, 2004. Tamil Nadu, India.

217. Citrus fruits in weight management: case studies – safety and efficacy of Citrus aurantium (bitter orange). S.J. Stohs and H.G. Preuss. WorldNutra 2004, San Francisco, CA.

218. Four-week exposure to a novel nutritional mixer containing a series of polyphenolic phytochemicals antagonizes acetaminophen-induced hepatotoxicity in vivo. S.J. Stohs, A.Nagori, A. Naqvi, N.Shah and S.D. Ray. Society of Toxicology, 2005. New Orleans, LA.

219. Activation of endonuclease or caspase-activated DNASE (CAD) as a marker of apoptosis rather than necrosis in drug- or chemical-induced oncosis in vivo. S.D. Ray, S. Stohs and G. B. Corcoran. Society of Toxicology, 2005. New Orleans, LA.

220. Development of web-based programs/pathways from an administrative perspective. S.J. Stohs. Symposium on the Virtual Frontier, 2005. Omaha, NE.

221. Selected nutrients in muscle atrophy and advancing age-related disorders. S.J. Stohs. WorldNutra 2005. Anaheim, CA.

222. Glycemic index: Impact on diabetes and metabolic syndrome. S.J. Stohs, WorldNutra 2006. Reno, NV.

223. In vivo exposure effects of a complex metabolic nutrition system on serum chemistry and histopathology: A one-year safety and efficacy study. S.D. Ray, J. Rathod, D. Zinkovsky, E.Bulku, S. Ismail, J. Gigliotti, R. Hackman and S. Stohs. American College of Nutrition, 2006. Reno, NV.

224. Long-term exposure effects of a novel phytochemical-nutraceutical mixture (metabolic nutrition system – platinum) on serum biochemistry and histopathology of seven vital target organs of B6 C3 F1 mice. S.D. Ray, S. Patel, J. Rathod, N. Patel, E. Bulku, D. Zinkovsky, R.M. Hackman and S.J. Stohs. Society of Toxicology, 2006. San Diego, CA.

225. Eight-week exposure effects of a novel dietary supplement – ThermoPlus on serum biochemistry and histopathology of vital target organs of fisher rats. S.D. Ray, M. Parmar, D. Zinkovsky, I. Syed, J. Rathod, K. Sajh, R.M. Hackman and S.J. Stohs. Society of Toxicology, 2007. Charlotte, NC.

226. Importance of proper hydration: A novel rehydration drink supported by research studies. S.J. Stohs, P. Snell, R. Ward and C. Kandaswami. Institute for Food Technology, 2007. Chicago, IL.

227. Dietary supplement ThermoPlus reduces the level of oxidative stress and stabilizes the genomic integrity in the liver and kidneys of fisher 334 rats. S.D. Ray, E. Bulko, M. Parmar, I. Syed, C. Patel, S. Bhatt, R.M. Hackman and S.J. Stohs. American College of Nutrition, 2007. Orlando, FL.

228. The AdvoCare experience in monitoring adverse events. S.J. Stohs and T. Dollar. American College of Nutrition, 2007. Orlando, FL.

229. Effects of an adaptogen-based supplement (A-Supreme) on stress parameters in healthy volunteers. M. Shara, S. J. Stohs and E. Kakish. American College of Nutrition, 2008. Arlington, VA.

230. Safety evaluation of Super CitriMax (HCA-SX), a natural extract of (-)-hydroxycitric acid derived from *Garcinia cambogia*. S. J. Stohs, S. E. Ohia, H. G. Preuss, M. Bagchi, F. C. Lau, and D. Bagchi. American College of Nutrition, 2009, Orlando, FL.

231. Are non-nutritive sweeteners as safe as sugar? S. J. Stohs. American College of Nutrition, 2009, Orlando, FL.

232. Efficacy and safety of Super CitriMax(HCA-SX), a novel weight loss supplement derived from *Garcinia cambogia*. International Society for Nutraceuticals and Functional Foods, 2009, San Francisco, CA

233. *p*-Synephrine safety study: A randomized clinical study. M. Shara and S. J. Stohs. American College of Nutrition, 2011, Morristown, NJ.

234. Nutritional supplements in the surgical patient. S. J. Stohs, S. J. Dudrick. American college of Nutrition, 2011, Morristown, NJ.

235. 60 Day human double blind, placebo-controlled safety study on Citrus aurantium (bitter orange) extract. S. J. Stohs, G. R. Kaats, H. Miller, H. G. Preuss. American College of Nutrition. 2012. Morristown, NJ.

236. Review of the safety and efficacy of banaba and bitter orange extracts. S. J. Stohs. International Society for Nutraceuticals and Functional Foods, 2012. Kona, Hawaii

237. Review of the safety and efficacy of banaba and bitter orange extracts. S. J. Stohs. International Society for Nutraceuticals and Functional Foods, 2012. Kona, Hawaii.

238. Nutraceuticals and Dietary Supplements in enhanced sports performance. S.J.Stohs. Institute of Food Technicians, 2014, New Orleans, LA

239. Role of Dietary Supplements in Disease Prevention and Management. S. J. Stohs, Fourth International Meeting of Pharmacy and Pharmaceutical Sciences, 2014, Istanbul, Turkey.

240. Major changes in clinical pharmacy education and practice. S.J.Stohs. Fourth International Meeting of Pharmacy and Pharmaceutical Sciences, 2014, Istanbul, Turkey.

241. The effects of supplementation with Advantra Z alone and in the combination with caffeine on lypolytic and cardiovascular responses during resistance exercise. N.A. Ratamess, J. A. Bush, W.J. Kraemer, S. J. Stohs, V. G. Nocera, M. D. Leise, K. B. diamond, L. Pigott, J. Pacifico, C. Noonan, A.D. Faigenbaum. International Society of Sports Nutrition. 2015. Austin, TX.

242. Effect of p-synephrine and caffeine supplementation on metabolic responses during heavy resistance exercise. J.A. bush, N>D. Ratamess, J. Kang, W.J. Kraemer, S.J. Stohs, V.G. Nocera, M.D. Leise, K.B. Diamond, L. Pigott, J. Pacifico, C. Noonan, A.D. Fagenbaum. National Strength and Conditioning Association. 2015. Orlando, FL.

COURSES DEVELOPED:

Pharmaceutical Biochemistry, 6 cr
Clinical Chemistry and Biochemistry, 2 cr
Chemotherapy and Immunotherapy, 4 cr
Immunologic Systems of Defense, 2 cr
Introduction to Nutrition and Diet Therapy, 2 cr
Basic Life Support for Pharmacists, 1 cr
Drug Metabolism and Disposition, 2 cr
Research Design and Biostatistics, 3 cr

TEACHING INTERESTS:

Clinical education and nutrition; toxicology; food and nutritional toxicology. chemotherapy; drug metabolism; clinical chemistry and biochemistry; biochemistry.

RESEARCH INTERESTS:

Clinical nutrition; extrahepatic drug metabolism; enzyme induction; xenobiotic metabolism in normal and disease states; drug toxicity as a function of age; glutathione metabolism; role of oxidative stress in xenobiotic toxicology; biochemistry of heavy metal toxicity; chemical carcinogenesis; dietary supplement safety and efficacy.

EDUCATIONAL AND SCIENTIFIC CONFERENCES ATTENDED:

American Society of Pharmacognosy (ASP)-Boulder, CO, 1960; Houston, TX, 1961; Chapel Hill, NC 1963; Minneapolis, MN 1966; Corvallis, OR, 1969; Columbus, OH, 1972; Jekyll Island, GA, 1973; Chicago, IL, 1974; Seattle, WA 1977.

Academy of Pharmaceutical Sciences - San Francisco, CA, 1971; Houston, TX, 1972; Chicago, IL, 1974; San Francisco, CA, 1975; Montreal, Canada, 1978; Kansas City, MO, 1979; Minneapolis, MN, 1985.

Federation of American Societies of Experimental Biology - Chicago, IL, 1967; Atlantic City, NH, 1968; Atlantic City, NJ, 1970; Chicago, IL, 1977; Dallas, TX, 1979; Anaheim, CA, 1980; Chicago, IL, 1983; St. Louis, MO, 1984; New Orleans, LA, 1989.

Nebraska Pharmacists Association - Omaha, 1962; Lincoln, 1964; Lincoln, 1973; Omaha, 1985; Kearney, 1989, 1990, 1991, 1992, 1993, 1994; Grand Island, 1995; Lincoln, 1996; Grand Island, 1997, 1998, 1999, 2000, 2002.

International Plant Tissue Culture Conference, State College, PA, 1963.

Sigma Xi Symposium on Mind Altering Drugs, Lincoln, NE 1970.

American Association of Colleges of Pharmacy - Columbus, OH, 1972; Seattle, WA, 1977; Orlando, FL, 1978; Denver, CO, 1979; Boston, MA, 1980; Scottsdale, AR, 1981; Washington, DC, 1983; Portland, OR, 1989; Salt Lake City, UT, 1990; Boston, MA, 1991; Jacksonville, FL, 1992; Washington, DC, 1992; Arlington, VA, 1993; Hilton Head, SC, 1994; Albuquerque, NM, 1994; Washington, DC, 1995; Philadelphia, 1995; Anaheim, CA, 1996; Reno, NV, 1996; Washington, DC, 1997; Indianapolis, IN, 1997; Savannah, GA, 1998; Snowmass, CO, 1998; Washington, DC, 1998; Washington, D.C., 1999; Richmond, VA, 2000; San Diego, CA, 2000; Toronto, Canada, 2001; Toronto, CA, 2001; San Francisco, CA, 2001, Houston, TX, 2002; Kansas City, MO, 2002.

AACP Board of Directors Meeting - Snowmass, CO, 1998; Boston, MA, 1999; Washington, D.C., 1999; Richmond, VA, 2000; San Diego, CA, 2000.

Governor's Workshop on Drug Abuse, Lincoln, NE, 1970.

West-Central States Biochemistry Conference - Manhattan, KS, 1970; Lawrence, KS, 1971; Iowa City, IA 1972.

Drug Abuse Education Workshop for Educators, Lincoln, NE, 1971.

University of Nebraska Management Conference, Lincoln, NE, 1971.

Midwest Regional American Chemical Society Meeting, October, 1973, Lawrence, KS.

Steroid Symposium of the American Oil Chemists Society, September, 1973, Philadelphia, PA.

Nebraska Society of Hospital Pharmacists' Symposium on Clinical Pharmacy, Omaha, NE, 1975.

International Congress of Pharmacology, Helsinki, Finland, 1975; London, England, 1984.

International Conference on Drug Metabolism and Disposition, Turku, Finland, 1975.

International Conference for Research on Medicinal Plants, Munich, West Germany, 1976.

Symposium on "The Pharmacists' Role in the Management of the Hypertensive Patient" sponsored by the Greater Omaha Pharmaceutical Association, Omaha, NE, 1977.

Scientific Sessions of the Annual Nebraska Heart Association Conference, Omaha, NE, 1977.

International Symposium on Microsomes and Drug Oxidations, Ann Arbor, MI, 1978; Brighton, England, 1984.

Symposium on Vitamins: Nutritional Use and Abuse, University of Nebraska Medical Center, Omaha, NE, 1978.

National Sigma Xi Meeting, Toronto, Canada, 1983.

International Symposium on Foreign Compound Metabolism, West Palm Beach, FL, 1983.

National Research Forum on Aging, Lincoln, NE, 1984.

Cold Spring Harbor Laboratory Conference on the Biological Mechanisms of Dioxin Action, Cold Spring Harbor, NY, 1984.

Free Radicals, Pathology and Aging, San Diego, CA, 1985.

Society of Toxicology, San Diego, CA, 1982, 1985; New Orleans, LA, 1986; Washington, D.C., 1987; Dallas, TX, 1988; Atlanta, GA, 1989; Miami, FL, 1990; Dallas, TX, 1991; Seattle, WA, 1992; New Orleans, LA, 1993; Dallas, TX, 1994; Anaheim, CA, 1996; Cincinnati, OH, 1997; Seattle, WA, 1998; New Orleans, LA 1999; Philadelphia, PA, 2000; Salt Lake City, UT, 2003; Baltimore, MD, 2004; New Orleans, LA, 2005; San Diego, CA, 2006; Charlotte, NC, 2007.

International Symposium on Biological Reactive Intermediates, Baltimore, MD, 1985; Tucson, AZ, 1990.

International Congress of Biomedical Gerontology, New York, NY, 1985.

Third Tokyo Symposium on Liver and Aging, Tokyo, Japan, 1986.

Symposium on Frontiers in Aging, Tokyo, Japan, 1986.

Genetic Screening in the Workplace, Omaha, NE, 1986.

International Society for the Study of Xenobiotics, Clearwater, FL, 1987.

19th Annual Magnetic Resonance Conference, NIEHS, Research Triangle Park, NC, 1987.

Tenth International Conference on Organohalogen Compounds, Bayreuth, Federal Republic of Germany, 1990.

Central States Society of Toxicology, Lawrence, KS, 1990; Kansas City, MO, 1992; Omaha, NE, 1993; Kansas City, MO, 1994.

National Association of Retail Druggists, Baltimore, MD, 1991; Seattle, WA, 1992; Boston, MA, 1994.

American Association of Health-System Pharmacists, New Orleans, LA, 1991; Las Vegas, NV, 1995; New Orleans, LA, 1996; Atlanta, GA, 1997; Las Vegas, NV, 1998; Orlando, FL, 1999; Las Vegas, NV, 2000; New Orleans, LA, 2001; Atlanta, GA, 2002.

International Conference on Dioxins, Chaired Session on Role of Hormones, Research Triangle Park, NC, 1991.

Jesuit Conference on Collaboration, Spokane, WA, 1992.

American Pharmaceutical Association, Dallas, TX, 1993; Seattle, WA, 1994; Miami, FL, 1998; San Antonio, TX 1999; Washington, D.C., 2000; San Francisco, CA, 2001; Philadelphia, PA, 2002; Seattle, WA, 2004.

Glaxo/AACP Dean's Leadership Institute, Durham, NC, 1993.

American Physical Therapy Association, Cincinnati, OH, 1993; Seattle, WA, 1999.

International Meeting on Pharmacy and Pharmaceutical Sciences, Istanbul, Turkey, 1994, 1998.

Association of Schools of Allied Health Professions, Galveston, TX, 1993; Richmond, VA, 1994; Milwaukee, WI, 1995; Charleston, SC, 1996; San Diego, CA, 1998; Atlanta, GA, 1999, Norfolk, VA, 2001; San Diego, CA, 2002.

Oxygen Radicals and Antioxidants in Biotechnology and Medicine, Calcutta, India, 1993.

Jesuit Higher Education in the Heartland, Chicago, IL, 1994; St. Louis, MO, 1997; Omaha, NE, 2000.

American Occupational Therapy Association National Conference, Denver, CO, 1995; Chicago, IL, 1996.

National Association of Chain Drug Stores Annual Conference, Palm Beach, FL, 1995; Maui, HI, 1996; Palm Beach, FL, 1998; Palm Beach, FL, 2000; Scottsdale, AZ, 2001; Palm Beach, FL, 2002, 2003.

Federation International Pharmaceutique, Stockholm, Sweden, 1995.

USP Open Conference on Botanicals for Medical and Dietary Uses: Standards and Information Issues, Washington, DC, 1996.

International Symposium on the Damaging Effect of Oxidants in Cigarette Smoke, Athens, Greece, 1997.

World Congress on Allied Health, Telford, England, 1997.

Nebraska Minority Health Conference (Session Moderator), Omaha, NE, 1997.

American Association of Colleges of Pharmacy Institute, Leesburg, VA, 1998.

Annual Meeting of the Society for Neuroscience, Los Angeles, CA, 1998.

Annual Meeting of the Oxygen Society, Washington, DC, 1998.

Association of Schools of Allied Health Professions - Midwest Deans, Memphis, TN, 1999; Atlanta, GA, 1999.

American College of Nutrition, Washington, D.C., 1999; Las Vegas, NV, 2000; Kiawah Island, SC, 2005; Reno, NV, 2006; Orlando, FL, 2007; Washington, DC, 2008; Orlando, FL, 2009; Morristown, NJ, 2011, 2012; San Diego, CA, 2013; San Antonio, TX 2014; Orlando, FL 2015.

Academic Organizational Approaches to Transforming Health Science Education - Northeastern University, Boston, MA, 2000.

World Allied Health Congress, San Juan, Puerto Rico, 2001.

Rite-Aid Educational Connection Conference, Philadelphia, PA, 2001.

World Congress III of Health Professions, San Juan, Puerto Rico, 2001.

Midwest Health Science Deans Occupational Therapy Research Conference, Omaha, NE, 2002, 2003.

World Conference of Occupational Therapists, Stockholm, Sweden, 2002.

World Conference on Physical Therapy, Barcelona, Spain, 2003.

Supply Side West, Ahaheim, California, 2004; 2005; 2006; 2007.

NNFA Natural Foods Convention, Las Vegas, Nevada, 2004.

The Virtual Frontier: Developing and Delivering Health Science Education from a Distance, Omaha, NE, 2005.

WorldNutra Conference, Anaheim, CA, 2005; Reno, NV, 2006.

International Society for Nutraceutical and Functional Foods Conference, San Francisco, CA, 2009; Kona, Hawaii, 2012.

Board for the Certification of Nutrition Specialists: Fredericksburg, VA 2010; Chicago, IL 2012, 2013, 2014, 2015.

International Society for Sports Nutrition: Austin, TX 2014.

International Meeting of Pharmacy and Pharmaceutical Sciences, Istanbul, Turkey, 1999, 2002, 2014.

Natural Products Expo, Las Vegas, NV 2013; 2015, 2016

INVITED PAPERS, PRESENTATIONS AND SEMINARS:

1. Over 40 speaking engagements since 1968 to a wide range of civic, social, educational and professional groups on the non-medical use of drugs, and over 100 speaking engagements since 1998 regarding the use of nutraceuticals in health, wellness, weight loss and sports performance.

2. Sigma Xi: Symposium, Center for Continuing Education, University of Nebraska, 1970. "Mind Altering Drugs - Actions and Reactions".

3. Presented a seminar to the College of Pharmacy, University of Pittsburgh on "Steroid Metabolism by Plant Tissue Homogenates", July 1970.

4. Regional Drug Abuse Education Workshop for Educators, Wesleyan University, October 1970. "Commonly Misused Drugs".

5. Hastings College, Interim Course on Drugs, January, 1971. "Pharmacology, Toxicology, Biochemistry, and Chemistry of Hallucinogenic Drugs".

6. Presented a seminar to the School of Pharmacy, University of Washington on "Metabolism of Steroids by Plant Tissue Cultures and Leaf Homogenates", June 1971.

7. Youth Workshop in Drugs, July 1971. Center for Continuing Education, "Pharmacology of Drugs".

8. Presented a seminar to the Department of Biochemistry, UNMC, on "Cardenolide Metabolism", March 1972.

9. Presented a seminar to the Department of Food Science and Technology, UNL, on "Labeling of Vitamin and Mineral Preparations", April 1974.

10. Central States Branch Tissue Culture Association, Inc., Lincoln, Nebraska, April, 1974. "Plant Tissue Culture Methods and Applications".

11. American Oil Chemists Society Steroid Symposium, Philadelphia, Pennsylvania, October 1974. "Plant Tissue Culture Sterols and Sterol Metabolism".

12. Presented a seminar to the Department of Pharmacology, University of Nebraska Medical Center entitled "Cardenolide Metabolism and the Influence of Enzyme Induction", December 1974.

13. Presented a seminar to the College of Pharmacy, University of Minnesota on "In Vitro Cardenolide Metabolism", April 1975.

14. Presented a seminar to Sandoz Toxicology Laboratories, Basle, Switzerland entitled "Stability of Mixed Function Oxygenases", August 1975.

15. Presented a seminar to the Karolinska Institute, Stockholm, Sweden entitled "Some Drug Interactions of Cardiac Glycosides", November 1975.

16. International Congress for Research on Medicinal Plants, Munich, West Germany, September 1976. "Metabolism of Steroids by Plant Tissue Cultures".

17. Presented a seminar to the faculty of UNMC entitled "Isolation of Intestinal Microsomes and Their Metabolism of Xenobiotics", September 1976.

18. Presented a seminar to the faculty of UNMC, November 1976. "Xenobiotic Metabolism by Isolated Intestinal Epithelial Cells."

19. Presented a seminar to the Student American Pharmaceutical Association on "Pharmacy in Sweden", December 1976.

20. Presented a seminar to the Department of Biochemistry, Iowa State University, January 1977. "Metabolism of Xenobiotics by Intestinal Microsomes and Cells".

21. Presented a seminar to the Institute on Aging, University of Nebraska, October 1978. "Tissue Glutathione Levels in Mice as a Function of Age".

22. Participant in Symposium on Vitamins and Nutrition, October 1978. Presentation on "Vitamins: Current Research and Controversy".

23. Presented a seminar to the College of Pharmacy, University of Minnesota on "Xenobiotic Metabolism in Streptozotocin-Diabetic Rats". December 1978.

24. American Association of Colleges of Pharmacy, Denver, Colorado, July, 1979. Symposium presentation entitled "Impact of Clinical Pharmacy on Biological Sciences Instruction: Course Offerings and Student Qualifications."

25. Presented a seminar to the Department of Pharmacology, UNMC, October 1979. "Effects of Various Hormonal Factors on Hepatic and Extrahepatic Mixed Function Monoxygenases."

26. Presented a seminar to the Eppley Institute, UNMC, March 1980. "Studies on Hormonal Factors Influencing the Regulation of Xenobiotic Metabolism".

27. American Association of Colleges of Pharmacy, Boston, Massachusetts, July 1980. Symposium presentation entitled, "Concurrent M.S. - Pharm. D. Program at the University of Nebraska".

28. Presented a seminar to the College of Pharmacy, Yarmouk University, Irbid, Jordan, July 1981 on "Drug and Glutathione Metabolism as a Function of Age".

29. A discussion of "The Effect of Medication on Sexual Functioning" at Immanuel Medical Center symposium on "Sexuality and the Disabled Person", Omaha, Nebraska, September 1981.

30. A lecture to the sophomore medical students, UNMC, in the course on Perspectives on Aging. Lecture entitled "Drugs: metabolism and distribution in the aging person", October, 1981, 1982, 1983, 1984.

31. A seminar presented to the Department of Biochemistry, Creighton University, November, 1981. "Changes in Glutathione and Xenobiotic Metabolism Relative to Age".

32. A symposium lecture entitled "Vitamins, Pseudovitamins, and Non-vitamins" given at the 6th annual Dorsey Symposium, Lincoln, Nebraska, September 1982.

33. Lipid Peroxidation as a Possible Cause of TCDD Toxicity, Eppley Institute, UNMC, April 1983.

34. Role of Glutathione Peroxidase in TCDD Toxicity, Eppley Institute, UNMC, March 1984.

35. Induction of Lipid Peroxidation and Inhibition of Glutathione Peroxidase by TCDD. Banbury Conference on the Biological Mechanisms of Dioxin Action, Cold Spring Harbor, New York, April 1984.

36. A seminar presented to the Chemistry Department, South Dakota State University, October 1984, on "The Human and Animal Toxicity of TCDD".

37. A seminar presented to the Lincoln Branch of the Nebraska Affiliate of the American Heart Association, October 1984, on "The Myocardial Effects of TCDD".

38. A seminar presented to Nebraska Wesleyan University, November 1984, on "Lipid Peroxidation, Glutathione Peroxidase and TCDD Toxicity".

39. Invited speaker at workshop on "Free Radicals, Pathology and Aging", San Diego, California, February 1985. Co-sponsored by the National Institute on Aging and University of California, San Diego. Topic of presentation "Glutathione, DNA Damage and Aging".

40. A seminar presented to the Department of Chemistry, University of Nebraska at Omaha, March 1985, on "TCDD (Agent Orange) Toxicity".

41. Presented a program at UNMC on "Food Facts and Fallacies" as part of the University Lunch and Learn Services, March 1985.

42. A seminar presented at the University of Wyoming on "The Roles of Lipid Peroxidation, Hydrogen Peroxide and Glutathione Peroxidase in Dioxin (TCDD) Toxicity", March 1985.

43. Lecture to the School of Pharmacy, Creighton University on "Graduate Educational Opportunities in the Pharmaceutical Sciences", November 1985, 1986.

44. Invited speaker at the Third Tokyo Symposium on Liver and Aging presentation on "The Role of Glutathione in Aging", August 1986.

45. Presentations to the Nebraska Pharmacists Association State Convention on "The Percutaneous Absorption of Iodochlorhydroxyquin". April 1986.

46. Invited speaker at Conference on Frontiers in Hepatic Aging, in Tokyo, Japan. Presentation on "Glutathione, Free Radicals Antioxidants and Aging", August 1986.

47. A seminar presented at Shionogi Pharmaceuticals Corporation in Osaka, Japan on "The Role of Glutathione Metabolism in Aging", August 1986.

48. A seminar presented to the Departments of Life Sciences and Chemistry at University of Kansas Medical Center on "Glutathione, Free Radicals, Antioxidants and Aging", October 1986.

49. A seminar presented to the South Dakota State University on "Graduate Studies in the Pharmaceutical Sciences", November 1986.

50. A seminar presented to Auburn University College of Pharmacy on "The Role of Iron in TCDD-Induced Toxicity", February 1987.

51. A seminar presented at the National Institute of Environmental Health Sciences on "Factors Involved in TCDD-Induced DNA Damage", November 1987.

52. A seminar presented at Burroughs Wellcome Co., Research Triangle Park, N.C., entitled "The Roles of Iron and Free Radicals in the Hepatotoxicity of TCDD", November 1987.

53. A seminar presented to the Department of Pharmacology, University of North Carolina, entitled "Oxidative Stress and the Toxicity of TCDD", March 1988.

54. A seminar at the University of Montana on "Health Sciences Education: Past, Present and Future", April 1988.

55. A seminar presented at the National Institute of Environmental Health Sciences entitled "AHH Induction and Glucocorticoid Receptor Binding in Skin and Liver of TCDD Treated Mice", June 1988.

56. A seminar presented at Smith Kline & French Laboratories, King of Prussia, PA, entitled "Enzyme Induction and Oxidation Stress by TCDD", July 1988.

57. A seminar presented to the U.S. Army Research Laboratory at Fort Dietrick, Frederick, MD, entitled "The Role of Free Radicals in Hepatotoxicity, and Therapeutic Approaches", July 1988.

58. A presentation of Creighton University entitled "Employment Opportunities in Academia for Pharmacists," 1988, 1989, 1990.

59. A seminar on "The Roles of Free Radicals in Toxicology and Disease" presented at Grinnell College, Iowa, November, 1988.

60. A seminar on "Free Radicals in Toxicology and Disease," Creighton University Health Sciences Center, October, 1989.

61. A seminar on "Production of an Oxidative Stress by TCDD," National Institute of Environmental Health Sciences, June, 1990.

62. A seminar on "Pharmacy Curriculum Development and Curricular Change," Marmara University, Istanbul, Turkey, November, 1991.

63. A seminar on "Pharmaceutical Care and the Clinical Practice of Pharmacy," Marmara University, Istanbul, Turkey, November, 1991.

64. A seminar entitled "The Importance of Bioavailability in Drug Treatment", Hoffmann-LaRoche Pharmaceutical Company, Istanbul, Turkey, November, 1991.

65. A seminar on "Pharmacy Curriculum Development and the Clinical Practice of Pharmacy," Ankara Pharmaceutical Society, Ankara, Turkey, November, 1991.

66. A seminar on "The Role of Free Radicals in Biology and Medicine," Ankara University, Ankara, Turkey, November, 1991.

67. A seminar entitled "Reactive Oxygen Species in Toxicity and Disease," Department of Biochemistry, University of Delhi, Delhi, India, December, 1993.

68. An invited symposium speaker at the International Symposium on Oxygen Radicals and Antioxidants in Biotechnology and Medicine, Calcutta, India, December, 1993. The seminar topic presented was "Oxidative Mechanisms in the Toxicity of Metal Ions."

69. An invited presentation entitled "Hiring for Mission," presented at the Jesuit Higher Education in the Heartland Conference, Chicago, IL, May, 1994.

70. A plenary lecture entitled "The Role of Free Radicals in Toxicity and Disease," presented at the First International Meeting on Pharmacy and Pharmaceutical Sciences, Istanbul, Turkey, September, 1994.

71. The keynote lecture entitled "Curricula for the Present and Future Practice of Pharmacy" at the First International Meeting on Pharmacy and Pharmaceutical Sciences, Istanbul, Turkey, September, 1994.

72. "Mechanisms associated with the toxicity of polyhalogenated cyclic hydrocarbons and heavy metals," presented at the Air Force Office of Scientific Research Predictive Toxicology Program Review, Fairborn, OH, May, 1995.

73. "Considerations involving the establishment of a physician assistant program," presented at the American Association of Colleges of Pharmacy, Philadelphia, PA, July, 1995.

74. "Political, economic, professional and academic realities of our rapidly evolving health care system," presented at the American Occupational Therapy Association, Chicago, IL, April, 1996.

75. "Mechanisms associated with the toxicity of naphthalene, chromium and cadmium," presented at the Air Force Office of Scientific Research Predictive Toxicology Program Review, Fairborn, OH, December, 1996.

76. "Toxicity of metal ions in cigarette smoke," presented at the International Symposium on Damaging Effects of the Oxidants in Cigarette Smoke, Athens, Greece, January, 1997.

77. "The standardization of herbal products," presented at AdvoCare International, Dallas, TX, July, 1998.

78. Invited participant at an American Physical Therapy Association Consensus Conference on Clinical Doctoral Education, Washington, DC, July, 1998.

79. "Toxicity and carcinogenicity of tobacco products: Role of reactive oxygen species and protective effects of antioxidants," presented at the Second International Meeting on Pharmacy and Pharmaceutical Sciences, Istanbul, Turkey, September, 1998.

80. "Successful Transition to the Clinical Doctorate", presented at the American Physical Therapy Association, Seattle, WA, February, 1999.

81. “Free Radical Biology and Chemistry”, presented at the Society of Toxicology, New Orleans, LA, March, 1999.

82. “Educational Requirements and Pharmacy Practice Expectations in the United States”, presented at the Second International Clinical Pharmacy Conference, Amman, Jordan, April, 1999.

83. A Seminar on “Recent Changes in Pharmacy Curricula in the USA” presented at Jordan University for Women, Amman, Jordan, April, 1999.

84. A Seminar on “Role of Free Radicals and Oxidative Stress in Disease and Toxicity” presented at Jordan University, Amman, Jordan, April, 1999.

85. A Seminar on “Toxicity and Carcingenicity of Tobacco Products” presented at Jordan University, Amman, Jordan, April, 1999.

86. “Innovative Collaborative Rural Practice Initiative Among Native Americans in Nebraska presented at NABP-AACP District 5 Meeting, Duluth, MN, August, 1999.

87. Stohs, S.J. and Threlkeld, A. J. The Entry-Level Clinical Doctoral Program in Physical Therapy (DPT). American Physical Therapy Association, 1999. Seattle, WA.

88. Invited speaker and panel member on a program entitled "The Impact of Technology on Creighton University", Omaha, Nebraska, April, 2000.

89. A radio interview on KIOS "Healthwise" concerned with Medical and Medication Errors, April, 2000.

90. Member of Organizing Committee for a Symposium entitled "Academic Organizational Approaches to Transforming Health Science Education". Also chaired session, made a presentation, and served on a panel. Northwestern University, Boston, MA, August, 2000.

91. "An Interdisciplinary Administrative Structure at Creighton University" presented at the Academic Organizational Approaches to Transforming

Health Science Education Conference, Northeastern University, Boston, MA, August, 2000.

92. Oxidative Stress and Toxicity. S. J. Stohs. Central States Society of Toxicology, 2001. Kansas City, Missouri.

93. A radio interview on KFAB "Community Health Watch" on Bioterrorism, Omaha, NE. October, 2001.

94. "Citrus Fruits in Weight Management: Case Studies – Safety and Efficacy of Citrus aurauntium (bitter orange)." S.J. Stohs and H.G. Preuss. WorldNutra 2004 Conference, San Francisco, CA, October, 2004.

95. Invited speaker and panel member for a Symposium entitled, "The Virtual Frontier: Developing and Delivering Health science Education from a Distance." Creighton University, Omaha, NE, June, 2005.

96. "Selected Nutrients In Muscle Atrophy And Advancing Age-Related Disorders" presented at the WorldNutra 2005 Conference. Anaheim, CA. October, 2005.

97. "Dietary Supplements: Safety and Efficacy" presented to the Long Island University College of Pharmacy, Long Island, NY, November, 2007.

98. "Are Non-nutritive Sweeteners as Safe as Sugars?" presented at the American College of Nutrition, Orlando, FL, October, 2009.

99. "Safety and Efficacy of Super CitriMax(hydroxycitric acid-SX), a Novel Weight Loss Supplement Derived from *Garcinia cambogia*," presented at the International Society for Nutraceutical and Functional Foods, San Francisco, CA, Novemberr, 2009.

100. "Weight Loss/Weight Management, Increased Vitamin D and Decreased Salt: Solutions to Rising healthcare Costs" presented at Long Island University School of Pharmacy, Brooklyn, NY, April, 2010.

101. "Are Non-Nutritive Sweeteners as Safe as Sugars or are Sugars as Safe as Non-Nutritive Sweeteners?" presented at Long Island University, Brooklyn, NY, April 2010.

102. "Perspectives on Non-Sugar Sweetreners" presented at Georgetown University, Washington, DC, April 2010.

103. "The Safety and Efficacy of Bitter Ornage Extract and its Primary Protoalkaloid p-Synephrine". Iovate Nutraceuticals, Toronto Canada, August 2011.

EDITORIAL BOARDS:

Research Communications in Molecular Pathology and Pharmacology
Archives of Gerontology and Geriatrics
Bulletin of Environmental Contamination and Toxicology
Quintessence
The Original Internist
Journal of the American College of Nutrition
Oxidative medicine and Cellular Longevity

REVIEW FOR THE FOLLOWING JOURNALS:

Journal of Pharmaceutical Sciences
Biochemical Pharmacology
Drug Metabolism and Disposition
Science
The Journal of Natural Products
Lipids
U.S. Pharmacist
Cancer Letters
Toxicology and Applied Pharmacology
Research Communications in Chemical Pathology and Pharmacology
Journal of Pharmacology and Experimental Therapeutics
Carcinogenesis
Archives of Environmental Contamination and Toxicology
Dirisat
Chemico-Biological Interactions
Age
Archives of Gerontology and Geriatics
Biochimica et Biophysica Acta
Toxicology Letters
Journal of Biochemical and Molecular Toxicology
Journal of Toxicology and Environmental Health
Environmental Pollution
Antioxidants and Redox Signaling
In-vitro and Molecular Toxicology
Free Radical Biology and Medicine
Molecular and Cellular Biochemistry
Journal of the American College of Nutrition
Toxicology Mechanisms and Methods
Journal of Inorganic Biochemistry
Journal of Medicinal Food
Cell Biochemistry and Biophysics
Nutrition Journal
Phytotherapy Research

OTHER ACTIVITIES:

1. Co-author of an article in the Supplement of the October 4, 1970 Lincoln Sunday Journal and Star on drug abuse entitled "Another View". Also in this supplement an outline of the commonly abused drugs, their effects and dangers.

2. Governor's Commission on Drug Abuse for the State of Nebraska, and co-chairman of the Education Subcommittee thereof, 1970-71. Nebraska State Commission on Drugs and member of the Grant Reviews and Evaluations Subcommittees, 1971-72. As a member of the Commission on Drugs:

   A. Established recommended films on drugs for primary and intermediate grades, junior high, high school, college, and adult and professional levels - for general distribution throughout the State, 1970. Revised 1971.

   B. Evaluated existing drug information materials and established a list of recommended reference materials, 1970. Revised 1971.

   C. Made available to the physicians, hospitals, and clinics of the State a manual entitled "Drug Abuse Acute Treatment Manual." 1971.

   D. Coordinated and compiled drug education materials for "DRUG ABUSE, An Authoritative Selected List of Materials," published by the Nebraska Public Library Commission and the Nebraska State Commission on Drugs, 1971. Revised in 1972.

3. Developed an outline on "Mind Altering Drugs - Actions and Reactions" which covers the chemistry, sources, pharmacology, psychopharmacology, toxicology, doses, dosage forms, and therapeutic applications of the commonly used and misused mind-altering drugs for continuing education.

4. Developed an outline of the potential symptoms of the use of heroin, barbiturates, glues, and solvents, marijuana, hallucinogens (LSD), and amphetamines for continuing education.

5. The establishment of a drug education committee, the teaching and training of interested pharmacy students to go out and speak on drugs, and a drug information service with Drs. E.B. Roche and R.W. Piepho, 1969-74.

6. Member of the University Lutheran Chapel Planning and Building Committee, University of Nebraska, Lincoln, Nebraska, 1968-71, which resulted in the building of a new facility on the UNL campus.

7. Development of an integrated three-semester course sequence in biochemistry and natural products for pharmacy students. Complete revision and updating of the laboratory equipment and facilities, 1968-71.

8. Drafted the By-Laws for the College of Pharmacy, 1973.

9. Coordinator of the Graduate Programs of the College of Pharmacy, 1972-75.

10. Coordinator of the Visiting Scientists Programs of the College of Pharmacy, 1972-75.

11. Developed a required three-credit course in Clinical Chemistry and Biochemistry, 1973, with Dr. H. Rosenberg.

12. Drafted the procedure whereby the three graduate degree granting departments from within the College of Pharmacy could be incorporated into the Interdepartmental Area of Medical Sciences, and successfully negotiated this incorporation, 1972-73.

13. Provided and supervised a tape-recorded, 2 credit, self-paced, elective course entitled "Systems of Defense," 1974-1985.

14. Developed and reorganized a required two quarter, 8 credit course in Pharmaceutical Biochemistry, 1976, with Dr. H. Rosenberg and Dr. W. Murray.

15. Reorganized the graduate level courses in the Department, 1976.

16. Final Judge of Student Presentations at UNMC Student Research Forum, September 1976.

17. Developed a 1 credit elective course "Modified Basic and Advanced Life Support for Pharmacists," 1977.

18. Final Judge of Student Presentations at UNMC Student Research Forum, 1977.

19. Discussion Leader on Drug Metabolism at Annual Midwest Student Medical Research Forum, Omaha, Nebraska, 1978.

20. Developed and coordinated a required 6 quarter credit hour course in Chemotherapy, 1978. Revised to 4 sem. credit hours in 1982.

21. Presented a continuing education course on New Drugs to Pharmacists at Scottsbluff, Nebraska and Lincoln, Nebraska, April 1978.

22. Member of Advisory Committee for Health Resources Administration Contract 232-7E 0908 entitled "Alternative Methods by Which Basic Sciences Pharmacy Faculty can Relate to Clinical Practice," College of Pharmacy, University of Minnesota, 1978-79.

23. Presented lectures on Cancer Chemotherapy for Lincoln Medical Education Foundation program for School Nurses Update. Lincoln, Nebraska, June 1979.

24. Abstract and Manuscript Judge for UNMC Student Research Forum, 1979.

25. Presented three lectures on drug metabolism to graduate students in Enzymes Course, Department of Biochemistry, Creighton University, 1979.

26. Vice President, First Lutheran Church, Omaha, NE 1978-79. President First Lutheran Church, Omaha, NE 1979-81 and 1985-87.

27. Member of Site Visit Team, NIH Minority Biomedical Support (MBS) Grant Application, School of Pharmacy, Florida A&M University, Sept 21-23, 1980.

28. Invited to Jordan to negotiate an exchange agreement between the College of Pharmacy, Yarmouk University and the University of Nebraska Medical Center College of Pharmacy.

29. Grant Reviewer for NCI National Large Bowel Cancer Project, January, 1982.

30. Developed a graduate level course in Drug Metabolism and Disposition, UNMC, 1982.

31. Developed a course in nutrition and diet therapy, UNMC, 1983.

32. Member of Review Panel, NIH Minority Biomedical Research Support (MBRS) Program, Washington, D.C. Sept. 28-30, 1983.

33. Session Moderator at the National Research Forum on Aging, Feb. 22-24, 1984, Lincoln, NE.

34. Invited Participant at the Banbury Center of Cold Spring Harbor Laboratory Conference on the Biological Mechanisms of Dioxin Action, April 1-4, 1984.

35. Final Judge of Student Presentations at Student Research Forum, UNMC, 1984.

36. Invited participant at a workshop on Free Radicals, Pathology and Aging, sponsored by the National Institute on Aging, February 16, 1985, San Diego, CA.

37. Special Grant Reviewer for National Institute on Aging, March, 1985.

38. Visiting Professor, Faculty of Pharmacy, Tanta University, Tanta Egypt, April 1985. Presented a series of research related seminars, assisted with cirriculum development, and worked with graduate students in the development of research projects. In Egypt, lectures were also presented at Cario University, Assiut University, Alexandria University and Zagazig University.

39. Invited to Jordan's Yarmouk University and Jordan University Faculties of Pharmacy, May 1985. Worked with faculty on curriculum development and research projects of mutual interest.

40. Member of Review Panel, NIH Minority Biomedical Research Support (MBRS) Program, Washington, D.C., July, 1985.

41. Radio interviews concerning proposed closing of College of Pharmacy, July, 1985; KFAB, KGRI, KNEB, KLIN, KRNY, and Light 96.

42. Interviews concerning vioform research, July, 1985. National Public Radio, KFAB, KFOR, Z92, KGRI, NBC-Chicago, UPI-Lincoln, Washington Post, Medical Letter, Omaha World Herald, Lincoln Star, Bruce Prentosil Channel 6, WOWT (Channel 6) Morning Show.

43. Radio Interview on Research in the College of Pharmacy, UNMC, July, 1985, Z92/KEDS.

44. Presentation to the Goodfellowship Club, Omaha, NE, August, 1985, on "The College of Pharmacy as an Integral Component of a Medical Center."

45. NIH Site Visit Team for Minority Biomedical Research Support Program at California State University, Long Beach, CA. Oct. 1985.

46. Expert witness before FDA Dermatology Panel, Washington, D.C. Nov. 1985. Testified concerning a petition on the removal of iodochlorhydroxyquin from the market. The petition submitted by the Health Research Group was based primarily on studies reported in Publications #100 and 102 by Stohs et al. The Panel voted unanimously to recommend removing the drug as an over-the-counter product and restrict its use to prescription only.

47. Member of Review Panel, NIH Minority Biomedical Research Support Program, Washington, D.C. January 1986.

48. NIH Site Visit Team and Review Panel for Research Centers in Minority Institutions (RCMI) Program at Xavier University, New Orleans, LA, April 1988.

49. Interview on Channel 6 TV "Alive at Five" Program with Gary Kerr and Dave Webber on "Steroid Use by Athletes," Sept., 1988.

50. NIH Site Visit Team and Chairman of Review Panel for Research Centers in Minority Institutions (RCMI) Program at Florida A&M University, Miami and Tallahassee, FL, Feb., 1990.

51. NIH Study Section for Research Centers in Minority Institutions (RCMI) Grant Program, Washington, DC, March, 1990.

52. Reviewed graduate program at Mercer University, Atlanta, GA, April, 1991.

53. N.I.H. Study Section for Research Centers in Minority Institutions (RCMI) grant program, Washington, D.C., March, 1992.

54. Initiated revision of the School Grade Appeals Policy, By-Laws, Scholastic Standing Policy, and Misconduct Policy, Creighton University (1991-92).

55. Established Industrial Advisory Committee, and re-established Alumni Advisory Board, Creighton University, 1991.

56. Established Post-B.S. Pharm.D. recruitment program, hired additional student recruiter and expanded student recruitment efforts, 1991.

57. Established grant review procedure and Grant Review Committee, and established a Faculty and Staff Development Committee, Creighton University, 1991.

58. Remodeled faculty offices, computer laboratory, and Admissions Department offices, Creighton University, 1991.

59. Developed rural health student recruiting agreements with Chadron State College and Wayne State College, 1991.

60. Established faculty, student and research exchange agreement with Marmara University, Istanbul, Turkey, and Creighton University, 1992.

61. Chaired the School Strategic Planning and Implementation Committee, Creighton University, for the Year 2000, being directly responsible for the implementation of the strategic goals which were set forth, 1990-91.

62. Initiated and promoted curriculum revision in pharmacy based on outcome objectives which were established by the faculty at a series of all-faculty retreats. Faculty approved new Pharm.D. curriculum for Creighton University, 1992.

63. Initiated the establishment of Phi Lambda Sigma, National Pharmacy Leadership Honor Society chapter, at Creighton University, 1992.

64. Initiated the development of an advanced degree program in occupational therapy, and hired new faculty in occupational therapy at Creighton University, 1992.

65. Worked with alumni to solicit funds for the Sebastian Pirruccello Scholarship (raised over $50,000), and organized the Sebastian Pirruccello Recognition Dinner at Creighton University, 1992.

66. Organized the establishment of a Department of Physical Therapy, and hired a Director in 1992. Secured approval to offer the first entry-level clinical doctorate (Doctor of Physical Therapy) in 1992, with the first class entering in the fall of 1993. First class of 48 students entered in the fall of 1993. Established the first entry-level Doctor of Physical Therapy program in the country at Creighton University.

67. Initiated the inclusion of Occupational Therapy students in the Creighton University Summer program in the Dominican Republic to provide service, cultural immersion and reflection beginning in 1993.

68. Secured approval to adopt the Doctor of Pharmacy degree as the sole professional degree with the entering class in 1994 at Creighton University.

69. Secured Creighton University approval to establish a Non-traditional Doctor of Pharmacy program, 1993. First students enrolled in May, 1994.

70. Member of Organizing Committee for International Symposium on Oxygen Radicals in Biotechnology and Medicine, Calcutta, India, 1993.

71. Member of Organizing Committee for International Symposium on Pharmacy and Pharmaceutical Sciences, Istanbul, Turkey, 1994. Gave keynote lecture.

72. Secured Creighton University approval to establish a post professional (baccalaureeate or masters degree) Doctor of Occupational Therapy (OTD) program with the first students being admitted in the fall of 1995. The program is one of the first clinical doctoral programs in occupational therapy in the country.

73. Guest editor of a special issue of the Journal of Basic and Clinical Physiology and Pharmacology 6, 1995.

74. Served as a consultant to the Assistant Secretary of Health, U.S. Department of Health and Human Services, regarding the future of academic health centers and the role of pharmacy and allied health professions in academic health centers, October, 1996.

75. Secured Creighton University approval to establish a M.S. degree in pharmaceutical sciences, December, 1996.

76. Initiated the inclusion of Physical Therapy students in the Creighton University Summer program in the Dominican Republic to provide service, cultural emersion and reflection beginning in 1996.

77. Appointed to the International Advisory Board, Research Institute of Preventive and Environmental Medicine and Biotechnology, Athens, Greece, 1997.

78. Raised approximately $165,000 toward the renovation of a clinical education center at Creighton University. Funds were raised through solicitations of alumni, pharmaceutical corporations and friends of the School. 1995-1998.

79. Solicited funds for the establishment of fourteen endowed scholarships (minimum of $25,000 each) for students in the School of Pharmacy and Health Professions, Creighton University. Funds solicited from corporations, alumni and friends of the University. 1993-2003.

80. Procured the establishment of an endowed scholarship from Hoffmann-La Roche Corporation in the amount of $250,000 for minority students, Creighton University. 1994-1998.

81. Member of Organizing Committee for International Symposium on Pharmacy and Pharmaceutical Sciences, Istanbul, Turkey, 1998. Gave initial lecture to start the conference.

82. Member of an organizing committee for an International Symposium on Clinical Pharmacy, Amman, Jordan, 1999.

83. Initiated the first entry-level Clinical Doctoral program in Occupational Therapy in the country with the class of students entering in the fall of 1999 at Creighton University.

84. Established an agreement with the College of Business Administration to offer a combined Doctor of Pharmacy/MBA program in 1999 at Creighton University. This program replaced a previously negotiated program which had attracted very few students because it was participant unfriendly.

85. Initiated the first Non-Traditional, Transitional Doctor of Physical Therapy program in the country in the fall of 1999 at Creighton University.

86. An editorial in the Omaha World Hearld concerning the role of Pharmacists in the prevention of medication errors, February 10, 2000.

87. An editorial in the Omaha World Hearld concerning the national shortage of Pharmacists, December 29, 2000.

88. Initiated the first non-traditional Doctor of Occupational Therapy pathway in the country in the fall of 2001 at Creighton University.

89. Initiated the nation's first entry-level web-assisted Doctor of Pharmacy pathway in the fall of 2001 at Creighton University. All didactic courses in the pathway are available via Internet and CD Rom technology.

90. An editorial in the Omaha World Herald concerning the failures of Medicare prescription discount card programs. Worked with U.S. Senator Chuck Hagels' office to develop legislation for a prescription drug benefit. August 10, 2001.

91. Consultant to the Scientific Advisory Board of the United States Environmental Protection Agency, 1996-2001.

92. Consultant to the National Institute of Environmental Health Sciences for the preparation of a background document on the carcinogenicity of naphthalene, 2001.

93. Member of the Creighton University Steering Committee for the design and building of the new integrated science building which included a new administrative suite for the School and approximately 60 offices for pharmacy faculty as well as two departmental administrative suites. Moved into new facilties in December 2002.

94. Established one of the first residency programs for physical therapy in the nation at Creighton University Medical Center, July, 2003.

95. Initiated the development of a B.S. in Phamacy, Physical Therapy and Occupational Therapy students enrolled in Doctoral programs at Creighton University Medical Center, beginning in the Fall of 2003.

96. Responsible for the development of a Research & Development program at AdvoCare International L.P., hiring individuals in product development, formulation, regulatory affairs and package engineering, 2003 – 2004.

97. Responsible for establishing and developing back-up manufacturers for over 50 AdvoCare products while developing new products for market, 2003 – 2005.

98. Site visits to the following manufacturing facilities during 2004 – 2007: Vitarich, Interactive Nutrition, Arizona Nutritional Supplements (ANS), American Nutritional Corporation (ANC), National Vitamin Company (NVC), Nellson Nutraceuticals, Natural Alternatives International (NAI), Custom Nutrition Laboratories (CNL), Biozone Laboratories, Protec Laboratories, Progressive Laboratories, Cornerstone Research & Development, ELAN Nutrition, and We-Pack-It-All.

99. From 1998-2014 participated in over 40 AdvoCare Success Schools, providing product training and interfacing with Distributors, speaking to as many as 25,000.

100. From 1998-2014, gave numerous AdvoCare product training programs around the country including in Texas, Colorado, Florida, Washington, California, Michigan, Nebraska, Iowa, Illinois, Tennessee, Georgia, South Carolina, South Dakota, Missouri, Mississippi, Alabama, Oklahoma, Kentucky, Virginia, Washington, Oregon, and Hawaii.

101. Established and coordinated a symposium on "dietary supplement safety" at the annual meeting of the American College of Nutrition, Orlando, FL, 2007.

102. Applied for patents on behalf of AdvoCare for 3 products between 2005 – 2006.

103. Between 2004–2008, was responsible for developing over 25 new AdvoCare products and making improvements to over 40 products based on the latest science.

104. Participated in a series of radio talk shows on KCFO (AM970) regarding nutrition, particularly for senior citizens, 2007.

105. Responsible for coordinating research on a series of AdvoCare products including MNS Orange, MNS Platinum, A-Supreme, ThermoPlus, Carb-Ease, SPARK Energy Drink, Rehydrate Electrolyte Replacement Drink, Muscle Fuel, and the glycemic indices of all AdvoCare drinks, shakes and bars, 2003-2008.

106. Responsible for ultimate development of quality assurance/quality control procedures, adverse events management and procedures, clinical assessment of products, product launch and elimination procedures and other general operating procedures within AdvoCare's R&D department, 2003-2005.

107. Appointed to the Certification Board for Nutrition Specialists, 2004 – 2007, a national nutrition certification program. Reappointed in 2008. Vice – Chair for Administration 2009-2010. Served as President from 2011-2014.

108. Participated in various AdvoCare distributor incentive trips: Australia and New Zealand, 2002; Maui, Hawaii, 2004; Lake Louise, Canada, 2004; Little Palm Island, FL, 2005; San Francisco, CA, 2006; New York City, NY, 2006; Alaska Cruise, 2007; Peter Island, 2009; Paris, 2013; Mediterranean, 2014.

109. For the American College of Nutrition (2008-2009), served as the chief scribe for revision of By-Laws and the Standing Committee Procedures document. Drafted a Strategic Planning document. Chaired the Publications Committee, resulting in a change in publishers for the Journal of the American College of

Nutrition with significant savings. Revised and updated the Editorial Board of the Journal, and instituted co-editors.

110. Consultant for Nutratech/Novel ingredients 2009-present. Traveled and gave presentations in California, New Jersey, New York, Utah, Colorado, Texas, Illinois.

COMMITTEES:

University of Nebraska-Lincoln:

Faculty Health Council, 1967 - 71
Marshalling Corps, 1968 - 70
Nebraska College Opportunity Ad Hoc Committee, 1970 - 71
Ad Hoc Committee to Recommend Changes in the Structure and Operation of the Senate, 1969
Liaison Committee, 1970-73, Secretary, 1971 - 72
Search Committee for Vice-Chancellor of Academic Affairs, 1972

University of Nebraska System:

Executive Graduate Council, 1973 - 75
Executive Graduate Council, 1985 - 87
Executive Committee of the Executive Graduate Council, 1986 - 87

College of Pharmacy - University of Nebraska:

Admission and Scholarship Review, 1968 - 71
Course of Study, 1967 - 72
Grade Appeals, 1968 - 72
Curriculum Committee, 1968 - 74
Departmental Graduate Committee, 1968 - 84
Pharmaceutical Sciences Area Graduate Committee, 1969 - 74
Search Committee for a Dean of the College, 1971
Ad Hoc Committee on Promotion and Tenure, 1971
Space Utilization Committee, 1972 - 76
Executive Committee, 1972 - 1987
Ad Hoc Faculty Policies and Procedures Committee, Chairman, 1973
Teaching Improvement Committee, 1974 - 75
Ad Hoc Committee on NCACS Accreditation, 1976
Search Committee for Chairman, Dept. of Pharmacodynamics and Toxicology, 1979
ACPE Accreditation Self-Study Review Committee, 1979
Continuing Education Committee, 1978 - 85, 1986 - 87
Self-Study Role and Mission Subcommittee, Chairman, 1985
Self-Study Budget and Finance Subcommittee, 1985
Safety Committee, 1984 - 87
GAPS Grant Review Committee, 1985 - 86
Strategic Planning Committee, 1986 - 87
Long-Range Planning Committee, 1983 - 85

Pharmaceutical Sciences Graduate Program Committee, 1983 - 87
Ad Hoc By-Laws Committee 1983 - 84

University of Nebraska Medical Center:

Pre-Clinical Seed Grant Review Committee, 1972
Ad Hoc Committee on the Academic Organization of the Medical Center, 1973 - 74
Medical Sciences Area Graduate Committee, 1973 - 1984
University Graduate Council, Medical Center Representative, 1973 - 74
Ad Hoc Committee for Organization of Medical Center Senate, 1974
Seed Grant Review Committee, 1974 - 76
Liaison Committee, 1975 - 76
Systems Office Task Force on Travel, Moving, and Recruitment Expenses, Medical Center Representative, 1975 - 76
Ad Hoc Committee on Graduate Fellowships, 1975 - 76
Graduate Council, 1977 - 79
Ad Hoc Committee on Selecting a Grants Manager, 1977
Chancellor's Ad Hoc Task Force on Research, 1977 - 79
Chancellor's Ad Hoc Oncology Program Committee, 1977 - 1979
Search Committee for a Dean of College of Medicine, 1978
Search Committee for a Dean of Graduate Studies and Research, 1978 - 79
Ad Hoc Continuing Education Committee on Gerontology, 1979
Faculty Senate Grievance Committee, 1979-82; Chairman 1981 - 82
Clinical Cancer Education Committee, 1980 - 1984
Cell Sorter Facility Policy Committee, 1980 - 1984
Search Committee for Director of Eppley Institute, 1981 - 82
UNMC Special Functions Committee 1981-1982, 1984 - 85
LB 506 Grant Reviews Committee, 1982, 1983, 1984, 1986
Chancellor's Task Force on Nutrition, 1984 - 85
Executive Graduate Council, 1985 - 87
Bulk Purchasing Committee, 1985 - 86
First Professional Degree Committee, 1985 - 86
Marshalling Corps, 1982 - 86
Tenure Review Committee, 1985 - 86
Board of Directors, Toxicology Program, 1985 - 1989
Faculty Senate Academic Freedom and Tenure Committee, 1986 - 87
Chancellor's Planning Committee, 1986 - 1987
Search Committee for Dean of College of Pharmacy, 1986

School of Pharmacy and Allied Health - Creighton University:

Administrative Committee, 1989 - 2003
Executive Committee, 1989 - 2003
Pharmacy Curriculum Committee, 1989 - 1992
Grade Appeals Committee, 1989 - 1990
Scholastic Standing Committee, Chairman, 1989 - 1990
Pharmacy Accreditation Self-Study Steering Committee, 1989 - 1991
Finance Subcommittee, Chairman

Faculty and Staff Subcommittee, Chairman
Creighton in the Year 2000 Planning Committee, Chairman, 1990
Occupational Therapy Admissions Committee, 1990 - 2003
Pharmacy Admissions Committee, 1990 - 2003
Alumni Advisory Board, Chair, 1990 - 2003
Strategic Planning and Implementation Committee, 1991 - 2003
Physical Therapy Admissions Committee, 1992 - 2003
Industrial Advisory Board, Chair, 1992 - 2003

Creighton University:

Health Sciences Center Safety Committee, 1989 - 1992
International Students Committee, 1989 - 1991
Planning Steering Committee, Creighton in the Year 2000, 1990 - 1992; 1994 - 1996
Academic Council, 1990 - 2003
Academic Leadership Committee, 1990 - 2003
Council of Deans, 1990-present; Chair – 1996 - 97
Grant Review Committee, 1990 - 1992
Search Committee for Academic Vice President, 1993
Search Committee for Assistant Grants Administrator, 1993
Search Committee for the Dean, School of Dentistry, Chairman, 1993 - 94
International Planning and Advisory Committee, 1994 - 96
Search Committee for Director of Annual Giving, 1995 - 96
Search Committee for Director of Alumni Relations, 1996
Search Committee for the Dean, College of Business Administration, Chairman, 1996 - 97
Strategic Planning Committee for Programs in the Dominican Republic, 1996 - 97
Process Redesign Advisory Committee, 1996 - 97
Steering Committee for the Center for Practice Improvement and Outcomes Research, 1996 - 2003
Budget Redesign Advisory Committee, 1997 - 98
Cancer Center Advisory Board, 1995 - 98
Budget Design Planning Advisory Committee, 1997
Academic Process Redesign Committee, 1997 - 98
Center for Health Policy and Ethics Deans Advisory Council, 1996 - 2003
Search Committee for the Dean, Chairman, School of Law, 1998 - 99
Cancer and Smoking Disease Research Program Advisory Committee, 1998 - 2003
Strategic Planning - Beyond Creighton 2000 Committee #1, Chair, 1998 - 99
Student Information Systems Coordinating Committee, 1999 - 2001.
Creighton University Strategic Planning Committee, 1999 - 2000.
Creighton University Strategic Planning Academic Excellence Subcommittee, 2001
Biomedical Research Advisory Council, 2002 - 2003
Science Complex Steering Committee, 2000 - 2003
HIPAA Steering Committee, 2002 - 2003
Search Committee for Vice President University Relations, 2002 - 2003

State:

Governor's Commission on Drug Abuse, 1970 - 71
State Commission on Drugs, 1971 - 72
Research Committee of the Nebraska Heart Association, 1973 - 76
Sigma Xi Faculty & Graduate Student Outstanding Scientist Awards Committee, 1979
Nebraska Chapter of Sigma Xi President-Elect, 1983-84; President, 1984 - 85
State Board of Pharmacy's Review Committee for Continuing Pharmacy Education 1984 - 87
Nebraska Pharmacy Strategic Planning Committee, 1994 - 96
Nebraska Pharmacy Coordinating Council, 1996 - 2003
Creighton Federal Credit Union, 1997 - 2001
Nebraska Pharmacists Association Legislative Committee, 1997 - 2002

National:

Scientific Program Committee of the Pharmacognosy and National Products, Section of the Academy of Pharmaceutical Sciences, 1970 - 71
Chairman of the Scientific Program Committee of the American Society of Pharmacognosy, 1972 - 73
Chairman of the Kilmer Prize Committee of the Pharmacognosy and Natural Products Section of the Academy of Pharmaceutical Sciences, 1973 - 74
Chairman, Special Projects Grant Committee, American Association of Colleges of Pharmacy, 1983 - 85
Resolution Committee, American Society of Pharmacognosy, 1973 - 74
American Association of Colleges of Pharmacy, Section of Teachers of Biological Sciences Nominating Committee Chairman, 1974 - 75
Committee on Long-Range Goals, Pharmacognosy and Natural Products Section of the Academy of Pharmaceutical Sciences, 1977 - 78
Chairman, Resolutions Committee, Biological Sciences Section, American Association of Colleges of Pharmacy, 1978 - 79
Chairperson-Elect, 1979 - 80, Biological Sciences Section of The American Association of Colleges of Pharmacy
Kilmer Prize Committee, Academy of Pharmaceutical Sciences, 1979
Advisory Committee for HRA Contract 232-7E 0908, University of Minnesota, 1978 - 79
Kilmer Prize Committee, Academy of Pharmaceutical Sciences, 1980 - 82
Chairman, Kilmer Prize Committee, Academy of Pharmaceutical Sciences, 1980 - 81
Chairman, Biological Sciences Section, American Association of Colleges of Pharmacy, 1980 - 81
Site Visit Team, NIH Minority Biomedical Research Support (MBRS) Grant Application for School of Pharmacy, Florida A & M Univ., Sept 21 - 23, 1980
Distinguished Pharmacy Education Award Committee, American Association of Colleges of Pharmacy, 1982 - 83
Chairman, Nominating Committee, Biological Sciences Section of the American Association of Colleges of Pharmacy 1982 - 83, 1983 - 84, 1984 - 85

Secretary, Council of Sections of the American Association of Colleges of Pharmacy, 1982 - 83
American Association of Colleges of Pharmacy Council of Faculties, Faculty Development Committee, 1984 - 85
Site Visit Team, NIH Minority Biomedical Research Support (MBRS) Grant Application for program at California State University, Long Beach, CA, 1985
Review Panel, NIA Minority Biomedical Research Support Program, Washington, DC, 1985, 1986
Review Panel, NIH Research Centers in Minority Institutions (RCMI), 1988
APhA Academy of Pharmaceutical Sciences Selection Committee for the Research Achievement Award in Pharmacodynamics, 1988 - 89
Society of Toxicology Central States Chapter Program Committee, 1990
Review Panel, Chairman, NIH Research Centers in Minority Institutions (RCMI), 1990
Nominating Committee, Biological Sciences Section of the American Association of Colleges of Pharmacy, 1990 - 91
Central States Chapter of the Society of Toxicology
Vice President, 1991 - 92
President-Elect, 1992 - 93
President, 1993 - 94
Technical Committee, Society of Toxicology, 1990 - 92
Environmental Protection Agency Dioxin Science Advisory Board, 1995 - 96
Ethics Committee, Association of Schools of Allied Health Professions, 1994 - 96
USP Division of Standards Development Advisory Panel on Identification and Standardization of Natural Products, 1995 - 2000.
American Association of Colleges of Pharmacy Special Committee to Study Structure and Process, 1995 - 98
American Association of Colleges of Pharmacy Institutional Research Advisory Committee, 1996 - 97
NIEHS Special Emphasis Panel, 1997, Chair
Board of Directors, American Association of Colleges of Pharmacy, 1998 - 2001
American Association of Colleges of Pharmacy Private Schools and Colleges Forum, Secretary – 1994 - 96; Chair, 1996 - 98
Site Visit Team for Accreditation in Physical Therapy, University of Washington, Seattle, WA, 2002.
Site Visit Team for Accreditation in Physical Therapy, Bellarmine University, Louisville, KY, 2004.
Elected member of the Certification Board for Nutrition Specialists, 2004, re-elected 2007.
American College of Nutrition 21st Century Steering Committee,subcommittees on By-Laws, Structure, and Development, 2008-2009.
Chairman, American College of Nutrition Publications Committee, 2008-2009

CONSULTING (PARTIAL LIST):

AdvoCare International

U.S. Army Medical Research Institute for Infectious Diseases
Wyeth-Ayerst
Sandoz Drug Company
Chemical Products, Inc.
InterHealth Nutraceuticals
U.S. Environmental Protection Agency
Meridrew Enterprises
Cape Cod Laboratories, Inc.
Hughes Enterprises
Air Force Office of Scientific Research
ConAgra Corporation
SmithKline Beecham
Proctor & Gamble
Greek National Institute of Health
Marmara University, Istanbul, Turkey
Jordan University, Amman, Jordan
Tanta University, Tanta Egypt
Yarmouk University, Yarmouk, Jordan
Wayne State University, Detroit, Michigan
Mercer University, Atlanta, Georgia
U.S. Pharmacopeial Convention,
Proliant Health & Biologicals
Custom Nutrition Laboratories(CNL)
SIO Healthcare Advisors
Nutratech Inc.
Various Law Firms

RESEARCH SUPPORT, GRANTS AND CONTRACTS RECEIVED:

1. Smith, Kline and French Foundation Award of $6750 for the purchase of a liquid scintillation counter, 1968.

2. University of Nebraska Research Council Award of $3190 for the purchase of a high speed refrigerated centrifuge, 1968.

3. Nebraska Heart Association Grant of $2070 for a study on "The Metabolism of 14C-Cholesterol in Dioscorea Tissue Suspension Cultures". July 1, 1968 - June 30, 1969.

4. Nebraska Heart Association Grant of $1665 for an investigation of the "Metabolism of Heart Glycosides by Cardiac and Hepatic Microsomes". September 1, 1968 - August 31, 1969.

5. National Science Foundation Grant of $11,200 for an investigation entitled, "Studies on Cholesterol Side Chain Cleavage Enzymes in Cardenolide Biosynthesis." September 1, 1969 - August 31, 1971.

6. Public Health Service Grant of $24,406 for an investigation on the "Production and Biosynthesis of Medicinals by Plants." November 1, 1969 - October 31, 1972.

7. Nebraska Heart Association Grant of $4300 for an investigation entitled "In Vitro Studies on Cardiac Glycoside Metabolism". July 1, 1971 - June 30, 1972.

8. National Science Foundation Grant of $12,000 for "Sterol Side Chain Cleavage and Cardenolide Biosynthesis in Cardenolide Producing Plants". January 1, 1971 - December 30, 1973.

9. Nebraska Heart Association Grant of $6600 for an investigation entitled, "In Vitro Studies on Cardenolide Metabolism". July 1, 1972 - June 30, 1973.

10. University of Nebraska Medical Center Seed Grant of $3820 for "Preliminary Studies on the Mechanism of Action of PCN." November 1, 1972 - June 30, 1973.

11. Nebraska Heart Association Grant of $5090 for an investigation entitled "PCN Mechanism of Protection from Digitalis Toxicity." July 1, 1973 - June 30, 1974.

12. Lincoln-Lancaster Drug Projects Contract for approximately $2500 to "Conduct Routine Urinalysis for Drugs for 'Full Circle' Staff and Residents." Co-sponsored with Dr. E.B. Roche, December 1, 1973 - June 30, 1974. Renewed for July 1, 1974 - December 31, 1974.

13. Nebraska Heart Association Grant of $4695 for an investigation entitled "Blood Level Determinations of Thiazide Diuretics." July 1, 1974 - June 30, 1975.

14. ConAgra Corporation contract of $9490 for a study on "The Growth of Selected Microorganisms on Oat Hulls as a Potential Source of Protein." September 1, 1974 - August 31, 1975. Co-principal investigator with Dr. H. Rosenberg.

15. University of Nebraska Medical Center Seed Grant of $4675 for an investigation on "The Growth of Microorganisms on Agricultural Wastes as a Source of Utilizable Protein." July 1, 1974 - June 30, 1975. Co-principal investigator with Dr. D. H. Rosenberg.

16. University of Nebraska Medical Center Seed Grant of $2100 to conduct "An Evaluation of the Physiological and Metabolic Changes Associated with

Chronic Methaqualone Abuse." July 1, 1974 - June 30, 1975. Co-principal investigator with Dr. R.W. Piepho.

17. Nebraska Heart Association Grant of $4852 for the study on "Blood Level Determinations of Thiazide Diuretics." July 1, 1975 - June 30, 1976.

18. ConAgra Corporation contract of $3090 for a study on "The Growth of Selected Microorganisms as a Potential Source of Protein on Carbohydrate Extracts Obtained from Oat Hulls." September 1, 1975 - March 1, 1976. Co-principal investigator with Dr. H. Rosenberg.

19. Nebraska Heart Association Grant of $5310 for an investigation on the "Metabolism of Quinidine-Like Antiarrhythmic Agents". July 1, 1977 - June 30, 1978.

20. University of Nebraska Medical Center Seed Grant of $4949 for an investigation entitled "The Mechanism and Significance of Intestinal Drug Metabolism". July 1, 1977 - June 30, 1978.

21. Center on Aging Grant of $1980 to initiate a project entitled "Glutathione Levels in Rat Hepatic and Extrahepatic Tissues as a Function of Age." March 1, 1978 - September 30, 1978.

22. Nebraska Heart Association Grant of $5840 to continue our investigation on the "Metabolism of Quinidine-Like Antiarrhythmic Drugs." July 1, 1978 to June 30, 1979.

23. University of Nebraska Medical Center Seed Grant of $7442 for a project entitled "Glutathione Levels and Xenobiotic Metabolism in Hepatic and Extrahepatic Tissues as a Function of Age." July 1, 1978 - June 30, 1979.

24. Center on Aging Grant of $1900 to initiate a project entitled "Xenobiotic Metabolism in Hepatic and Extrahepatic Tissues as a Function of Age." November 15, 1978 - September 30, 1979.

25. Public Health Service Grant for $68,238 entitled "Childhood Lead, Pyrimidines and Metabolic Debris." April 1, 1978 - March 31, 1980. Associate Investigator. Dr. Carol Angle - Principal Investigator.

26. Nebraska Heart Association Grant of $5965 for a study entitled "Lithium and Cardiac Rhythm, Electrolytes and ATPase." July 1, 1979 - June 30, 1980.

27. National Institute on Aging (PHS) Grant for $75,859 entitled "Glutathione Levels and Drug Metabolism Relative to Age." July 1, 1979 - November 31, 1982.

28. Public Health Service Grant for $97,741 entitled "Childhood Lead, Pyrimidines and Metabolic Debris." April 1, 1980 - March 31, 1983. Associate Investigator. Dr. Carol Angle - Principal Investigator.

29. American Heart Association Nebraska Affiliate Grant-in-Aid for $7000, entitled "Hemodynamic Properties of 6'-Hydroxycinchonine," July 1, 1980 - June 30, 1981.

30. University of Nebraska Medical Center Seed Grant of $1618 for a project entitled "Intestinal Tract as the Target Organ for TCDD Toxicity," July 1, 1980 - June 30, 1981.

31. University of Nebraska Medical Center Seed Grant of $5000 for a project entitled "Effects of Dilantin on Estrogen Metabolism," July 1, 1981 - June 30, 1982. Dr. H. Lemon - Principal Investigator.

32. State of Nebraska Cancer Research Grant for $30,500 entitled "Cancer and Aging: Role of Glutathione and its Metabolism," July 1, 1982 - June 30, 1983.

33. Public Health Service Grant for $119,290 entitled "Lead and the Red Cell Membrane." July 1, 1983 - June 30, 1986. Co-investigator. Dr. Carol Angle - Principal Investigator.

34. American Heart Association (Nebraska Affiliate) Grant for $23,821 entitled "Lipid Peroxidation: Cause of Myocardial Toxicity to TCDD." July 1, 1983 - June 30, 1985.

35. State of Nebraska Research Grant for $31,911 entitled "Cancer and Aging: Roles of Glutathione and DNA Damage." July 1, 1983 - June 30, 1984.

36. March of Dimes Birth Defects Foundation for $50,000 entitled "Role of Lipid Peroxidation in TCDD-induced Testicular Atrophy." December 1, 1983 - November 30, 1985.

37. Ayerst Laboratories contract for $31,800 entitled "Efficiency of Betacor in Mild-to-Moderate Hypertension (One Step Therapy)." October 1984 - March, 1985.

38. Ayerst Laboratories contract for $31,800 entitled "Efficiency of Betacor in Mild-to-Moderate Hypertension (Two Step Therapy, Post-Thiazide)." October 1984 - March, 1985.

39. National Cancer Institute Laboratory Cancer Research Support (CORE) Grant, 1984-86. Co-Investigator. Dr. E. Bresnick, Principal Investigator.

40. March of Dimes Birth Defects Foundation for $40,000, entitled "Mechanism of TCDD-Induced Testicular Atrophy." December 1, 1985 - November 30, 1987.

41. National Institute of Environmental Health Sciences Area Grant for $50,000, entitled "Free Radicals and Iron in TCDD-Induced Lipid Peroxidation." July 1, 1987 - April 30, 1990.

42. National Institute of Environmental Health Sciences Sabbatical Award for $28,336 for September 1, 1987 - June 30, 1988.

43. American Aging Association, Inc. grant of $500, to support a study entitled "DNA Single Strand Breaks in Peripheral Lymphocytes of Human Subjects as a Function of Age." February 1, 1989 - January 30, 1990. Co-Investigator - Dr. T. Lawson.

44. Health Future Foundation Grant of $5000, to support a study entitled "Macrophage Activation by TCDD." December 1, 1989 - November 30, 1990.

45. William Randolph Hearst Foundation Grant of $30,000, to establish a graduate fellowship. December, 1989.

46. Air Force Office of Scientific Research Grant for $288,897 entitled "Production of Reactive Oxygen Species by Polyhalogenated Cyclic Hydrocarbons (PCH)." June 15, 1990 - June 14, 1993.

47. Ciba-Geigy Grant of $2500 for support of Graduate Students, March, 1990.

48. Smith-Kline-Beecham Grant of $32,660 for support of graduate student George Baker. September, 1990.

49. U.S. Army Medical Research and Development Command Grant for $61,869 entitled "Specific Mechanism-Based Glycosidase Inhibitors as Chemoprotectants Against Ricin Toxicity." June, 1991 - May, 1992.

50. Creighton University BRSG for $2,000 with graduate student Dan Muldoon for a project entitled "Potential Chemoprotectants Agains Ricin Toxicity." June, 1991 - May, 1992.

51. Ciba-Geigy grant of $2,500 for support of the computer laboratory, February, 1992.

52. State of Nebraska Department of Health grant for $59,902 for a project entitled "Smokeless Tobacco: Macrophage Activation and Cytotoxicity." July, 1992 - June, 1994.

53. Health Future Foundation grant of $10,000 to support a study entitled "Production of Reactive Oxygen Species by Cadium and Chromium." July 1, 1993 - June 30, 1994.

54. California Aging Research Institute, Inc. grant of $4,500 to support a study entitled "Age-Related Oxidative Damage in the Retina." October, 1993 - March, 1996.

55. Air Force Office of Scientific Research grant for $321,732 entitled "Mechanisms associated with the toxicity of polyhalogenated cyclic hydrocarbons and heavy metals." November 1, 1993 - October 31, 1996.

56. State of Nebraska Department of Health grant for $151,004 entitled "Smokeless tobacco, reactive oxygen species, oral cancer and collagen synthesis." Dr. M. Bagchi - Principal Investigator. October 1, 1994 - July 1, 1996.

57. Procter & Gamble research grant for $24,998 entitled "Restraint stress, diet, and chemically induced oxidative stress in rats, and protective ability of bismuth subsalicylate (BSS)." Dr. D. Bagchi - Principal Investigator. January 1, 1996 - September 30, 1996.

58. Air Force Office of Scientific Research grant for $339,097 entitled "Common pathways in the toxicity of structurally diverse xenobiotics." November 1, 1996 - October 31, 1999.

59. NACDS Education Foundation grant for $25,000 for a project entitled "A certificate program in diabetes care for pharmacists." Dr. Ken Keefner - Principal Investigator. September, 1997 - July, 1998.

60. NIEHS grant for $101,243 for a project entitled "TCDD-induced oxidative stress in tissues of rats." Dr. Ezdihar Hassoun - Principal Investigator, Dr. S.J. Stohs - Co-Investigator. April, 1998 - March, 1999.

61. NIH grant for $70,000 for a project entitled “Helicobacter pylori, oxidative stress and gastric injury”. April, 1998 - March, 2001.

62. Institute for the Advancement of Community Pharmacy to create "an asynchronous web-based Doctor of Pharmacy Pathway at Creighton University". July 1, 2000 - June 30, 2003, $1 million.

63. Support for a web-based Doctor of Pharmacy pathway at Creighton University. July 1, 2000 – June 30, 2002: ShopKo - $10,000; Walgreen Corporation - $50,000; J.M. Long Foundation - $50,000; Eckerd Corporation - $10,000; AstraZeneca - $1,000; BD Pharmaceutical Corp. - $10,000; CVS Corporation - $25,000.

64. Research grant from AdvoCare International for $95,435 for a project entitled "Effects of long term chronic exposure to MNS orange on serum chemistry and histopathology in mice." Dr. S.D. Ray – principal investigator, Dr. S.J. Stohs – co-principal investigator. July, 2001 - June, 2003.

65. Research grant from AdvoCare International for $5,000 for a project entitled "Quantitation of ephedra and caffeine in MNS Orange dietary supplement." Dr. Alekha Dash – principal investigator, Dr. S.J. Stohs – co-principal investigator. September, 2001 - June, 2003.

66. Research grant from InterHealth Nutraceuticals Inc. for $20,000 for a project entitled "Determination of testicular atrophy (lipid peroxidation, physical observations and histopathology) in rats following supplementation with Super CitriMax. Dr. S.E. Ohia – principal investigator, Dr. S.J. Stohs – co-principal investigator. January, 2002 - December, 2002.

67. Research grant from the Health Future Foundation for $850,000 for the establishment of a Toxicology Center. Dr. S.J. Stohs – principal investigator, Dr. S.E. Ohia – co-principal investigator, Dr. D. Bagchi – co-principal investigator. July, 2002 - June, 2005.

68. Research grant from InterHealth Nutraceuticals Inc. for $50,000 for a project entitled "ChromeMate 90-day safety study." Dr. Michael Shara – principal investigator, Dr. S.J. Stohs – co-principal investigator. January, 2002 - September, 2003.

69. Over $800,000 in research grants funded by AdvoCare International between 2003 – 2014 for various studies in animals and human subjects on the efficacy and safety of selected AdvoCare products. Dr. S. J. Stohs served as Co-principal investigator on these studies.

70. Research grant from Nutratech Inc. for $22,000 for "A comparative effectiveness study evaluating changes in resting metabolic rate associated with four different nutritional supplements in human subjects." Dr. S. J. Stohs Co-principal investigator with Dr. Gil Kaats, Dr. Harry Preuss-Co-Investigator, June, 2010 – July, 2011.

Updated 04-03-16

EXHIBIT 3

COMMENTARY ON THE REPORT OF DR. SIDNEY J. STOHS CONCERNING THE HEALTH CLAIMS FOR NTX, A PRODUCT COMPOSED OF GLYCYRRHIZIN AND MANNITOL, IN CONJUNCTION WITH ALCOHOL CONSUMPTION

Harry G. Preuss MD, MACN, CNS
Professor of Biochemistry, Medicine, Physiology, and Pathology
Georgetown University Medical Center
Washington, DC 20057

SOURCES USED IN ADDITION TO THE STOHS REPORT

MATERIAL PROVIDED BY COMPANY
PATENT (REF 14)
ADDITIONAL REFERENCES (102-105)

INTRODUCTION

The excellent, extensive report of Dr Sidney J Stohs was the primary basis for my commentaries. I found his report to be quite reasonable and complete in covering the safety and efficacy of the NTX product that is intended to prevent and/or ameliorate the adverse effects of alcohol consumption. In my account, I take highlights from the Stohs' report that were most meaningful to me in making my conclusions.

Previous evidence indicates that the major elements of NTX, namely glycyrrhizin and mannitol individually and combined, favorably decrease reactive oxygen species (ROS) and metabolites, lipid peroxidation (MDA formation) as well as carbonyl protein formation secondary to alcohol-induced production, while positively increasing the levels of the endogenous antioxidant reduced glutathione (GSH)[14,16-49]. In other words, these elements, alone or together, have the ability to lessen hepatic damage from alcohol. In addition, mannitol being an osmotic diu-

retic would lessen the alcohol challenge in a secondary manner by allowing for more rapid renal excretion of the consumed liquid – thus lessening the alcohol challenge[105]. Accordingly, data obtained from the patent[14] indicates that the two elements together have additive effects, and in some parameters studied, even synergistic tendencies.

The Stohs' report states that the patent for NTX points out that glycyrrhizin is used at a concentration of 0.05-0.3 % while mannitol is used in the range of 0.5-3.0 %[14]. Someone consuming 60 ml of an alcoholic beverage (4 shots) containing NTX, would consume in the range of 30-180 mg of glycyrrhizin and 300 mg-1.80 grams of mannitol. For the individual consuming 60 ml of an alcoholic beverage (4 shots) containing NTX, he/she would consume 30-180 mg of glycyrrhizin and 300 mg-1.80 grams of mannitol. The dose of glycyrrhizin would be theoretically lessened by the presence of an osmotic diuretic like mannitol[105]. Long-term experiences using intravenous levels of glycyrrhizin at daily doses of 200 mg without major complications would suggest that the NTX concentration taken orally is safe[98,103].

BACKGROUND

[REFERENCES 1-19]

It is generally recognized that excess chronic alcohol consumption is frequently associated with severe liver perturbations and is quite commom[1-19]. Alcoholic liver disease is composed of various histological stages -- from hepatic fat accumulation to more advanced stages such as alcoholic steatohepatitis, cirrhosis, hepatocellular carcinoma and liver failure [3,4]. A typical pathological progression includes: metabolism of

alcohol to very toxic acetaldehyde along with a variety of free radical species, creation of harmful reactive oxygen (ROS) and nitrogen species with resulting oxidative stress, discharge of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), deviant lipid metabolism, DNA damage, and ultimately cell necrosis with general systemic malfunctions[3-9].

The status of hepatic health can be assessed by numerous so called biomarkers. Biomarkers indicating disturbed hepatic function include measurements of the blood serum levels of the enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT) and alkaline phosphatase (ALP). Importantly, elevated circulating levels of these enzymes are found when the liver is injured from excess alcohol intake and are important biomarkers of the health status of the liver. On the positive side, reduced serum levels of these markers occur as the liver begins to repair[8-12].

In addition to enzymes, other established markers signifying the status of hepatic function are available. For example, the hepatic metabolism of alcohol leads to excess production of tissue-injuring free radicals and reactive oxygen species (ROS) as mentioned above. Markers of oxidative stress and oxidative tissue damage include reactive oxygen species (ROS) production, the lipid metabolite malondialdehyde (MDA), protein carbonyl formation, and DNA damage. Also, reduced glutathione (GSH) content is a key intracellular antioxidant and chemoprotectant in human tissues, because a diminution in GSH is associated

with augmented oxidative stress as occurs in the case of alcoholic liver disease[8-12]. Accordingly, GSH content is an important marker of the status of ROS and free radicals.

Because of its proclivity, there is a vital need to develop safer strategies to ameliorate the effects of chronic alcohol overindulgence and to develop strategies that reduce alcohol-associated morbidity and mortality. NTX is a patented product composed of the primary ingredients glycyrrhizin and mannitol that act effectively when combined. Numerous studies in humans and animals have demonstrated the hepatic protective effects of NTX as well as its individual components during acute and chronic alcohol consumption. Importantly, the safety of the ingredients in NTX is well established.

HEALTH CLAIMS FOR NTX

1. PROVIDES ANTIOXIDANT AND ANTI INFLAMATORY SUPPORT

References [14, 16-49]

A plethora of animal studies have shown that licorice extract and mannitol alone favorably decrease reactive oxygen species (ROS) and metabolites, lipid peroxidation (MDA formation) as well as carbonyl protein formation secondary to alcohol-induced production, while positively increasing the levels of the endogenous antioxidant reduced glutathione (GSH)[14,16-49]. Many of these findings with glycyrrhizin and mannitol have been corroborated in clinical studies [15-17,45-47]. In addition, NTX containing both ingredients decreases the alcohol-induced DNA damage in human peripheral lymphocytes[16]. NTX

also enhances the levels of the enzymes catalase, GPX and SOD that decompose ROS[14] and, in turn, protects hepatocytes from the damaging influences of ROS. Worth reemphasizing, these findings emanate from over 30 published animal, *in vitro*, and clinical studies that confirm the antioxidant and anti-inflammatory properties of the major of individual components of NTX --glycyrrhizin and mannitol as well as NTX itself.

2. HELPS PROTECT AGAINST OXIDATIVE DAMAGE TO THE LIVER.

References [14-16,19,30,35,36,38-44,50-69]

A multitude of published human studies and animal studies have revealed that NTX and a major active constituent (glycyrrhizin) provide a shield against oxidative injury to the liver. No doubt this is due, at least in part, to overcoming primary mechanisms behind alcohol toxicity and liver disease, i.e., oxidative stress and damage[14,-16,19,38-44,50-69] .

A meta-analysis of 12 randomized controlled trials summarizes the ability of a glycyrrhizin product to ameliorate liver dysfunctions in 838 patients with alcoholic liver disease[53]. Because the study found that glycyrrhizin significantly decreases serum ALT and AST levels, the authors concluded that the glycyrrhizin product can significantly improve liver function in alcoholic liver disease[53].

Similar findings occurred when studies examined the combination of glycyrrhizin with mannitol (NTX). A randomized, double-blind, placebo-controlled clinical trial involving human subjects reported that

NTX provided significant liver protection during 12 days of alcohol consumption as evidenced by decreases in the biomarkers of liver toxicity ALT, AST, GGT and ALP[15]. A second randomized, double-blind, placebo-controlled cross-over clinical trial was conducted in which all participants received alcohol with and without NTX[16]. Consumption of alcohol alone caused the following: increased serum ROS and its metabolites, decreased serum GSH, and elevations in MDA, protein carbonyl, and DNA damage in peripheral lymphocytes. However, consuming the alcohol with NTX significantly reduced levels of ROS and several oxidative markers -- demonstrating the tissue protective effects of NTX.

3. HELPS MAINTAIN NORMAL LIVER ENZYME PRODUCTION AND FUNCTION

References [14-16,19,30-34,38,54-67]

Several published human, animal and cell culture studies have discovered that NTX and its primary active constituents glycyrrhizin and mannitol will permit normalization or, at least, near-normalization of a wide range of liver enzymes. These include ALT, AST, GGT, ALP, SOD, GPX and catalase. In addition, they have the ability to ameliorate alcohol-induced inflammation and creation of products signifying oxidative tissue damage. The latter include ROS, carbonyl protein and MDA. Therefore, glycyrrhizin and mannitol singly and in combination protect the liver, and support and facilitate normal liver function in the presence of many challenges including alcohol.

Glycyrrhizin has been shown to reduce, at least to 35%, serum ALT and AST in patients with chronic hepatitis[54-67]. The results depended upon duration of glycyrrhizin administration[54].

A combination of glycyrrhizin and mannitol (NTX) in a randomized, double-blind, placebo-controlled clinical trial involving 12 human subjects who received alcohol daily for 12 days with or without NTX was carried out. When NTX was co-administered with alcohol, it provided significant decreases and normalization in the examined biomarkers of liver toxicity ALT, AST, GGT and ALP[15,16]. Accordingly, NTX overcame to some extent alcohol toxicity and severe liver dysfunction. A second randomized, double-blind, placebo-controlled cross-over clinical trial involving human 31 subjects was conducted whereby all participants received a single dose of alcohol with and without NTX[16]. Co-consumption of alcohol with NTX significantly reduced the levels of ROS to facilitate development of normal liver enzyme circulating concentrations.

4. SUPPORT NORMAL LIVER DEFENSES AND REGENERATIVE MECHANISM

References [14-19,21,22,53,68,69]

Almost 50 published human clinical studies and a wide variety on animal studies have demonstrated that NTX and its individual components glycyrrhizin and mannitol support normal defense mechanisms against alcohol and other hepatotoxins, allowing normal function and regeneration of the liver.

A published meta-analysis of 12 randomized controlled trials (RCTs) has summarized the effects of a glycyrrhizin product on liver function in 838 patients with alcoholic liver disease[53]. The analysis showed that glycyrrhizin significantly decreased serum ALT and AST levels, and the authors concluded that the glycyrrhizin product significantly improved liver function in alcoholic liver disease[53].

A study has examined the beneficial effects of a glycyrrhizin product on alcohol-induced fibrosis in rats[21]. Pathological liver fibrosis was observed when rats were treated daily with alcohol for 16 weeks. Fibrosis was assessed and demonstrated by measuring serum levels of hyaluronic acid, laminin, procollagen III and collagen type IV as well as examining histopathology. All indices of fibrosis were significantly decreased in rats co-administered the glycyrrhizin product, thus demonstrating that the glycyrrhizin exhibits tissue protective effects and supports normal liver function[21].

A double blind, comparative, cross-over clinical trial was conducted which examined the effects of alcohol on blood levels of ROS in the absence and presence of co-administered NTX[17]. The results demonstrated that NTX significantly decreased the alcohol-induced generation of ROS and ROS metabolites, and decreased serum lipid peroxidation product MDA, while increasing serum levels of the endogenous antioxidant GSH. NTX administration with alcohol also significantly decreased

serum protein carbonyl levels, which is another marker of alcohol-induced oxidative stress[17]. Thus, NTX co-administration with alcohol decreased oxidative tissue damage and supported normal liver function.

5. REDUCES THE RISK OF LIVER DISEASES INCLUDING FIBROSIS AND CIRRHOSIS

References [14-16,19,21,22,50-53,54-70,73-75]

Several clinical trials and laboratory studies have establish that the individual components glycyrrhizin and mannitol alone and combined in NTX support normal defense mechanisms and ameliorate alcohol liver diseases lessening the potential fibrosis and cirrhosis, thereby providing healthier function and allowing the potential for hepatic regeneration.

Magnesium isoglycyrrhizinate is a magnesium salt form of glycyrrhizin that improves hepatic function in humans with alcoholic liver[70], disease, chronic hepatitis[71,72], and cirrhosis[73]. This was revealed by decreases in the circulating levels of ALT, AST and total bilirubin. A meta-analysis summarized the ability of a glycyrrhizin to influence liver function in 838 patients with alcoholic liver disease. Data was obtained from 12 randomized controlled trials (RCTs)[53]. The authors concluded that a glycyrrhizin product significantly improved liver function in alcoholic liver disease, because administration markedly decreased serum ALT and AST levels[53].

In a randomized, double-blind, placebo-controlled cross-over clinical trial, it was found that NTX provides significant hepatic protection during 12 days of alcohol consumption when compared to receiving only alcohol. Decreases in the hepatic enzyme biomarkers of liver toxicity ALT, AST, GGT and ALP provided the evidence[15].

A different randomized, double-blind, placebo-controlled cross-over clinical trial involving human 31 subjects was conducted in which a single dose alcohol with and without NTX was given. Duration of washout was one week between cross-over treatments[16]. Alcohol increased circulating ROS and ROS metabolites, lowered serum GSH, elevated MDA, raised protein carbonyl, and increased DNA damage in peripheral lymphocytes. However, consumption of alcohol with NTX significantly lowered concentrations of ROS and all the oxidative markers and increased serum GSH. The results indicated that NTX may "be effective in reducing alcohol-induced pathophysiological changes"[16].

6. AMELIORATES THE SYMPTOMS ASSOCIATED WITH LIVER DISEASE INCLUDING FIBROSIS AND CIRRHOSIS

REFERENCES [14-16,19,21,22,34,50-67,68,69,70-74]

A number of clinical investigations and laboratory studies have shown that NTX and its individual components glycyrrhizin and mannitol support normal defense mechanisms and ameliorate liver diseases including fibrosis and cirrhosis, thereby facilitating improved function and improved regeneration of the liver.

Glycyrrhizin has been found in a number of clinical studies to significantly reduce and normalize serum ALT and AST in patients with chronic hepatitis[54-67].

A meta-analysis assessing liver function in 838 patients with alcoholic liver disease reported the influence of a glycyrrhizin product[53]. Data were derived from 12 randomized controlled trials (RCTs)[53]. Glycyrrhizin administration significantly lowered circulating ALT and AST concentrations. The investigators reported that the glycyrrhizin product significantly made the symptoms better in the alcoholics subjects [53].

Laboratory studies involving animals have corroborated that NTX provides significant protection against alcohol-induced oxidative hepatic damage exemplified by lower circulating levels of ALT, AST, GGT and ALP[14,19]. Oxidative hepatic cellular damage ultimately causes hepatitis, fibrosis and cirrhosis. The combination of glycyrrhizin and mannitol, better than either one alone, ameliorated alcohol-induced presence of reactive oxygen species (ROS) by enhancing the activity level of the enzymes catalase, GPX and SOD that decompose tissue damaging ROS[14], leading to improved liver function. Also, consumption of mannitol and glycyrrhizin (NTX) contributed to healthy functioning of the liver by preventing alcohol-induced depletion of endogenous GSH levels [14].

7, HELPS MAINTAIN NORMAL RENAL FUNCTIONS

References [14-16,19-22,30-35,38,45-54,67-69]

Several laboratory and clinical studies have verified the ability of NTX and its constituents to protect the liver exposed to excess acid challenge acutely and chronically [14-16,19-22,30-35,38,45-54,67-69]. Mannitol , the second major component of NTX has been shown to improve blood flow, contain antioxidant and free radical scavenging properties, and possess anti-inflammatory and antioxidant properties in tissue baths to protect renal functions prior to transplant[46,47]. Similarly, mannitol showed anti-inflammatory and antioxidant activity in rodents after traumatic brain injury, whereby it decreased production of MDA and improved levels of the antioxidant enzymes catalase and GPx[49].

In respect to the workings of glycyrrhizinate alone, a meta-analysis involving 24 randomized control trials examining 3201 patients with chronic hepatitis was published[53]. Diammonium glycyrrhizinate significantly lowered circulating ALT and total bilirubin, and lessened fibrosis, therefore supporting normal liver function.

A meta-analysis summarized the effects of a glycyrrhizin product on liver function in 838 patients with alcoholic liver disease involving 12 randomized controlled trials (RCTs)[53]. Glycyrrhizin administration significantly decreased serum ALT and AST levels. The authors concluded that the glycyrrhizin product significantly improved liver function in alcoholic liver disease[53].

The co-administration of NTX with alcohol significantly lowers and tends to normalize the enzyme biomarkers of liver toxicity -- ALT,

AST, GGT and ALP[15], supporting the hepatoprotective effects of NTX. For example, a randomized, double-blind, placebo-controlled cross-over clinical trial involving 12 human subjects received alcohol daily for 12 days with or without NTX. NTX decreased biomarkers indicating toxicity and revealed that NTX helped maintain normal hepatic function.

In a second clinical trial involving 31 subjects, all participants received a single dose of alcohol with and without NTX[16]. Alcohol by itself elevated circulating levels of ROS and ROS metabolites, lowered serum GSH, increased MDA, raised protein carbonyl, and increased DNA damage in peripheral lymphocytes. When NTX was added to the alcohol challenge, the combination significantly reduced ROS concentrations and all other oxidative markers. The results indicated that NTX may "be effective in reducing alcohol-induced pathophysiological changes"[16], with the distinct possibility of preserving normal liver function.

8. HELPS PROTECT DNA FROM ALCOHOL-INDUCED DAMAGE

9. REDUCES ALCOHOL-INDUCED DNA DAMAGE

REFERENCES [16,38,76-94]

The influence of excess alcohol challenge contributes to the production of oxidative damage to DNA that creates strand breaks and fragmentation[76, 77]. ROS can also activate or repress epigenetic elements as chromatin remodeling, micro-RNAs, DNA (de)methylation and histone modification that affect gene expression and has the potential to bring about several liver disorders[77,78]. In addition, alcohol can form adducts

with DNA, inhibiting the formation of various proteins essential for healthy hepatic function[79,80]. Accordingly, various human, animal and *in vitro* studies have shown that NTX and it principal components glycyrrhizin and mannitol restrain the formation of alcohol-induced DNA damage.

Thirty-one subjects in a randomized, double-blind, placebo-controlled cross-over clinical trial received alcohol with and without NTX[16]. Alcohol challenge resulted in apparent DNA damage in peripheral lymphocytes as determined by the single cell electrophoresis comet assay, the cytokinesis-block micronucleus assay, and 8-hydroxy-2-deoxyguanosine formation. When the alcohol challenge was combined with NTX , a significant reduction in DNA damage occurred. Various studies using human and animal tissues have shown that individually glycyrrhizin and mannitol can prevent oxidative damage to DNA[81-94]. Accordingly, NTX and its major components glycyrrhizin and mannitol have the potential to reduce DNA damage from DNA single and double strand breaks induced by alcohol and other ROS generating systems in liver.

SAFETY

References [15,19,54,97-104]

Licorice and glycyrrhizin are Generally Recognized as Safe (GRAS) for food use in the US.

Over four week, NTX co-administration with alcohol provided significant decreases and normalization in the enzyme biomarkers of liver toxicity ALT, AST, GGT and ALP[15]. Importantly, NTX had essentially no adverse effects on heart rate, blood pressures or respiratory rate, or for that matter any other adverse events as reported in these human subjects[15].

In another trial over 28 days, at no time points did NTX treatment at any of three doses compared to placebo result in significant changes with respect to leukocyte count, erythrocyte count, lymphocyte count, monocyte count, eosinophil count, basophil count, hemoglobin, differential count, blood clotting time, packed cell volume, serum sodium and potassium, plasma AST, ALT, ALP, blood urea nitrogen (BUN), creatinine or total protein. Microscopic examination of the urine sediment of control and NTX treated groups did not reveal the presence of any epithelial casts, bacterial cells, erythrocytes or any other abnormalities at any of the time points over the 28 days of treatment[19]. In another 8 week study involving 39 healthy volunteers, the no effect level for glycyrrhizin was conservatively determined to be 2 mg/kg/day or 160 mg for an 80 kg individual[97].

Several clinical studies using doses up to 200 mg per day, primarily given intravenously were reported[97,102-104]. Most of the studies reported no adverse effects, including a study that continued approximately 10 years. Hypertension with skin rash was reported in one study that lasted for up to 14.5 years and involved daily intravenous injections which

results in much higher blood and tissue levels than achieved by oral administration due to the gastro-intestinal metabolism of glycyrrhizin. Two studies reported headache and three studies reported an increase in blood pressure[99], which cannot be correlated with similar doses given orally.

The subchronic oral toxicity of mannitol was studied in rats that were given approximately 12 grams of mannitol per kg per day for 90 days[101]. This dose would be equivalent to an 80 kg individual consuming about 155 grams of mannitol per day. The authors wrote that mannitol did not produce signs of toxicity. Mannitol is used commonly to produce volume expansion and reperfusion[105].

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97. Cosmetic Ingredient Review expert Panel. 2007. Final report on the safety assessment of glycyhrretinic acid, potassium glycyrrhetinate, disodium succinoyl glycyrrhetinate, glyceryl glycyrrhetinate, glycyrrhetinyl stearate, stearyl glycyrrhetinate, glycyrrhizic acid, ammonium glycyrrhizate, dipotassium glycyrrhetinate, disodium glycyrrhizate, trisodium glycyrrhizate, methyl glycyrrhizate, and potassium glycyrrhizate. Int J Toxicol 26: Suppl. 2: 79-112.
98. Ikeda K, Arase Y, Kobayashi M, Saitoh S, Someya T, Hosaka T, Sezaki H, Akuta N, Suzuki Y, Suzuki F, Kumada H. 2006. A long-term glycyrrhizin injection therapy reduces hepatocellular carcinogenesis rate in [patients with interferon-resistant active chronic hepatitis C: a cohort study of 1249 patients. Dig Dis Sci 51: 603-609.
99. Li JY, Cao HY, Liu P, Cheng CGH, Sun MY. 2014. Glycyrrhizic acid in the treatment of liver diseases: Literature review. BioMed Res Int 2014: Article ID 872139. doi: 10.1155/2014/872139.
100. Meena M, Prasad V, Zehra A, Gupta VK, Upadhyay RS. 2015. Mannitol metabolism during pathogenic fungal-host interaction under stressed conditions. Front Microbiol 6: 1019. doi: 10.3389/fmicb.2015.01019.
101. Til HP, Kuper CF, Falke HE Bar A. 1996. Subchronic oral toxicity studies with erythritol in mice and rats. Regul toxicol Pharmacol 24: S221-231.
102. Kumada H: Long-term treatment of chronic hepatitis C with glycyrrhizin for preventing liver cirrhosis and hepatocellular carcinoma. Oncology 62:82 (Supl 1):94-100, 2002.

103. Arase Y, Ikeda K, Murashima N, et al: The long term efficacy of glycyrrhizin in chronic hepatitis C patients. Cancer 79:1494-1500, 1997.
104. Asi MN, Hosseinzadeh H: Review of pharmacological effects of Glycyrrhiza sp and it bioactive compounds. Phytother Res 22:709-724, 2008.
105. Moore J Jr, Johnson JP: Acute renal failure. In: The Clinical Practice of Nephrology, Preuss HG (ed) Fields and Wood, New York, NY. pp 40-45, 1989.

EXHIBIT 4

CURRICULUM VITAE

Harry G. Preuss, M.D., M.A.C.N., C.N.S.

GENERAL INFORMATION

RANK Professor of Biochemistry, Physiology, Medicine, and Pathology

ADDRESS Georgetown University Medical Center
Basic Science Bldg, Room 231 B
4000 Reservoir Rd NW
Washington, D.C. 20057

TELEPHONES

Work:	Home:
Phone (202) 687-1441	(703) 323-7638
Fax: (202) 687-8788	(703) 764-8058
E Mail: preusshg@georgetown.edu	

MARRIED October 8, 1960 - 4 children

MD LICENSE District of Columbia #21388
Pennsylvania 1966-1971

EDUCATION

1945 - 1948 Binghamton West Junior High School
Valedictorian
Mathematics and History Prize

1948 - 1952 Binghamton Central High School
Valedictorian
Mathematics & French Prize

1952 - 1955 Cornell University, New York
Dean's list, 1953, 1954, 1955 - BA received in 1956 after 1 year medical school. Three academic scholarships including New York State Regent's Scholarship for College (competitive)

1955 - 1959 Cornell University Medical School, M.D., 1959
New York State Regent's Scholarship (Medical School-competitive)

TRAINING AND ACADEMIC POSITIONS

1959 - 1960 Internship (Straight Medicine)
under Dr. David E. Rogers, Chairman Dept of Medicine

	Vanderbilt University Hospital, Nashville, TN
1960 - 1962	Resident (Straight Medicine) under Dr. David E. Rogers, Chairman Dept of Medicine Vanderbilt University Hospital, Nashville, TN
1962 - 1964	Fellowship - Renal Physiology under Dr. Robert F. Pitts, chairman Dept of Physiology Cornell University Medical School New York, N.Y.
1964 - 1966	Fellowship Nephrology (Clinical & Research) under Dr. George E. Schreiner, Chief of Nephrology Georgetown University, Washington, D.C.
1966 - 1970	Assistant Professor of Medicine under Dr. Jack Meyers, Chairman Dept of Medicine University of Pittsburgh School of Medicine Pittsburgh, PA,
1970 - 1971	Associate Professor of Medicine (Tenured) under Dr. Jack Meyers, Chairman Dept of Medicine University of Pittsburgh School of Medicine, Pittsburgh, PA,
1971 - 1976	Associate Professor of Medicine & Pathology (Tenured 1976) under Dr. Dudley Jackson, Chairman Dept of Medicine Georgetown University Medical Center, Washington, D.C.
1971 - 1994	Director of Research Renal Division Head, Dr. George E. Schreiner
1974 - 1978	Consultant Lecturer to Nephrology Branch National Naval Medical Center, Bethesda, MD
1976 -	Professor of Medicine & Pathology (Tenured) Georgetown University MedicaL Center, Washington, D.C.
1993-1994	Sabbatical in Molecular Biology under Maurice Burg, Arlyn Garcia-Perez, David Sheikh-Hamad Kidney Laboratory, NHLBI, NIH Bethesda MD
1997	Certified Nutrition Specialist (CNS) designated by Certification Board of Nutrition Specialists, New York, NY.
2000	Professor of Physiology, Georgetown Medical Center (Tenured)

2012	Professor of Biochemistry,Georgetwon Medical Center (Tenured)

JOURNAL ADVISORY EDITORIAL BOARDS or SPECIAL EDITORSHIPS

1977 - 1978	American Journal of Medicine
1978 - 2005	Nephron, S Karger, Basel, Switzerland
1979 - 1982	Nephrology Review
1981	Guest editor, Nephron Festschrift for George E. Schreiner, MD
1982	Guest editor, "Compensatory Renal Growth" Symposium Kidney International
1984 -1986	Series Editor, Nephrology Today Co-Editor, Geriatric Nephrology Editor, Management of Common Problems in Renal Editor, The Clinical Practice of Nephrology
1997-1998	Women's Health Journal--Editorial Advisory Board
1989-2003	Clinical Nephrology, Dustri-Verlag, Germany
1989 -	Geriatric Nephrology and Urology, Klewer Academic Publishers,
1991	Guest Editor for Clinics on Laboratory Evaluation: Renal Function, Electrolytes and Acid-Base Homeostasis Saunders Co, Philadelphia, PA
1992	Guest Editor - Symposium on Nutrition and Diseases of Women published in the Journal of the American College of Nutrition
1992 -	Kidney, Springer-Verlag, New York, NY
1992 -	Journal of the American College of Nutrition, American College of Nutrition, New York, NY
1998-2000	Women's Health Alternative Medicine Report. Mary Ann Liebert, Inc, Larchmont NY.
1999-	Original Internist, Clinton Publications, Rolla, MO
	Research Communications in Pharmacology and Toxicology, PJD Publications LTD, Westbury, NY

2002	Consultant to Arthritis Today, Supplement Guide 200
	Council of Chief Editors of RCPT -- Research Communications in Pharmacology and Therapeutics
	Current Nutrition and Food Science- Editorial Board
2009	Natural Medicine Journal – Editorial Board
2010	International Journal of Green Pharmacy Editorial Board
2010	International Journal of Physiology, Pathophysiology and Pharmacology, Editorial Board

AWARDS, HONORS, POSITIONS HELD

1962 - 1965	NIH Postdoctoral Fellowship
1966-1967	NIH Special Research Fellow
1967 - 1972	Established Investigator, American Heart Association
1968	Interstate Postgraduate Medical Association Research Prize for young investigators
1971	Member, Site Visit Team Cincinnati Veterans Administration Hospital Cincinnati, Ohio Core Research Grant
1972 - 1976	National Kidney Foundation, Washington, DC Peer Review Board
1978 -	Beta Chapter, AOA, Washington, D.C.
1979 - 1981	Clinical Science 3, NIH Study Section (member), Bethesda MD
1981 - 1985	Research Committee, Peer Review, Virginia Chapter of American
1982	Research Task Force, Northern Virginia Chapter American Heart
1983 - 1987	Member, National Advisory Council on Aging, Bethesda, MD Data Planning Committee, NIA Program Committee, NIA
1984	Ad Hoc Member, Biochemistry Study Section N I H, Bethesda, MD

1984 - 1986	Member, Director's Advisory Council NIH (Wyngaarden), Bethesda, MD - NIA Representative
1985 - 1989	American Heart Association, DC Chapter, Research Committee, Peer Review, Washington, DC
1986	Member, NIH Site Visit Team Polycystic Kidney Disease Program Grant Albuquerque, New Mexico
1988 - 1990	AHA, Nation's Capital Affiliate Development Committee
1990 - 1997	Chairman Nephrology-Hypertension Council, Am College of Nutrition
1991 - 1997	ACN - Coordinator Cardiovascular-Nephrology-Hypertension
1992-1993	Elected Board of Directors American College of Nutrition
1993 - 1997	Member of Board Human Kurdish Right Committee, Washington DC
1993 - 1996	American College of Nutrition Secretary-Treasurer Chairman Finance Committee By-Laws Committee
1994	American College of Nutrition Program Committee
1994	ACN representative ILSI meeting: “Sodium and Health”, Washington, DC
1994	American College of Nutrition Representative to ILSI Conference on Sodium: Health and Disease
1995-	Representative of ACN to National Cholesterol Conference at NIH
1995	American College of Nutrition Representative to Partners for Heart Disease and Stroke Prevention. A Vision for Action. sponsored by Assoc of State and Territorial Health Officials, Washington, DC.
1995	Member Advisory Board for Certifying Examination (CBNS)
1995	Chairmen: Washington Welcoming Committee ACN Meeting in

Oct 1995

1995	ACN Representative: ASTHOS meeting on Prevention of
1996 - 1997	ACN, Vice President
1996 -	ACN Representative to the Nutrition and Health Council of America, Georgetown University
1997-1998	President-Elect American College of Nutrition Program Director for 1998 Annual Meeting ACN
1997-	Director of the National Research Council for Health (NRCH) Boise, ID
1997	Selected as laboratory for 1997 Science Teacher Summer Research Fellowship Award -- Douglas Tyson American Society for Clinical Investigation: Committee on Science Education
1997	Nominated for FDA Commissioner by Senators Daschle and Harkin
1997	JACN Advisory Board for Richardson Prize for Research Excellence.
1997-1999	Member of Alternative Medicine Program Advisory Council, NIH. Appointed by Secretary of Health -- Donna Shalala
1997	Invited Participant to National Institute of Aging Conference in Tucson AZ “Micronutrients and Aging”.
1998	Board of Directors -- National Research Council for Health (NRCH)
1998	President American College of Nutrition
1998	Board of Directors, American Preventive Medical Association, Falls Church, VA
1998	Member for the National Cholesterol Education Program Coordinating Committee Meeting. NHLBI. Renaissance Hotel. Washington DC Dec .
1998	Co Chairman ACN Session-- Free Radicals, Apoptosis, and Human Health: Role of Antioxidants

	Invited Lecture: Natural Therapies for Osteoarthritis: Non Drug Invited Lecture: Aging in Relationship to Insulin Resistance in The Cutting Edge of Aging Research in Nutrition Session
1999	Office of Dietary Supplements Advisor Quarterly Conference Calls.
1999 ences,	ACN representative to USNC/IUNS if National Academy of Sci- Institute of Medicine
1999	ACN Representative to Calcium Summit: Developing a public health strategy to insure America's optimal calcium intake. International Trade Center/Ronald Reagan Bldg, Washington DC June 25
1999	Office of Dietary Supplements, NIH, Delegate to Chromium Meeting in November 1999
1999-	Permanent ACN representative to National Cholesterol Education
1999	Invited Member: Nutrition Research Academy
2000 -2002	Chairman of the ACN Council -- "Dietary Supplements, Nutraceuticals, and Functional Foods
2001-2003	Treasurer American Association for Health Freedoms
2001	Fellow of the American Association of Integrative Medicine Diplomate of the College of Physicians Diplomate of the College of Nutrition
2001	Elected the Ninth Master of the American College of Nutrition
2001-2003	Treasurer American Association for Health Freedoms
2002-2003	Elected Board of Directors American College of Nutrition
2003-2004	Nutrition Policy Institute of ACN – President
2002	James Lind Award for excellence in supplement research
2004	Mark Bieber Award of the American College of Nutrition for promoting unity between industry and academia
2004	Advocare Award for Best Research Poster, American College of Nutrition, annual meeting

2004	Member of Expert Panel Diabetes 2020. Report prepared for TSN 109225 Nov 2004 Healthcare.
2006	ACN Charles Ragus Award – Best research paper JACN 2006
2007-	Adjunct Professor, Union Institute and University, Cincinnati Ohio. (Joan Ifland and Shoshona Shinnar
2009	President ACN (second time)
2010	American College of Nutrition Award for outstanding senior investigator in Nutrition.
2011	President ACN (third time)
2011	Chairman, Scientific Advisory Board NutraSpace Internet
2011	Contributing Editor Heart MD Institute
2011	IMBC consultant, Chairman Scientific Advisory Board
2012	Alliance for Natural Health, Scientific and Medical Collaboration Committee Member
2012	CBNS Job Analysis Task Force (member)
2014	Emord Sacred Fire of Libertky Awards – 3 awards Excellence award in Medical Research Excellence award in Integrative Medical Research Excellence award in Nutrition Research
2014	American College Nutrion Presidentail Recognition Award

MEMBERSHIP IN PROFESSIONAL SOCIETIES.

1966 -	American Federation for Clinical Research
1967 -	International Society of Nephrology
1968 -	American Society of Nephrology
1968 - 1982	Central Society for Clinical Research
1969 -	American Society of Physiology

1970 -	Society for Experimental Biology & Medicine
1974 -	American Society for Clinical Investigation
1979 - 1982	New York State Academy of Science
1983 - 1985	American Association for the Advancement of Science
1984 -	American Society of Renal Biochemistry
1985 -	Association of Clinical Scientists
1985 -	American Society of Hypertension
1988 -	Fellow, American College of Nutrition
1999	American Society for Pharmaceutical and Experimental Therapeutics
	Fellow of the Institute for Integrative Medicine
2004	Member North American Association for the Study of Obesity (NAASO)

UNIVERSITY COMMITTEES AND DUTIES

1971 -	Georgetown Representative to Washington VA Research Board
1978 - 1979	Committee to Review Pharmacology Department
1979 - 1980	Chairman, Committee to Review Biochemistry Department
1979 - 1997	Subcommittee on Scientific Merit (IRB) Chairman, 1986 -1997
1979 - 1985	Member, Student's Financial Appeals Board Chairman, 1981 - 1985
1981 - 1982	Member, Search Committee Biochemistry Department Chairman
1981 - 1983	Member, Graduate Committee Department of Pathology
1981 - 1990	Member, Ad Hoc Committee on Teaching Department of Patholo-

	gy
1984 - 1991	Interdepartmental Research Group Georgetown University Medical Center Chairman
1984 - 1986	Animal Welfare Committee
1984 - 1985	Georgetown University Committee on Aging Subcommittee on Research
1984 - 1987	Georgetown University Research Committee (Chairman, 1984 – 1 985) (Vice Chairman, (1985 - 1986)
1985 - 2003	Director, Student's Research Day - GUMC
1986 - 1997	GUMC Institutional Review Board (Vice Chairman)
1987 - 1995	Member, Gertrude Maegwyn-Davis Award Committee
1987 - 1995	Ad Hoc, Student Academic Appeals Committee
1987 - 1988	Dean's Task Force on Incentives for Obtaining Extramural Funds (Chairman)
1997 -2000	JSHS - Judge
1998 -2008	Chairman Institutional Review Board
2002	Co-Director of Nutrition Course at Georgetown with Dr. Eugene Davidson
1998	Pharmacy Committee for IRB
2008	Georgetown External Awards Committee

CONSULTATIONS

1987 - 1988	Expert Witness-Patent Infringement Moduretic Patent Merck, Sharp & Dome vs. Biocraft
1995	Consultant for Ciba-Geigy Pharmaceuticals. Use of Auriculin (Atrial Natriuretic Protein) in Acute Tubular Necrosis.
1995	Ciba Pharmaceuticals, Special Consultant --Obesity and Type II

	Diabetes Mellitus"- Use of Bromocriptine”
1995	Research Project for InterHealth Concord CA
1995	Expert Witness Electromedical Products, Mineral Wells Texas
1996-	Birkmayer Inc, Vienna Austria: Use of NADH in Syndrome X Effects of NADH on Chronic Fatigue Syndrome
	Emords Associates, Washington: DC Role of Fiber in Health
	Medical Products Laboratories, Philadelphia PA --Benzocaine as Local Anesthetic.
	Great Salt Lakes Mineral Corporation, Ogden Utah Toxicity of Armak 1225
	Ciba Pharmaceuticals, Summit NJ, ACE inhibitors and renal protection
	Richardson Laboratories, Boise ID -- Saw Palmetto and Ginkgo Biloba
1997-	Sci-Med Board of Advocare, Inc, Dallas Texas
1998 - 2000	Rexall-Sundown consultant
1998	Consultant to Clinical Trial on the Use of Glucosamine in the Treatment of Osteoarthritis. National Institutes of Health Jan.
1998	Optimum Lifestyle Inc., Mill Valley CA.
1998	Novartis Pharmaceutical. Aminoguanindine.
2000 -2003	Venable Law Firm (Enforma Nutraceuticals)
	Sunny Health Enhancement Center (Taipei and USA) Scientific Advisory Board
2002-2004	Pharmaton (Boeringer-Ingelheim). Representative of SAMe and glucosamine for production of Flexium
2005-	Fuji Chemical. Japan
2006	Manett Law Firm (Shari Lieberman Chitosan)

2006-	Pharmachem, NJ (Phase 2)
	Stop Aging Now, Washington, DC
2008	Sarcona Case in Florida. Witness for defense. Chitosan
2009-2013	Neways International, Scientific Advisory Board
2013-2014	IsaGenix, Scientific Advisory Board
2013-2014	Expert Witness, Pfizer vs. Nutrition 21

LECTURESHIPS, TEACHING, & OTHER ACTIVITIES

1975	Visiting Professor University of Michigan Medical Center Ann Arbor, Michigan
1978	Visiting Professor Loyola University Medical Center, Chicago, IL
1978	Invited Lecturer First International Workshop on Renal Ammoniagenesis, Montreal, Canada
1981	Visiting Professor, St. Louis University School of Medicine St. Louis, MO
1981	Invited Lecturer Second International Workshop on Ammoniagenesis, Athens, Greece
1984	Invited Lecturer Third International Workshop on Ammoniagenesis, Carmel, CA
1985	Visiting Professor, Nagasaki University Medical Center Nagasaki, Japan
1986	Invited Lecturer Biochemistry of Systemic Acid-Base Balance Titisee, Germany
1986	Invited Lecturer 8th Annual European Renal Biochemistry Meeting Dubrovnic, Yugoslavia
1987	Invited Lecturer New York Academy of Medicine

	Geriatric Nephrology Symposium, New York, NY
1987	Faculty (Invited Lectureship) 2nd Annual Meeting The American Society of Hypertension, New York, NY
1987	Invited Lecturer, Session Chairman 4th International Workshop on Ammoniagenesis Cadarache, France
1987	Faculty, Seminar on Geriatric Nephrology Lenox Hill Hospital, New York NY
1988-	Invited Lecture, Seventh Annual St Louis GRECC Symposium: Endocrine Function and Aging
1990	Visiting Professor Loyola University Medical Center
1991	Co-Course Leader PMA Symposium- Hypertension January 1991
1991-1994	Appointed course leader for PMA Symposium on Hypertension to be held October 1991, 1992
1991	Appointed course leader for PMA symposia on Laboratory Evaluation held in December 1991 and 1992
1993	Co-chairman of Symposium on Nutrition and Cardiovascular Diseases and Hypertension - ACN Annual Meeting Chicago, IL
1992	Invited Lecturer-American College of Nutrition. Subject: Lead influence on mentation and the cardiovascular system.
1992	Invited lecturer- Lenox Hill Hospital Exogenous Factors Affecting Blood Pressure
1993	Invited faculty member and speaker Conference on the Inclusion of Women and Minorities in Clinical Research sponsored by PPRR, Georgetown University
1993	Invited speaker "Advances in Perinatal and Pediatric Nutrition" on "Lead Toxicity-Effect on Intellect and Hypertension" Stanford University, Palo Alto CA
1993	Co-chairman of symposium on Nutrition and Cardiovascular Diseases and Hypertension - ACN Annual Meeting, Chicago, IL

1993	Invited Author "Nutrition" for Encyclopedia Americana
1994	Invited Speaker USDA -The Role of Macronutrients in Blood Pressure Regulation
1994	Visiting Professor Lenox Hill Hospital - “Sugar and Hypertension”
1995	Invited speaker Washington Area Micronutrient Club, "Insulin Resistant Disorders"
1995	Co Chairmen: Interaction of Nutrition and Genetics on Chronic Diseases. ACN Meeting in Washington DC Invited speaker Dairy Council. Interaction of Genetics and Nutrition
1995	Invited speaker Expo East Antioxidant conference, Baltimore MD "Effects of chromium on blood pressure and free radical formation (Sept 1995).
1995	Guest speaker Treatment of Hypertension Martinsburg West VA and Patuxant Naval Base MD
1996	Invited speaker Fourth International Conference 0n Geriatric Nephrology and Urology. "Macronutrients and Trace Elements as They Affect Blood Pressure of the Elderly." Toronto Canada (April, 1996)
1996	Invited Speaker, Japanese Society of Urology, Special Lecture "Aspects of Geriatric Nephrology" Nagasaki, Japan (May 1996).
1996	Invited Speaker. Second International Congress on Alternative and
1996	Co-Chairmen Symposium Nutritional Factors Affecting the Glucose-Insulin System: Role in Chronic Diseases and Aging ACN meeting Invited speaker ACN annual Meeting in San Francisco CA: Insulin
1996	Co-Chairmen Symposium Controversies in the Nutritional Management of Chronic Fatigue Syndrome ACN meeting San Francisco CA
1996	Taped Interview on Nutritional and Exogenous Factors Affecting Overall Health Nutrition and Blood Pressure Regulation Preventive Medicine Update, Clinician of the Month (May 1996)
1996	ACN Vice President 1996, President-Elect 1997, President 1998 Vice Chairman Program Committee 1996

1997 Invited speaker to Chiropractors' Meeting, Scottsdale, Arizona July 1997 "Natural means to control blood pressure."

1997 American College for Advancement of Medicine (ACAM)- Invited speaker to Anaheim CA. Harold Harper Lecture "Antioxidants and Hypertension) and workshop on "Role of Antioxidants in Aging."

1998 Invited speaker to Calcutta India. Antioxidants and Stress: Hypertension January, 1998.

1998 Invited lecturer to Chromium Conference in Boston Mass, May 1-2, 1998 Topic: Chromium Effect on BP

1998 Invited Speaker -- Natural Sensations Symposium on Syndrome X May 10, 1998

1998 Invited Speaker "Nutrition and Cancer" and commentator on session "New Biologicals to Treat Cancer, Comprehensive Cancer Care: Integrating Complementary and Alternative Therapies, Washington, DC June 12-14, 1998

1998 Invited Speaker - Meeting Regulatory and Legal Challenges in the Promotion of Dietary Supplements Washington DC Dec 1998.

1998 Invited Speaker: Conference on Cardiovascular Health. Role of Maitake in Lowering Cardiovascular Risk Factors. Expo East, Baltimore MD 1998.

1998 Invited Speaker to Texas Board on Diabetes Mellitus. Natural means to prevent or treat type 2 diabetes.

1998 Invited Speaker to International College for Advancement of Longevity Medicine (InCALM) Reno Nevada. Insulin Resistance in Aging

1998 Invited Speaker to Night of Innovation, ACAM Meeting Phoenix AZ. New Findings Concerning the Clinical Relevance of Maitake Mushroom and Its Extracts.

1999 Invited Speaker to Natural Means to Prevent Aging , Expo West Anaheim CA. Clinical Relevance of Maitake Mushroom in Cancer Treatment.

1999 Three Clinical Conferences via phone satellite on the "Use of glucosamine sulfate in the treatment of osteoarthritis. In: The Emerging Role of Complementary Therapy in the Clinical Setting" National CME/CE Audioconference/Audiocassette Series. Pragmaton, Chicago IL.

1999	Invited Lecturer -- Michigan Pharmacist Association's 2000 Annual Convention and Exposition, Dearborn, Michigan Use of herbs to treat Prostatitis and BPH (Feb 1999)
1999	Invited to write chapter for ILSI: Present Knowledge in Nutrition, 8th Edition (Sodium, Chloride and Potassium)
2000	Invited Speaker 4th Annual International Symposium on Aging Skin Fort Lauderdale FL "The Role of Insulin Resistance in Aging."
2000	Invited speaker -- Modified Foods in the Marketplace, Univ of North Carolina JJB Anderson Chairman
2000	Invited judge 38th National Junior Science and Humanities Symposium-- Paper Competition - Academy of Applied Science 4H Center, Bethesda MD
2000-2001	Consultant to Reader's Digest Book on "Looking after Your Body. An Owners' Guide to Successful Aging." The Philip Leif Group and Reader's Digest Association, Pleasantville, NY, pp 1-416, 2001.
2000	Invited Speaker to symposium on Natural Therapy for Diabetes. University of Guelph "Effects of chromium and other natural substances on glucose-insulin metabolism". Toronto Canada (Sept 2000).
2000	Invitation "Strategies in Internal Medicine and Beyond" Examining the Benefits of Nutraceuticals. Saint Joseph Mercy Hospital, Ann Arbor, MI (Oct 2000).
2000	Invitation to speak at 10th Biennial Meeting of International Society for Free Radical Research. "Effects of a novel grape seed extract and a niacin bound chromium supplement on the lipid profile of hypercholesterolemic patients. Kyoto Japan O 18-4, SFRRI 2000 p 81.
2001	Invited speaker. Expo West Antifungal Action of Oil of Origanum (March)
2001	Invited participant in "Alcohol and Wine in Health and Disease," Palo Alto, CA in April, 2001. Topic: Grape Seed Extract: Effects on Aging and Various Aspects of Syndrome X.
2001 -- (May)	Invited Speaker to Nick Perricone Conference on Aging, San Diego CA "Natural Means to Overcome Syndrome X"

2001 Invited Speaker to ACAM Conference in Nashville TN (May). "Scientific Method to Evaluate Natural Products"

2001. Member Steering Committee for Alternative Medicine Summit. Georgetown University October 2001

2002. Invited Lecturer: First Annual Nutrition Week San Diego "Interesting Nutraceuticals" February

2002. Invited Lecturer to Expo West, Anaheim CA March
Overview of Maitake
Effects of Cernitin on Weight Loss

2002. Invited Lecturer Mini Med School, GUMC March "Interesting Nutraceuticals"

2002. Invited Lecturer to Perricone Conference on Aging. May-June in Chicago, "Chipping Away at Syndrome X"

2002 Invited Lecturer to NABC. Food for Health Conference, University of Minnesota, Minneapolis, Minnesota May 2002

2002 Invited lecturer – training camp of Jacksonville Jaguars " Ephedra and other substances banned by the NFL

2002 Co Chairman session of Am Coll Nutr--Perspectives on Nutraceutical and Functional Foods. San Antonio October

2002 Chairman of session of Am Coll Nutr -- Controversies in Nutrition. Risk/Benefit Ratios of Controversial Supplements. San Antonio (October)

2002. Invited speaker, 2nd ICMAN Conference, Vienna, "Maitake Mushroom and Essential Oils: Immune Enhancers and Antimicrobials" 2nd Icman Conference, Vienna Austria

2003 Invited Speaker to Expo West Anaheim CA "Fraction SX of Maitake"

2003 Invited Keynote Speaker for Japanese Alternative Medicine Society, Tokyo Japan June

2003. Co Chair session on Nutraceutical to Treat Obesity, Nashville TN (October)

FDA Task Force on "labeling," 511 Paint Branch Parkway, College Park Maryland, March 13, 2003

2003 National Academies of Science. Invited Speaker for Food for Health Conference June 2003

2004 Virginia College of Emergency Medicine. Hot Springs, VA 2004 Scientific Assembly "Evaluation of Fluid and Electrolytes (Feb)

2004 Co Chair ACN meeting "Intricate Issues of food and supplement labeling guidelines. (Oct)

2004 Invited Speaker and session co chair World Nutra Conference, San Francisco, November "Chitosan, Hydroxycitric Acid, and Chromium in Weight Management: New Clinical Findings"

2004 Member National Center for Complementary and Alternative Medicine, Special Emphasis Panel -- NIH

2005 Co Chair ACN meeting "Sarcopenia and related issues. Co chair of open session, Kiawah Island, SC Oct 2005

2005 Invited Speaker and session co chair World Nutra Conference, Anaheim, November "Sarcopenia"

2006. Japanese Natural Products Fair - Keynote Speaker, Tokyo Japan, March "Treating the Metabolic Syndrome with Natural Dietary Supplements"

2006 American Oil Chemistry Society, Invited Speaker, Kansas City, KA May 2006 "Influence of monolaurin and essential oils as antimicrobial agents"

2006 World Nutra Co-chair session. Presentation: Chromium, an overview of laboratory and clinical studies. Reno, NV, November.

2006 Caloric Control Council - invited speaker, Carb blockers: Effects and safety of Phase 2. Naples, Florida, November.

2006- NIH Study Section NCCAM

2007 Expo West: The science behind carb blockers. February

2007 Co Chair ACN Meeting, Dietary Supplement Safety. Orlando FL

2007 Invited speaker Webinar Audio Conference: Weight Management. Virgo Publishing, November 15.

2007 A4M Anti Aging Conference -Invited Speaker: Antiaging Nutraceuticals December

2008 Expo West – Invited Speaker - Focus on Maitake SX, Anaheim, CA March

2008 Supply Side East- Invited Speaker -Diabesity - Secaucus, NJ April

2008 IFC- Invited Speaker - Globesity, New Burden for a New Millenium, New Orleans, LA June

2008 American College of Nutrition, President

2008 Co Chair session ACN

2009 American Society of Bariatric Physicians, Charleston SC. Invited for 2 lectures – Analyzing Clinical Studies on Dietary Approaches in Weight Management and The Multiple approach Hypothesis in Weight Management. April

2009 Supply Side East, Secaucus NJ. Invited by Virgo Publishing to speak. Weight Management: The Multiple Approach Hypothesis, April.

2009 IFC- Invited Speaker - The "Multiple Target Hypothesis" in Weight Management. Anaheim, CA, June

2009 ISNFF – Invited Speaker – Weight Loss Supplements, San Francisco, CA Nov

2010 Nutracon, Anaheim CA – Invited Speaker- Carb Blockers, Effectiveness and Safety, March 10, 2010.

2011 Invited speaker Expo West, "Beyond the Metabolic Syndrome" March 2011

2011 Contributing Editor www.heartmdinstitute.com

2011 Chairman Scientific Advisory Board of NutraSpace.com

2012 2012 Tea Symposium Steering Committee - Jeff Blumberg, Director Sept 2012

2012 XVI Congresso Brasileiro de Nutarologia, Sao Paulo, Brazil, Sept 2012, Two presentations -- Niacin-bound Chromium increases life span in Zucker rats and Astaxanthin carotenoid and the rennin-angiotensin system.

2012 CBNS Job Analysis Task Force. (Member)

2012 NHRI Symposium Chicago, IL Oct 20 Invited Speaker "Managing Obesity with Natural Dietary Supplements: Lessons Learned from Clincal Re-

search Studies."

2012 ACN Annual Meeting, Morristown, NJ Nov -- Invited lecture "Strategies for the Prevention of Type 2 Diabetes and moderator for session, "New Frontiers in Diabesity and Nutrition Research."

2013 35th Turkish Endocrine and Metabolism Diseases Congress and Hypophysis Symposium, Antalya, Turkey, May 16-18, 2013. "What is the place of herbal products to treat obesity, what is not?"

2013 ACN Annual Meeting, San Diego, CA Nov -- Invited lecture "Weight Loss Studies: Emphasis on *Garcinia cambogia* "2013.

2014 Chairman – Program Committed ACN Annual Meeting, San Antonio TX October. Moderator symposium on GMO and Metabolonics

2014 Invited panelist to AMAC Healthcare Symposium. Washington DC, Promoting Preventive Health Care

2015 Lecture Harvard Medical School Sept 15, 2015 "Lowering circulating glucose evels that are in the non-diabetic range is important for long-term optimal health." At Proceedings of the 18th International Conference. Chronic Diseases: Bioactive Compounds and Biomarkers.

2015 Initiated into National Academy of Inventors. Sponsored by by Georgetown University

PEER-REVIEWED RESEARCH PAPERS

1. Grossman LA, Kaplan JH, Preuss HG, Harrington JL: Mesenteric panniculitis. JAMA 183:318-323, 1963.

2. Denis G, Preuss HG, Pitts RF: The PNH3 of renal tubular cells. J Clin Invest 43:571-582, 1964.

3. Preuss HG, Bise BW, Schreiner GE: The determination of glutamine in plasma and urine. Clin Chem 12:329-337, 1966.

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ABSTRACTS
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EXHIBIT 5

Synthesis and *in Vitro* Antioxidant Activity of Glycyrrhetinic Acid Derivatives Tested with the Cytochrome P450/NADPH System

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Five glycyrrhetinic acid (Ib) derivatives have been synthesized to try to improve the antioxidant activity. Their *in vitro* antioxidant activities were studied using a cytochrome P450/NADPH reductase system from rat liver microsomes. The generation of microsomal free radicals was followed by oxidation of the DCFH-DA probe, while evaluating the capacity to inhibit reactive oxygen species (ROS) formation. Two hydroxylated derivatives, 18β-olean-12-ene-3β,11α,30-triol (II) and 18β-olean-12-ene-3β,11β,30-triol (IV), exhibited strong antioxidant activities. At a concentration of 1.0 mg/ml, these derivatives inhibited ROS formation by 50% and 51%, respectively. Moreover, two homo- and heterocyclic diene derivatives, 18β-olean-11,13(18)-diene-3β,30-diol (III) and 18β-olean-9(11),12-diene-3β,30-diol (V), were also effective in ROS-scavenging activity (inhibition of 41% and 44% of ROS activity, respectively). In the same conditions, the lead compound (Ib) and the reference vitamin E inhibited ROS activity by 31% and 32%, respectively. Our results suggest that the chemical reduction of the 11-keto and 30-carboxyl groups into hydroxyl function (example, II, IV) can increase the antioxidant activity of Ib significantly. In view of these results, our study represents a further approach to the development of potential therapeutic agents from Ib derivatives for use in pathologic events in which, free radical damage could be involved.

Key words glycyrrhetinic acid derivative; antioxidant activity; cytochrome P450/NADPH reductase

The involvement of free radicals in the pathology of human diseases such as atherosclerosis, cardiovascular diseases, and diabetes, has been recognized by a number of authors.[1—3] Protective therapeutic intervention might include natural or synthetic pharmacologic agents with antioxidant activity.[4] Therapeutic agents with multiple mechanisms of protective action, including antioxidant prophylactic properties, may become valuable in attempts to minimize tissue injury in human disease. This may be helpful in pharmaceutical R&D strategies.[4]

Due to multiple protective actions of a triterpenoid from licorice,[5] we have developed some semisynthetic derivatives of glycyrrhetinic acid (**Ib**), the aglycone of glycyrrhizin (**Ia**) which is a major triterpenoic saponin of licorice, and examined their antioxidant capacity. A derivative of licorice root (*Glycyrrhiza glabra* L.) and its main water-soluble constituent glycyrrhizin (**Ia**), a pentacyclic triterpene derivative of the *beta*-amyrin type (oleanane), has been widely used as an antidote, a food additive, and a folk medicine for generations in Asia and Europe.[6] **Ia** can be hydrolyzed into active aglycone, 18-*beta*-glycyrrhetinic acid (**Ib**). Both **Ia** and **Ib** have been shown to have several beneficial pharmacologic activities, such as antiinflammatory activity,[7] antiulcerative effect,[8] antiviral activity,[9] and antihepatitis effect.[10]

Recently, Zakirov and Abdullaev[11] have demonstrated the effective antioxidant and hypolipidemic properties of ammonium salt of **Ib**. It increases superoxide dismutase (SOD) activity and decreases the concentration of malondialdehyde (MDA) and lowers the cholesterol and triglyceride levels by 51% and 71%, respectively, at a concentration of 25 mg/kg in atherosclerotic and hypercholesterolemic rabbits. As a result, it effectively inhibits the progression of atherosclerotic lesion.[11]

Hye *et al.*[12] reported the protective effects of **Ib** against carbon tetrachloride-induced hepatotoxicity and the possible mechanisms involved in this protection. Shibata *et al.*[13] synthesized some new derivatives of **Ib** which suppressed its pseudoaldosteronism side effects, and Kiso *et al.*[14] demonstrated the effective antiinflammatory properties of those derivatives. Nevertheless, the antioxidant effects of these derivatives have not been systematically reported.

The multiple beneficial effects such as antioxidant, hypolipidemic, anti-inflammatory, and antiatherosclerosis effects of **Ib** point to the possibility of developing new active cardiovascular agents from this structural family. We structurally modified **Ib** and prepared five synthetic derivatives to improve the antioxidant activity. We performed a comparative study of their *in vitro* antioxidant properties in a microsomal free radical-generating system and found significant results with increased antioxidant activity for some. In addition, we carried out a computerized hypolipidemic pharmacophore identification of these derivatives with receptor mapping methods and propose a hypothesis to explain their possible hypolipidemic mechanism of action.[15]

Chemistry All derivatives were prepared by the methods previously described[13] (Fig. 1). Many preparative procedures have also been optimized. Other chemicals were obtained from the usual commercial sources. **Ia** was purified from licorice root and hydrolyzed into **Ib** (step a). We reduced **Ib** into the two stereoisomers **II** and **IV** (step b) with an optimized procedure. Dehydration of triol (**IV**) into diene (steps $c\text{-}_1$, $c\text{-}_2$) was conducted to obtain **V** and **III** in a 3 : 2 ratio. The product **VI** was obtained by a reduction of the mixture **II**+**IV** by catalytic hydrogenation in Pd–C at ambient temperature (step $c\text{-}_3$) and in absolute ethanol presaturated with $NaHCO_3$, while rigorously controlling hydrogen

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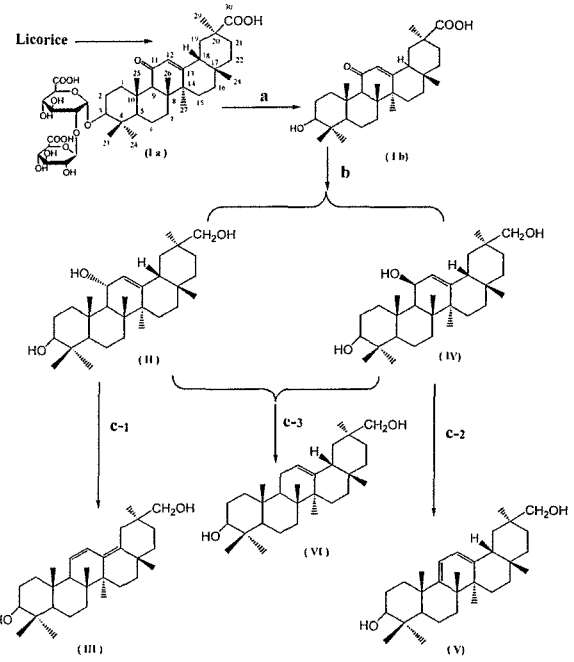

Fig. 1. Synthesis Pathway of Glycyrrhetinic Acid Derivatives

Ia, glycyrrhizin; **Ib**, 3β-hydroxy-11-oxo-18β-olean-12-en-30-oic acid; **II**, 18β-olean-12-ene-3β,11α,30-triol; **III**, 18β-olean-11,13(18)-diene-3β,30-diol; **IV**, 18β-olean-12-ene-3β,11β,30-triol; **V**, 18β-olean-9(11),12-diene-3β,30-diol; **VI**, 18β-olean-12-ene-3β,30-diol.

consumption.

Biochemical The *in vitro* antioxidant activities of these derivatives were tested with a major free radical-generating system, cytochrome P450/NADPH reductase, from rat liver microsomes. The typical antioxidant vitamin E served as a positive control.

The rate of reactive oxygen species (ROS) generation was quantified using the fluorescent probe precursor, 2,7-dichlorofluorescein diacetate (DCFH-DA), a probe that has been utilized extensively for the measurement of microsmal ROS production.[16,17] This assay has been shown to detect the fluorescence associated with the oxidation of DCFH by several reactive intermediates, including superoxide anions, H_2O_2, and hydroxyl radicals in microsomes.[16] Microsomes (rate, 0.5 mg/ml) were preincubated for 15 min with 4 μM of DCFH-DA for deesterification by endogenous esterase in phosphate buffer 50 mM, pH 7.4, at 37 °C. Then the mixture was incubated for an additional 30 min in the presence or absence of NADPH 0.5 mM and a sample (20 μl) using the method described by Choi *et al.*[18] At the end of incubation, fluorescent DCF formation was monitored using an FL 600 Microplate Fluorescence Reader (Bio-Tek, Highland Park, U.S.A.) at an excitation and emission wavelength of 485 and 530 nm, respectively. The rate of ROS generation was linear over the incubation period. A standard curve using DCF 0.01—0.10 μM was prepared and the results are expressed as picomoles of DCF formed per minute per milligram of protein.

The experiment was conducted at least three times. Statistical analysis was performed using one-way ANOVA (SAS software, SAS Institute Inc., NC). A value of $p<0.05$ was considered statistically significant. All biochemical results

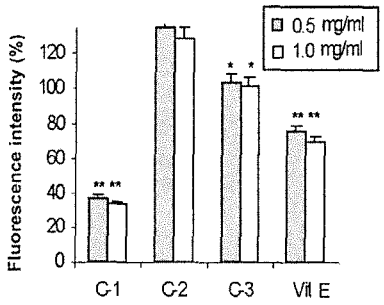

Fig. 2. NADPH-Initiated Microsomal Oxidation of DCFH-DA and Its Modification in the Presence of Vitamin E

The reaction mixture was composed of the microsome suspension (0.5 mg/ml), NADPH-generating system, DCFH-DA and vitamin E. After incubation at 37 °C for 30 min, microsomal oxidation was measured by the methods described. Values are the mean±S.D. of three individual determinations. Asterisks indicate a significant difference from the NADPH control (one-way ANOVA test, SAS software) $*p<0.05$, $**p<0.01$. Control 1 (C1), microsome alone; control 2 (C2), microsome+NADPH; control 3 (C3), microsome+NADPH+DMSO/ethanol; Vit E, vitamine E+NADPH+DMSO/ethanol.

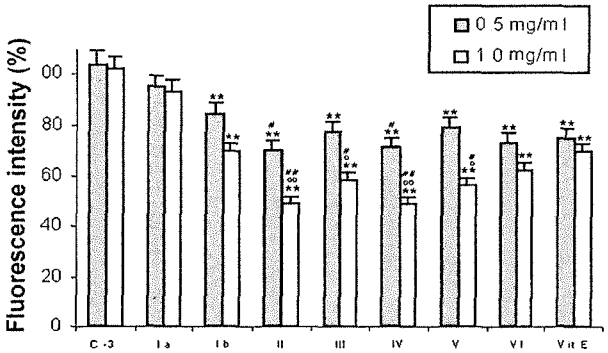

Fig. 3. NADPH-Initiated Microsomal Oxidation of DCFH-DA and Its Modification in the Presence of Glycyrrhetinic Acid Derivatives (**Ia**—**VI**) and Vitamin E (Vit E)

The reaction mixture was composed of the microsome suspension (0.5 mg/ml), NADPH-generating system, DCFH-DA, and the derivatives. After incubation at 37 °C for 30 min, microsomal oxidation was measured. Values are the mean±S.D. of three individual determinations. Asterisks indicate a significant difference from the NADPH control. C3, microsome+NADPH+DMSO/ethanol. * Significant difference *vs.* C3 ($*p<0.05$, $**p<0.01$); ° significant difference *vs.* vitamin E ($\circ p<0.05$, $\circ\circ p<0.01$); # significant difference *vs.* **Ib** ($\#p<0.05$, $\#\#p<0.01$) (one-way ANOVA test with SAS software).

are reported in Figs. 2 and 3.

Results and Discussion

Chemistry We applied a destruction method of aluminum salt with a calculated quantity of NaOH in reaction step b to minimize the rate of the second reaction (dehydratation of triols) and to facilitate the separation of product **II** from **IV** (the two stereoisomers) and from the byproduct aluminum salt. With this method, we achieved good separation of isomer **IV** with high purity with a simple crystallization method in chloroform and then in THF/AcOH. The isomer **II** was purified using the silica gel chromatographic method.

Dehydration of the triol **IV** into a diene (reaction step C-2) allows us to obtain a mixture of **V** and **III** in a 3 : 2 ratio. **III** was purified by recrystallization in absolute ethanol with $NaHCO_3$ under pressure, and **V** was purified by recristallization in chloroform. The separation process was performed using TLC and HPLC methods.

The product **VI** was obtained by reduction of a mixture of **II**+**IV** by catalytic hydrogenation in Pd-C, at ambient temperature and in absolute ethanol presaturated with $NaHCO_3$. It was purified by recrystallisation in ethyl acetate with a yield of 90%.

Biochemistry Samples were subsequently added to the biological oxidative system at a concentration of 0.50 and 1.00 mg/ml. The addition of NADPH to rat liver microsomes initiated DCFH-DA oxidation. This oxidation was elevated more than four-fold in the presence of 0.5 mM of NADPH (428±46 pmol/min/mg protein). At first, the addition of vitamin E (a known ROS scavenger) at an concentration of 1.0 mg/ml to the oxidation system resulted a diminution of DCFH-DA oxidation by 32%, as shown in Fig. 2. Second, the addition of 25 μM of DPI (an inhibitor of flavin oxidoreductase such as NADPH-cytochrome P450 reductase)[19] to the system resulted in suppression of NADPH-dependent electron transfer, and the oxidation of DCFH-DA was inhibited by 85%. These results demonstrate that ROS scavengers and inhibitors of microsomal enzyme can prevent the NADPH-dependent oxidation of DCFH-DA. We used this successful oxidation system to evaluate the NADPH-dependent ROS inhibition properties of our derivatives. We subsequently observed the inhibitory properties of NADPH-generated ROS production by the prepared derivatives. We found that the two hydroxylated derivatives **II** and **IV** have the most potent antioxidant activity. At a concentration of 1.0 mg/ml, they inhibited ROS formation by 50% and 51%, respectively, in the microsomal system. Moreover, two homo- and heterocyclic diene derivatives **III** and **V** inhibited ROS activity by 41% and 44%, respectively. Under the same conditions, the lead compound **Ib** inhibited ROS activity by 31%. The ROS inhibitory capacity of these derivatives increased with increasing concentration. We found that the saponin **Ia** had less antioxidant activity. All the results are shown in Fig. 3. Furthermore, we observed that the addition of an ethanol and DMSO mixture used to solubilize the products decreased the DCFH-DA oxidation levels relative to the control 2.

In the present study, we examined the initial structure–activity relationships of these derivatives by comparing the antioxidant actions monitored by the inhibition of microsomal ROS formation. Compounds **II** and **IV** showed the most intensive activity. The activity of all derivatives was in the decreasing order **IV**≥**II**>**V**>**III**>**VI**>**Ib**>**Ia**. From these results, it can be concluded that chemical reduction of 11-keto and 30-carboxyl functions into alcohol groups can increase the free radical-scavenging activity of the lead compound (**Ib**). However, it is also known that dehydratation of the 11-hydroxyl group gives the homo- and heterocyclic diene derivatives with relatively potent antioxidant activity.

Our findings indicate that the antioxidant property of some synthetic derivatives of **Ib** could represent potential therapeutic activity with multiple protective mechanisms against free radical damage, such as antioxidant, anti-inflammatory, and hypolipidemic properties.

Experimental

Chemistry Melting points were determined on a Kofler apparatus. Reaction courses were routinely monitored using thin-layer chromatography (TLC) on silica gel 60 F254 (Merck) with detection under a 254-nm UV lamp. ^{1}H-NMR spectra were determined in $CDCl_3$ solution with a Bruker DRX-400 (400 MHz) spectrometer and chemical shifts are presented in ppm from internal tetramethylsilane as a standard. Mass spectroanalyses were performed with a quadrupolar spectrometer VG Platform II. HPLC analysis was performed on a Merck L-3000/diode array with a Lichrospher-100 diol column (5 μm, 25 cm). All compounds showed a purity higher than 99% following analytical HPLC. Other analytical parameters including UV (UV Krontron) and IR (with KBr) spectra were also examined.

18β-Glycyrrhetinic Acid. 3β-Hydroxy-11-oxo-18β-olean-12-en-30-oic (Ib) Glycyrrhizin (**Ia**), a diglucuronide saponin of licorice root (*G. glabra*), colorless needless, mp 335 °C, was hydrolyzed to prepare **Ib**, which was used as a starting compound for chemical modification. **Ib**: Colorless needles, yield 70%, mp 296 °C (EtOH). ^{1}H-NMR ($CDCl_3$) δ ppm: 0.83 (3H, s, C-28 H_3), 0.86 (3H, s, C-24 H_3), 1.03 (3H, s, C-26 H_3), 1.15 (3H, s, C-23 H_3), 1.16 (3H, s, C-25 H_3), 1.25 (3H, s, C-29 H_3), 1.39 (3H, s, C-27 H_3), 2.37 (1H, s, C-9 H), 2.81(2H, dt/3.20, 13.6, C-1 H_2), 3.26 (1H, dd/5.80, 10.80, C-3 H), 5.73 (1H, s, C-12 H). IR (KBr) cm^{-1} ν: 3440 (OH), 3046 (C sp^2), 2943 (C sp^3), 1666 (C=O, Cα=Cβ), 1018 (C-3 OH); δ: 1446 (CH_2, α CH_3), 1375 (s CH_3).

18β-Olean-12-ene-3β,11α,30-triol (II) Colorless needles, yield 1%, mp 222—225 °C. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.83 (3H, s, C-28 H_3), 0.86 (3H, s, C-24 H_3), 0.93 (3H, s, C-26 H_3), 1.02 (3H, s, C-25 H_3), 1.03 (3H, s, C-29 H_3), 1.08 (3H, s, C-23 H_3), 1.28 (3H, s, C-27 H_3), 3.26 (1H, dd/5.80, 10.80, C-3 H), 3.55 (2H, AB/-10.96, C-30 H_2), 4.21 (1H or 2H, dd/3.62, 8.16, C-11 H), 5.26 (1H, dd/3.58, C-12 H). Not detected: C-1 H_2, C-9 H_1. IR (KBr) cm^{-1} ν: 3368 (OH), 3061 (C sp^2), 2924 (C sp^3), 1655 (C=C), 1029 (C-3 or 11 OH), δ: 1461 (CH_2, α CH_3), 1375 (s CH_3). MS *m/z* Calcd for $C_{30}H_{50}O_3$: M$^+$ 458.3760. Found: M$^+$ 458.3706. TLC *Rf*=0.19 (ethyl acetate–*n*-hexane, 70 : 30).

18β-Olean-11,13(18)-diene-3β,30-diol (III) Colorless needles, yield 31%, mp 228—230 °C. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.73 (3H, s, C-26 H_3), 0.87 (3H, s, C-25 H_3), 0.87 (3H, s, C-28 H_3), 0.92 (3H, s, C-24 H_3), 0.98 (3H, s, C-29 H_3), 1.07 (3H, s, C-23 H_3), 1.09 (3H, s, C-27 H_3), 3.26 (1H, dd/4.95, 11.28), 3.38 (2H, s, C-30 H_2), 5.56 (1H, d large/10.72, C-12 H), 6.39 (1H or 2H, dd/2.66, 10.36, C-11 H). Not detected: C-1 H_2, C-9 H_1. IR (KBr) cm^{-1} ν: 3353 (OH), 3040 (C sp^2), 2856—2974 (C sp^3), 1637 (C=C), 1022 (C-3 or 11 OH), 926 (C-30 OH), δ: 1478 (CH_2, α CH_3), 1360 (s CH_3). MS *m/z* Calcd for $C_{30}H_{48}O_2$: M$^+$ 440.3654. Found: M$^+$ 440.3658. TLC *Rf*=0.43 (CH_2Cl_2–MeOH, 96 : 4). HPLC t_R=12.8 min.

18β-Olean-12-ene-3β,11β,30-triol (IV) Colorless needles, yield 77%, mp 238—239 °C. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.85 (3H, s, C-28 H_3), 0.89 (3H, s, C-24 H_3), 0.93 (3H, s, C-26 H_3), 1.02 (3H, s, C-25 H_3), 1.13 (3H, s, C-23 H_3), 1.26 (3H, s, C-29 H_3), 1.42 (3H, s, C-27 H_3), 3.26 (1H, dd/5.41, 10.59, C-3 H), 3.55 (2H, AB/-10.77, C-30 H_2), 4.33 (1H or 2H, s large pic, C-11 H), 5.33 (1H, d/3.94, C-12 H). Not detected: C-1 H_2, C-9 H_1. IR (KBr) cm^{-1} ν: 3412 (OH), 2927 (C sp^3), 1634 (C=C), 1022 (C-3 or 11 OH), δ: 1456 (CH_2, α CH_3), 1382 (s CH_3). MS *m/z* Calcd for $C_{30}H_{50}O_3$: M$^+$ 458.3760. Found: M$^+$ 458.3728. TLC *Rf*=0.31 (ethyl acetate–*n*-hexane, 70 : 30).

18β-Olean-9(11),12-diene-3β,30-diol (V) Colorless needles, yield 63%, mp 236 °C. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.83 (3H, s, C-28 H_3), 0.91 (3H, s, C-26 H_3), 0.92 (3H, s, C-24 H_3), 1.03 (3H, s, C-27 H_3), 1.05 (3H, s, C-25 H_3), 1.15 (3H, s, C-23 H_3), 1.21 (3H, s, C-29 H_3), 3.26 (1H, t/5.87, C-3 H), 3.57 (2H, AB/-10.64, C-30 H_2), 5.56 (1H or 2H, dd/5.96, 25.22, C-11 H and C-12 H). Not detected: C-1 H_2, C-9 H_1. IR (KBr) cm^{-1} ν: 3390 (OH), 3038 (C sp^2), 2978—2853 (C sp^3), 1628 (C=C), 1029 (C-3 OH), 985 (C-30 OH), δ: 1449 (CH_2, α CH_3), 1360 (s CH_3). MS *m/z* Calcd for $C_{30}H_{48}O_2$: M$^+$ 440.3654. Found: M$^+$ 440.3663. TLC *Rf*=0.43 (CH_2Cl_2–MeOH, 96 : 4). HPLC t_R=12.2 min.

11-Deoxo-glycyrrhetol (18β-Olean-12-ene-3β,30-diol) (VI) Colorless needles, yield 90%, mp 250—254 °C. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.81 (3H, s, C-28 H_3), 0.85 (3H, s, C-26 H_3), 0.92 (3H, s, C-25 H_3), 0.96 (3H, s, C-24 H_3), 0.98 (3H, s, C-29 H_3), 1.02 (3H, s, C-23 H_3), 1.17 (3H, s, C-27 H_3), 3.25 (1H, dd/4.41, 11.93, C-3 H), 3.54 (2H, AB/-10.82, C-30 H_2), 5.21 (1H, t/3.21, C-12 H). Not detected: C-1 H_2, C-9 H_1, C-11 $H_{1\text{ or }2}$. IR (KBr) cm^{-1} ν: 3268 (OH), 3081 (C sp^2), 2971—2860 (C sp^3), 1691 (C=C), 1022 (C-3 OH), 926 (C-30 OH), δ: 1471—1390 (s CH_3). MS *m/z* Calcd for $C_{30}H_{50}O_2$: M$^+$ 442.3811. Found: M$^+$ 442.3842. TLC *Rf*=0.23 (CH_2Cl_2–MeOH, 96 : 4).

Biochemistry Animals and Microsome Preparation: Male Sprague-Dawley rats (150—180 g, Iffa-Credo, France) were housed in a controlled environment room with a 12-h light/dark photoperiod. The rats were fasted overnight prior to killing. Liver microsomes were prepared by differential centrifugation as previously described.[20] Protein concentrations were determined using the method of Lowry *et al.*[21] with bovine serum albumin as a standard.

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Oxidative Stress and Inflammation: Essential Partners in Alcoholic Liver Disease

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Abstract

Alcoholic liver disease (ALD) is a multifaceted disease that is characterized by hepatic steatosis or fat deposition and hepatitis or inflammation. Over the past decade, multiple lines of evidence have emerged on the mechanisms associated with ALD. The key mechanisms identified so far are sensitization to gut-derived endotoxin/lipopolysaccharide resulting in proinflammatory cytokine production and cellular stress due to oxidative processes, contributing to the development and progression of disease. While oxidative stress and inflammatory responses are studied independently in ALD, mechanisms linking these two processes play a major role in pathogenesis of disease. Here we review major players of oxidative stress and inflammation and highlight signaling intermediates regulated by oxidative stress that provokes proinflammatory responses in alcoholic liver disease.

1. Introduction

The pathogenesis of alcoholic liver disease (ALD) is a consequence of chronic alcohol abuse and approximately 44% of the 26,000 deaths from cirrhosis are due to ALD in the United States [1]. Alcoholic hepatitis, the clinical presentation of ALD, remains to be a common life threatening cause of liver failure, especially when it is severe. Chronic alcohol consumption has long been associated with progressive liver disease from steatosis to inflammation, development of hepatic cirrhosis, and the subsequent increased risk of hepatocellular carcinoma. Several studies have attempted to identify the molecular pathways, direct or indirect, affected by alcohol exposure in the liver. These pathways range from oxidative stress, metabolism-related effects, and inflammation to apoptosis. Induction of oxidative stress and activation of the inflammatory cascade are identified as key elements in the pathophysiology of ALD [2]. While these intracellular mechanisms affected by alcohol are studied exclusively, the interplay of signaling molecules between pathways leading to alcoholic liver disease has received less attention. Unraveling these interactions of oxidative stress mediators and inflammatory signaling in the liver will aid in identification of new integrative approaches as it relates to alcoholic liver injury and provide potential new directions to develop therapeutic target intervention. The goal of this concise paper is to first review alcohol-induced reactive oxygen species and oxidative stress generated by alcohol metabolism, endoplasmic reticulum stress, mitochondrial ROS in the liver, protein adduct formation, and autophagy and chaperone function and then to describe stress-mediated activation of receptors, kinases, and transcription factors resulting in proinflammatory signaling in ALD.

2. Classical Mechanisms of Alcoholic Liver Disease

Research done, so far, on the effects of pathophysiological mechanisms of alcoholic liver disease suggests the involvement of two main liver cell types, resident macrophages, or Kupffer cells and hepatocytes. The role of gut-derived endotoxin and liver macrophage activation is clearly established in ALD by Thurman and colleagues [2]. The deleterious effects of alcohol, attributed to its metabolism, primarily occur in hepatocytes [3]. Alcohol metabolism pathway including induction of cytochrome P450 2E1 [3] results in adduct formation and generation of reactive oxygen radicals respectively creating an oxidative microenvironment and damage in the liver [2]. In the currently accepted model of ALD, chronic alcohol induces oxidative stress and sensitization to endotoxin, which activates the CD14/TLR4 pathway and downstream signaling resulting in proinflammatory cytokine production [4]. The proinflammatory cytokines, particularly TNFα, then provoke hepatocellular injury and death by extrinsic, via TNFR1 [5] and intrinsic death pathways [6] leading to ALD. While the role of oxidative stress and macrophage activation, the two main pathophysiological processes affected in ALD, were studied independently in the past, recent studies suggest that these pathways are interconnected in ALD improving our understanding of the disease.

3. Reactive Oxygen Species (ROS) and Alcohol

While activation of inflammatory responses are central to alcoholic liver injury, excessive generation of reactive oxygen species plays an equally significant role in alcohol-induced cellular damage [7]. Alcohol-induced liver disease is associated with a state of "oxidative stress". The metabolism of alcohol by alcohol dehydrogenase [ADH] leads to formation of acetaldehyde. Further, the acetaldehyde is metabolized to acetate by acetaldehyde dehydrogenase [ALDH]. Acetaldehyde, a reactive intermediate has an ability to form adducts with DNA [8, 9]. Whether acute or chronic, alcohol metabolism increases production of acetaldehyde and enhances formation of DNA adducts leading to tissue injury. On the other hand, metabolism of alcohol via cytochrome P4502E1 induces production of reactive oxygen species which facilitates adduct formation, activates stress proteins, induces endoplasmic reticulum stress, and affects lysosomal function and autophagy leading to mitochondrial injury and hepatocellular death.

3.1. Alcohol Metabolism and ROS

Ethanol is primarily metabolized in the liver by oxidative enzymatic pathways. The classical pathway of alcohol metabolism involves enzymatic breakdown of alcohol by the enzyme, alcohol dehydrogenase (ADH) and its subsequent conversion to acetaldehyde and formation of acetate. ADH is predominantly expressed in liver [10] but other tissues like gastric mucosa express ADH and contribute to metabolism of alcohol [10]. Aldehyde dehydrogenase (ALDH) contributes to oxidation of aldehyde intermediates resulting in acetate which is unstable and breaks down to water and carbon dioxide. The second major pathway for ethanol degradation is the microsomal system catalyzed by cytochrome P450 enzymes. The 2E1 isoform of the cytochrome P450 (CYP2E1) system is induced during chronic alcohol consumption. Activation of CYP2E1 leads to ROS generation and highly reactive free radicals including superoxide anions and hydroxyl radicals resulting in oxidative stress and cell death [11]. The role of CYP2E1 in hepatocyte injury has been elucidated using HEPG2 cells overexpressing CYP2E1 [12], CYP2E1 knockout mice, and transgenic mice [13]. Increased oxidative stress from induction of CYP2E1 in vivo sensitizes hepatocytes to LPS and TNFα toxicity [14] and CYP2E1 knock-in mice showed elevated hepatic steatosis and liver injury after alcohol feeding [13]. On the other hand, CYP2E1 knockout mice showed decreased oxidant stress, upregulation of PPARα and were protective to alcohol-induced liver injury. Peroxynitrite, activation of p38 and JNK MAP kinases, and mitochondrial dysfunction are downstream mediators of the CYP2E1-LPS/TNF potentiated hepatotoxicity [15]. Oxidation of ethanol by alcohol dehydrogenase and subsequent metabolism of acetaldehyde results in increased NADH/NAD+ ratio in the cytoplasm and mitochondria [16]. The increase in NADH results in inhibition of mitochondrial β-oxidation and accumulation of intracellular lipids [17]. Alcohol/CYP2E1-mediated ROS has the potential to peroxidize lipids and inhibit mitochondrial and peroxisomal β-oxidation enzymes such as acyl-CoA dehydrogenases, carnitine palmitoyl transferase-1 (CPT-1), and peroxisomal proliferator-inducing pathways, respectively [18]. This disruption leads to increased fatty acids, substrates of these enzymes, and their accumulation resulting in development of hepatic steatosis. Oxidative stress and ROS generation due to alcohol metabolism not only increase accumulation of lipids in hepatocytes but also sensitize the liver to subsequent insults by cytokines.

3.2. Mitochondria and Oxidative Stress

In mitochondria, ROSs are generated as undesirable side products of the oxidative energy metabolism. An excessive and/or sustained increase in ROS production has been implicated in the pathogenesis of ALD, ischemia/reperfusion injury, and other diseases [19]. Oxidative stress induced by alcohol is closely associated with alterations in mitochondrial function resulting in cellular death. Hepatic mitochondria either acutely or chronically exposed to ethanol generate increased levels of reactive oxygen species (ROS) [20]. The induction of mitochondrial dysfunction is also linked to the metabolism of alcohol by CYP2E1 and increased oxidative stress [11]. Primary hepatocytes and rat hepatoma cells when treated with ethanol led to an increase in ROS/RNS and loss of mitochondrial function due to damaged mitochondrial DNA and ribosomes and subsequent inhibition of mitochondrial protein synthesis [21, 22]. Studies have shown that alcohol-induced ROS generation leads to alteration in mitochondrial membrane permeability and transition potential that in turn initiates the release of proapoptotic factors such as cytochrome c [21]. Transition of mitochondrial permeability results in increased caspase-3 activation in hepatocytes and this depends on p38 MAPK activation but is independent of caspase-8 [5]. Various studies show that decreased ATP synthesis accompanied by reduced mitochondrial protein synthesis, inhibition of the oxidative phosphorylation system (OxPhos), and damage to mitochondrial DNA leads to dysfunctional mitochondria and oxidative stress in alcoholic liver disease [23]. Peroxisome proliferator activated receptor gamma (PPARγ)-coactivator 1 alpha (PGC-1α), a transcription coactivator involved in mitochondrial biogenesis, is involved in defenses against ROS by inducing many ROS-mediated detoxifying enzymes. PGC-1 gene expression was lower in hepatic tissues of rats exposed to ethanol [24]. In vitro exposure of hepatoma cells to 500 mM ethanol significantly decreased hepatic SIRT-1; PGC-1α leads to ROS-induced mitochondrial and cellular injury [25]. Certain sirtuins, a family of protein deacetylases, were found to regulate glucose and fat metabolism in mammals [26, 27] and to enhance mitochondrial biogenesis in liver and muscle through PGC-1α and to influence cell survival [28].

Recent studies used an antioxidant peptide targeted to mitochondria to show that altered ROS metabolism facilitates enhanced expression of HIF-1alpha [29], which, in turn, increases TNF-alpha secretion. These findings provide in vivo evidence for the action of mitochondrial ROS on HIF-1alpha activity and demonstrate that changes in mitochondrial function within physiologically tolerable limits can modulate the immune response [29]. These studies suggest that alcohol-induced mitochondrial stress pathways set the stage for proinflammatory cytokine-induced cell death and liver injury.

3.3. Protein Adducts and Lysosomal Dysfunction

Alcohol metabolism and oxidative stress result in the formation of reactive aldehydes such as acetaldehyde, malondialdehyde (MDA), and 4-hydroxy-2-nonenal (HNE) that can bind to proteins to form adducts [30]. In vivo models of chronic alcohol consumption have shown that acetaldehyde, MDA, and HNE adduct formation are increased in various organs including the liver [30]. A strong corelation between 4-HNE adducts and expression of CYP2E1 in patients with ALD was recently shown [31]. Acetaldehyde and MDA react with proteins synergistically to form hybrid protein adducts called malondialdehyde-acetaldehyde (MAA) adducts [32]. Recognition of MAA-adducts by Kupffer cells, endothelial, and stellate cells via the scavenger receptor resulted in upregulation of cytokine and chemokine production and increased expression of adhesion molecules [32]. Circulating antibodies to MAA-adducts were detected in patients with alcoholic hepatitis and cirrhosis and correlated with the severity of liver injury [33]. Chronic alcohol feeding also induces formation of gamma-ketoaldehyde protein adducts in mouse livers [34]. These adducts are formed in a TNFR1/CYP2E1 dependent, but cyclooxygenase-independent manner in mouse liver [34]. Existence of protein adducts during chronic alcohol consumption and their identification in animal models has been challenging, limiting investigation of their precise role in ALD.

Increased ROS and lipid peroxidation rate in microsomal and lysosomal membranes with a simultaneous decrease in the levels of glutathione sulfhydryls and glutathione-S-transferase activity was observed during alcohol exposure [35]. Elevation of cathepsin B in hepatic cytosol fractions, indicating lysosomal leakage, was reported in ethanol-fed rats [36]. Lysosomal leakage was increased in alcohol-fed mice deficient in superoxide dismutase (SOD) indicating that oxidative stress correlated with loss of lysosomal function increased hepatic fat and inflammatory cell infiltration [37]. The exact mechanisms responsible for ethanol-induced changes in lysosomal function are not clear but there is evidence of enhanced lysosomal membrane fragility, which could result from either altered lipid peroxidation, oxidative stress, or both [38]. More recently, degradation of a cell's own cytosolic components in the lysosomes as a protective mechanism against the damaging effects of oxidative stress has been described and is termed autophagy [38]. Alcoholic liver injury is associated with decreased autophagy resulting in accumulation of damaged proteins and liver cell death [38]. Recent studies show that macro-, micro- and chaperone-mediated autophagy is linked to innate and adaptive immune responses [39]. While autophagy acts as an effector and regulator of pattern recognition receptors including TLR4 signaling in macrophages, loss or defective autophagy results in accumulation of cytosolic components and chronic inflammatory responses [40]. How loss of autophagy after chronic alcohol consumption contributes to proinflammatory responses in alcoholic liver disease remains to be investigated.

3.4. Endoplasmic Reticulum (ER) Stress

The unfolded protein response (UPR) is a protective response of the cell also referred to as the ER stress response during pathological conditions. In alcoholic liver disease, increased expression of glucose regulatory protein (GRP)78, GRP94, CHOP, and caspase-12 indicated a UPR/ER stress response [41]. Upregulation and activation of ER-localized transcription factors such as SREBP-1c and SREBP-2 were associated with increased lipid accumulation and induction of fatty liver during chronic alcohol exposure [42]. Another important inducer of ER stress, homocysteine, was increased in alcoholic human subjects leading to hyperhomocysteinemia, also observed in alcohol feeding rodent models [43]. The role of ER stress in triglyceride accumulation and fatty liver comes from studies showing that betaine increases an enzyme, betaine homocysteine methyltransferase (BHMT) and reduces homocysteine levels to inhibit lipid accumulation [43]. Recent studies suggest that ER/UPR stress pathways intersect with innate immune signaling determining the duration and intensity of inflammatory response [44]. Additional mechanistic studies to link ER/UPR stress and innate immune responses as a pathophysiological contributor in ALD are warranted.

3.5. Alcohol, Stress, and Molecular Chaperones

Stress or heat shock proteins (hsps) are ubiquitous and highly conserved proteins, functioning as molecular chaperones, whose expression is induced by oxidative stress stimuli and in response to accumulation of unfolded cellular proteins. Oxidative stress induces heat shock proteins via activation of the heat shock transcription factor (HSF) [45]. Male Wistar rats fed with acute as well as chronic alcohol showed induction of hsp70 in the various regions of the brain and the liver [46, 47]. However, the intensity of induction of hsp70 in the liver, the principal organ of ethanol oxidation, was much lower than the hippocampus or striatal areas of the brain [47]. Hsp90 levels, on the other hand, were increased in cultured rat hepatocytes exposed to acute alcohol [47, 48]. Acute and chronic alcohol treatment of monocytes/macrophages showed alterations in hsp70 and hsp90 mRNA and protein levels based on the length of alcohol exposure [49]. Acute alcohol induces HSF and hsp70, whereas chronic alcohol induces hsp90 but not hsp70 protein, through activation of HSF [49]. Hsp90 functions as a molecular chaperone controlling activity of various kinases and signaling molecules of the LPS signaling pathway such as CD14 [50], IKK [51], and IRAK [52]. Comprehensive studies on the effect of acute and chronic alcohol exposure on chaperone function of hsps in inflammatory responses in the alcoholic liver could provide novel mechanistic insights in ALD.

4. Inflammatory Response and ALD

Extensive studies over the past two decades have identified the importance of macrophage activation in the liver by gut-derived endotoxin after prolonged alcohol consumption [2]. Central to this activation is the sensitization of macrophages due to alcohol exposure and is associated with mechanisms ranging from upregulation and engagement of surface receptors on innate immune cells, intracellular kinases and transcription factors contributing to induction of proinflammatory cytokines.

4.1. Pattern Recognition Receptors, Alcohol, and Immune Cells

Pattern recognition receptors (PRRs) are expressed on liver nonparenchymal and parenchymal cells and function as sensors of microbial danger signals enabling the vertebrate host to initiate an immune response. The complexity of cellular expression of PRRs in the liver provides unique aspects to pathogen recognition and tissue damage in the liver [53]. Toll-like receptors (TLRs) that are membrane associated or endosomal recognize distinct microbial components and activate different signaling pathways by selective utilization of adaptor molecules [54]. TLRs such as TLR4 and TLR2 that detect PAMPs like LPS and lipoproteins, respectively, are located on the cell surface whereas; TLRs such as TLR3, TLR7, and TLR9 that detect viral RNA and DNA are located in the endosome [55]. The pivotal role of TLR4 as well as other TLRs has been extensively studied in alcoholic tissue injury [56–59].

The interaction of oxidative stress and TLR signaling is emerging. TLR4 is capable of inducing ROS leading to oxidative stress [59–61]. Kupffer cells or hepatic macrophages produce reactive oxygen species (ROS) in response to antigenic stimuli and chronic alcohol exposure as well as endotoxin [62, 63]. Alcohol-induced sensitization of macrophages to LPS has been attributed to ROS production [59, 60, 64]. Previous studies from Nagy and colleagues [64, 65] also show that chronic ethanol feeding increases the sensitivity of Kupffer cells to lipopolysaccharide (LPS), leading to increased tumor necrosis factor alpha (TNFα) expression. NADPH oxidase and ROS generation exhibit direct interaction with the TLR4 receptor and activation of down-stream kinases and transcription factors [61]. Studies by Gustot et al. [59] show that oxidative stress regulates TLR 2, 4, 6, and 9 mRNA expression in alcoholic liver. Thus, it appears that TLR mRNA, protein expression, and immune signaling can be strongly influenced by oxidative stress in ALD making these two events dependent on each other and not mutually exclusive. Besides ROS, TLRs also mediate responses to host molecules including intracellular mediators [66]. Amongst the well-characterized DAMPs, high-mobility group box 1 (HMGB1), S100 proteins, hyaluronan, and heat shock protein 60 (hsp60) are known to be recognized by TLR2 and TLR4 [66, 67]. In addition, necrotic or apoptotic cells are also recognized as DAMPs by TLRs [67]. In alcoholic liver injury, apoptotic bodies, generated due to alcohol-induced oxidative stress, could be recognized by DAMPs [68] and contribute to inflammatory responses in the liver.

Activation of TLR4 recruits IRAK-1 to the TLR4 complex via interaction with MyD88 and IRAK-4 [69]. The role of MyD88, the common TLR4 adaptor molecule, was evaluated in a mouse model of alcoholic liver injury [60]. These studies showed that MyD88 knockout mice were highly

susceptible to alcohol-induced fatty liver [60]. While alcohol feeding in TLR4 deficient mice prevented activation of NADPH oxidase, alcohol-fed MyD88 deficient mice showed high NADPH oxidase activity and increased oxidative stress resulting in liver injury [60].

Increasing evidence suggests that downstream signaling components activated by TLRs as well as cytokines and chemokines produced can be regulated by oxidative stress pathways. These interactions of stress pathways leading to inflammation could contribute largely to initiation and perpetuation of alcohol-related injury in the liver. The cross-talk of stress regulated intracellular molecules with TLRs, intracellular kinases and transcription factors resulting in alterations in cytokines/chemokines in ALD are of great importance.

4.2. MAPKs and IKKs

LPS/TLR4-induced ROS activation [61] plays an important role in activation of downstream signaling molecules such as IRAK1/4, TRAF6 leading to activation of MAP kinases and NFκB during chronic alcohol exposure [69]. Mitogen-activated protein kinase [MAPK] signaling cascade plays an essential role in several cellular processes including proliferation, differentiation, and apoptosis. Acute alcohol exposure results in activation of baseline p42/44 MAPK in hepatocytes [70] while chronic alcohol exposure causes potentiation of endotoxin-stimulated p42/44 MAPK, and p38 MAPK signaling in Kupffer cells leading to increased synthesis of TNFα [71, 72]. LPS stimulation of Kupffer cells in vitro exposed to chronic alcohol in vivo exhibited increased p38 activity and decreased JNK activity [71, 73]. Inhibition of p38 activation impaired alcohol-mediated stabilization of TNFα mRNA likely via interaction with tristetraprolin (TTP) [74]. On the other hand, ERK1/2 inhibition did not alter TNFα mRNA stability but affected mRNA transcription in chronic alcohol-exposed macrophages via Egr-1 binding to the promoter [75]. Whether alcohol-induced ROS plays a role in MAPK activation in ALD is not yet determined.

TLR4-induced MyD88-dependent and independent pathways lead to IKK kinase activation resulting in proinflammatory cytokine production [71]. Oxidative stress and ROS-mediated molecular chaperones such as hsp90 are shown to facilitate IKK kinase activity and downstream NFκB nuclear activation [51]. Studies show that chronic alcohol-induced NFκB activation in macrophages is due to increased hsp90 resulting in elevated IKK kinase activity [49]. Inhibition of hsp90 in chronic alcohol-exposed macrophages resulted in decreased IKK kinase activity and NFκB binding suggesting a cross-talk between cellular stress and inflammatory pathways [49].

4.3. Transcription Factors in ALD

The transcription factor NFκB is a ubiquitous transcription factor that can be activated by a large number of extracellular stimuli such as cytokines, chemokines, growth factors, and bacterial or viral products [76]. NFκB activation triggers the induction of inflammatory genes and plays an important role in initiation and progression of alcoholic liver disease [69, 77]. While TLR-mediated activation of NFκB is well established, ROS-induced activation of NFκB occurs but remains poorly understood. Chronic alcohol exposure induces LPS/TLR4-mediated NFκB activation in human monocytes and macrophages contributing to production of proinflammatory cytokine, TNFα [77]. Whether ROS mediates activation of NFκB directly during ALD is unclear. TLR4-induced MyD88-independent signaling leads to activation of IKKε and interferon regulatory factor 3 (IRF3) and downstream Type I IFN activation [78, 79]. Previous studies show that ROS mediates LPS-induced IRF3 activation [80]. Investigators found that IRF3 binds to the TNFα promoter in macrophages after chronic alcohol administration [81] and induces TNFα production. Whether alcohol-induced ROS mediates IRF3 induction to increase proinflammatory cytokines and liver injury needs further investigation.

Alcohol-mediated fatty liver injury is associated with increased expression of genes regulating fatty acid synthesis and suppression of genes involved in fatty acid oxidation [82]. Transcription factors like SREBP and PPARα play a pivotal role in fatty acid metabolism and rodent models as well as in vitro treatment studies with alcohol show downregulation of PPARα mRNA [83]. Further, DNA-binding activity of PPARα is significantly reduced resulting in decreased expression of target genes involved in fatty acid metabolism after alcohol exposure [83]. Decreased PPARα activity was accompanied by increased oxidative stress in the liver resulting in increased sensitization of TNFα-induced liver injury [83].

Another transcription factor, STAT3, in alcoholic liver injury was recently investigated in hepatocyte-specific STAT3 knockout (H-STAT3KO) mice and macrophage/neutrophil-specific STAT3 KO (M/N-STAT3KO) and endothelial STAT3 mice [84]. Compared with wild-type mice, Kupffer cells from alcohol-fed hepatocyte-specific STAT3KO mice produced similar amounts of ROS and hepatic proinflammatory cytokines compared to control mice [85]. On the other hand, Kupffer cells from M/N-STAT3KO mice produced higher ROS and TNFα compared with wild-type controls. These results suggest that STAT3 in hepatocytes promotes ROS production and inflammation whereas myeloid cell STAT3 reduces ROS and hepatic inflammation during alcoholic liver injury [85]. Thus, STAT3 may regulate hepatic inflammatory cytokines via ROS production.

5. Stress and Immune Signaling: How Are They Linked in ALD?

Cellular stress responses during alcohol exposure include oxidative stress due to metabolism of alcohol in the liver, ER stress, mitochondrial imbalance, heat shock protein induction, and inflammatory processes. Numerous mouse models have been used to study the discrete role of each of the stress responses in alcohol-mediated liver pathology. Yet, accumulating evidence suggests that these pathways cannot be regarded separately and are tightly interrelated. Similar to other inflammatory diseases [86], alcoholic liver disease is multifactorial and it is important to take into account interactions between various cellular responses for a better understanding of the pathogenesis of ALD. Based on studies so far, a clear relationship between oxidative stress and inflammation is emerging in ALD. It is increasingly apparent that in addition to gut-derived endotoxin, alcohol-induced upregulation of oxidative stress mediators plays a major part in activation of receptors, intracellular kinases, and transcription factors in innate immune cells (Figure 1). Pathways described above that are interrelated in ALD include ROS-mediated activation of TLR4 in macrophages, mitochondrial ROS regulation of transcriptional activators such as PGC-1α and HIF-1α promoting TNFα induction, ROS and autophagy associated enhancement of proinflammatory cytokine production [86], ER stress-associated innate immune cell activation, hsp-mediated activation of proinflammatory signaling kinases, and finally direct activation of transcription factors such as NFκB and STAT3 by ROS. Alcoholic liver disease exhibits enhanced inflammatory responses and exaggerated TNFα production leading to liver injury. While TNFRI knockout mice are protected from alcohol-induced liver injury [87], alcohol-induced ROS production was unaffected in TNFRI knockout mice indicating that ROS predominantly serves as a redox signal for proinflammatory cytokine production and may not be a direct toxicant to hepatocytes [87]. These studies argue against the direct role of ROS or oxidative stress in alcoholic liver injury and in fact support the notion that oxidative stress/ROS primarily affects and is indispensable to proinflammatory activation and cytokine induction in ALD creating a vicious cycle of the two pathways (Figure 1). Thus, attempts to further clarify the importance of oxidative stress and its cross-talk with inflammatory pathways will provide an insight into pathogenesis of ALD and open avenues for novel therapeutic targets.

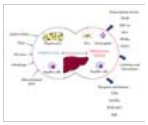

Figure 1: Oxidative stress and inflammation: interacting mechanisms in ALD. The development of alcoholic liver injury is a complex process involving oxidative stress microenvironment in the liver contributed by hepatocytes and macrophages. In addition to the activation of macrophages by gut-derived endotoxin, cellular stress responses contribute to proinflammatory cytokine production creating a tightly interrelated network in ALD.

6. Conclusion

This paper clearly implicates the role of oxidative stress in proinflammatory signaling and macrophage activation during liver injury providing a feed-forward mechanism in ALD. Therefore, targeting redox-sensitive inflammatory pathways and transcription factors offers great promise for treatment of ALD. Investigation of agents that interfere with oxidative stress mediators directly hampering inflammatory cytokine production is needed. Whether these agents will then alleviate alcoholic liver disease in patients should be tested.

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The Long Term Efficacy of Glycyrrhizin in Chronic Hepatitis C Patients

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BACKGROUND. Hepatocellular carcinoma (HCC) occurs in patients with hepatitis C virus-RNA positive chronic liver disease. It is important to prevent HCC with drug administration.

METHODS. A retrospective study was undertaken to evaluate the long term preventive effect of Stronger Neo-Minophagen C (SNMC) on HCC development. SNMC is a Japanese medicine that is commonly administered to patients with chronic hepatitis C to improve the serum alanine aminotransferase (ALT) level. Of 453 patients diagnosed with chronic hepatitis C retrospectively in the study hospital between January 1979 and April 1984, 84 patients (Group A) had been treated with SNMC; SNMC was given at a dose of 100 mL daily for 8 weeks, then 2–7 times a week for 2–16 years (median, 10.1 years). Another group of 109 patients (Group B) could not be treated with SNMC or interferon for a long period of time (median, 9.2 years) and were given other herbal medicine (such as vitamin K). The patients were retrospectively monitored, and the cumulative incidence of HCC and risk factors for HCC were examined.

RESULTS. The 10th-year rates of cumulative HCC incidence for Groups A and B were 7% and 12%, respectively, and the 15th-year rates were 12% and 25%. By Cox regression analysis, the relative risk of HCC incidence in patients not treated with SNMC (Group B) was 2.49 compared with that of patients treated with SNMC (Group A).

CONCLUSIONS. In this study, long term administration of SNMC in the treatment of chronic hepatitis C was effective in preventing liver carcinogenesis. ***Cancer* 1997; 79:1494–500.** © *1997 American Cancer Society.*

KEYWORDS: chronic hepatitis C, Stronger Neo-Minophagen C, glycyrrhizin, hepatocellular carcinoma.

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. Recently, greater than 80% of patients with HCC were found to have RNA for the hepatitis C virus (HCV).[1] The yearly incidence of HCC in patients with HCV-RNA positive cirrhosis ranges from 5–7% in Japan.[1–3] Kiyosawa et al.[1] reported that patients with long term abnormal serum levels after transfusion had a high probability of histological *aggravation* as well as HCC. However, there are few reports of chemopreventive studies of HCC.[5] In this study, the authors analyzed the chemopreventive action of Stronger Neo-Minophagen C (SNMC) in HCC patients. In Japan, SNMC has been used in the form of an intravenous solution, comprised of 0.2% glycyrrhizin (GL), 0.1% cysteine, and 2.0% glycine in physiologic solution. It is made by dissolving GL (200 mg), cysteine (100 mg), and glycine (2 g) in 100 mL of physiologic saline. GL is an aqueous extract of licorice root (glycyrrhizae radix), which is antiallergic and has detoxicating effects. As has been reported, the antiinflammatory mechanism of

SNMC is believed to be due to its protective effect on the hepatic cellular membrane, which may explain its ability to lower the serum transaminase level in patients with chronic hepatitis.[6,7] Glycine prevents an aldosteron-like action of GL[8] and cysteine has been found to be detoxicative as well as antiallergic through cysteine conjugation in the liver.[9] Therefore, SNMC has been developed with the expectation of the joint beneficial effect of these three components. Yamamoto et al.[10] first treated patients with chronic hepatitis with SNMC and found a distinct improvement in their ALT level. Suzuki et al.[11] confirmed its ability to suppress serum transaminase in patients with chronic hepatitis in a randomized, double blind controlled trial. Hino et al.[12] and Yasuda et al.[13] proved SNMC to be useful in the improvement of transaminase and liver histology. However, the long term effects of SNMC on patients with chronic HCV have not been confirmed. Therefore, in this study, the authors retrospectively analyzed the long term effects of SNMC on patients with chronic HCV.

MATERIALS AND METHODS

Patients

The number of patients at the study hospital diagnosed with chronic hepatitis by laparoscopy and liver biopsy between January 1979 and April 1984 was 874. Of these 453 patients had the following criteria: 1) ALT level more than twice the upper limit of the normal range (50 IU) within 6 months before liver biopsy; 2) anti-HCV (second-generation anti-HCV and enzyme-linked immunoadsorbent assays) was positive in sera preserved at −80 °C at the time of initial liver biopsy; 3) no corticosteroids, immunosuppressive agents, or antiviral agents were administered 3 months before liver biopsy; and 4) no hepatitis B surface antigens, hepatitis virus DNA, or antinuclear antibodies were detectable in the serum using radioimmunoassay and spot hybridization. In these 453 patients, 84 patients were treated with SNMC (Group A). SNMC was given at a dose of 100 mL daily for 8 weeks and 2–7 times a week for 2 to 16 years (median, 10.1 years). One hundred nine patients were not treated with SNMC (Group B) because there were no home health care professionals available to give the intravenous injection. Therefore Group B patients were given other herbal medicines (e.g., vitamin K) for 1 to 16 years (median, 9.2 years) and did not receive corticosteroids, immunosuppressive agents, or antiviral agents. The remaining 210 patients were treated with a small amount of SNMC, corticosteroids, or immunosuppressive agents. In this study, a comparison of Group A with Group B was performed with regard to hepatocarcinogenesis.

Follow-up began on the first day of SNMC treatment in Group A patients and on the day of the liver biopsy in Group B patients.

SNMC was prepared by Minophagen Pharmaceutical Company, Tokyo, Japan.

Patient Follow-Up

Clinical evaluation and biochemical and hematologic tests were performed at monthly intervals. One patient in Group A dropped out during the 7 years of follow-up. The remaining 83 patients continued to be treated with SNMC. Sixteen patients in Group B were lost to follow-up. Because the appearance of HCC was not identified in these 17 patients, they considered as censored data in the statistical analysis.[14] Deaths unrelated to HCC and patients who received treatment with antiviral agents were also classified as withdrawals and were regarded as censored. Moreover, patients treated with interferon (IFN) were regarded as withdrawals at the start of IFN treatment.

Moreover, the appearance rate of HCC was studied in 193 patients from both groups. Diagnosis of HCC was made by the typical hypervascular characteristics observed on angiography, in addition to certain features of computed tomography (CT) and ultrasonography. Microscopic examination by fine-needle biopsy was performed in patients whose angiograms did not demonstrate a typical image of HCC. Pathologic confirmation using surgically resected specimens was made in 18 patients.

Examination of HCV Genotype

HCV genotype was determined by polymerase chain reaction (PCR) with a mixed primer set derived from nucleotide sequences of the NS5 region.[15]

Liver Histology

Liver histology of chronic hepatitis was classified from the extent of fibrosis into three stages Stage I: periportal expansion; Stage II: portoportal septa; and Stage III: portocentral linkage or bridging fibrosis.[16]

Statistical Analysis

Groups A and B were compared using the Kruskal-Wallis test to assess differences in background factors; $P < 0.05$ was considered significant. ALT response to SNMC was studied by the chi-square test. HCC appearance rates were analyzed by the log rank test. A Cox proportional hazards model was used to analyze the factors contributing to the HCC appearance rate; factors examined included age, gender, histologic findings, HCV genotype, transfusion, indocyanine green retention rate at 15 (ICG R15) and SNMC admin-

TABLE 1
Profiles and Laboratory Data of the Patients at the Time of First Liver Biopsy

Parameters/Group	Group A (SNMC+; N = 84)	Group B (SNMC−; N = 109)	*P* value
Age (years)[a]	47 (31–64)	48 (30–64)	0.579
Gender (male/female)	73/11	92/17	0.833
Transfusion (+/−)	39/45	48/61	0.801
Liver histology staging (I/II or III)	51/33	61/48	0.507
HCV genotype (1b/2a or 2b)	60/16	62/21	0.486
ALT (IU)[a]	200 (100–726)	186 (104–698)	0.422
ICG R15 (%)[a]	14 (9–24)	15 (8–26)	0.767

SNMC: Stronger Neo-Minophagen C; HCV: hepatitis C virus; ALT: alanine aminotransferase; ICGR15: indocyanine green retention rate at 15 minutes.
[a] Data are expressed as the median value (range).

TABLE 2
Average ALT Levels during Follow-Up Periods between Group A and Group B

Average ALT	Normal (≦50 IU)	Abnormal (>50 IU)
Group A (SNMC (+))	30 (35.7%)	54 (64.3%)
Group B (SNMC (−))	7 (6.4%)	102 (93.6%)

ALT: alanine aminotransferase; SNMC: Stronger Neo-Minophagen C.

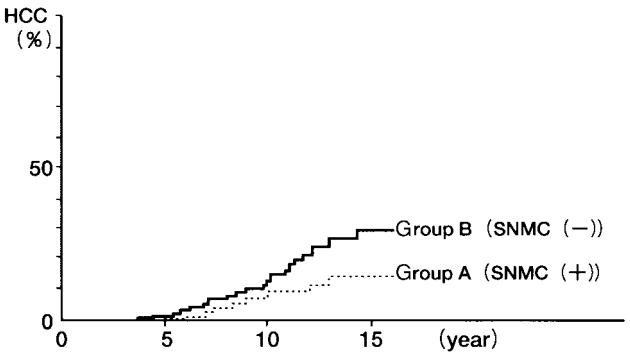

FIGURE 1. Cumulative hepatocellular carcinoma (HCC) appearance rate of Group A with Stronger Neo-Minophagen C (SNMC) administration and Group B without SNMC administration.

istration. The analysis was made using Biomedical Computer Program-p (BMDP).[17,18]

RESULTS

At the first liver biopsy, there was no significant differences between the two groups in age, gender ratio, source of infection, stage of liver histology, HCV genotype, serum ALT level, and ICG R15 (Table 1).

Serum ALT Activity during the Follow-Up Period

Table 2 shows the average ALT levels during the follow-up period. The average ALT levels declined to normal levels range, (6–50 IU) in 30 (35.7%, of the 84 patients in Group A treated with SNMC, compared with 7 (6.4%) of the 109 patients in Group B not treated with SNMC. The trend toward stabilization of ALT in Group A was statistically significant when compared with that in Group B by the chi-square test (P = 0.0000).

Cumulative HCC Appearance Rates

Figure 1 shows the cumulative HCC appearance rates in both groups. The 10-year rates in Groups A and B were 7% and 12%, respectively and the 15 year rates were 12% and 25%, respectively. The HCC appearance rate in Group A was significantly lower than that in Group B (P = 0.0319).

Figure 2 shows the cumulative HCC appearance rates based on the average ALT level for patients receiving SNMC administration. The HCC appearance rate in the 30 patients with a normal ALT level was slightly lower than that in the 54 patients with an elevated ALT level (P = 0.08).

Figure 3 shows the cumulative HCC appearance rates based on the average ALT level in Group B patients without SNMC administration. The HCC appearance rate in the 67 patients with an ALT level < 100 IU was slightly lower than that in the 42 patients with an ALT level > 100 IU (P = 0.054).

Figures 4 and 5 show the cumulative HCC appearance rates of patients based on histologic stage. In patients with histologic Stage I, there was no significant difference between patients who did or did not receive SNMC (P = 0.34). However, patients with histologic Stages II and III, the HCC appearance rate in the 33 patients treated with SNMC (Group A) was

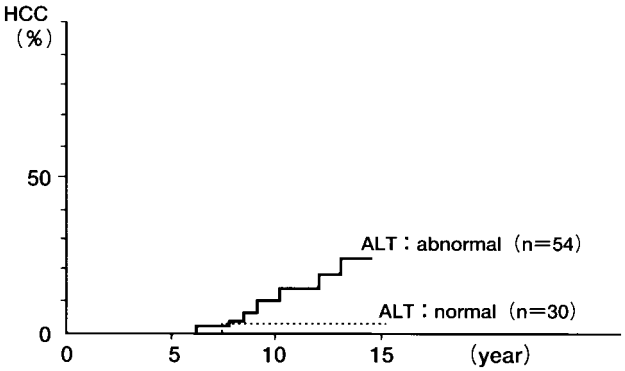

FIGURE 2. Cumulative hepatocellular carcinoma (HCC) appearance rate based on the average alanine aminotransferase level (ALT) after Stronger Neo-Minophagen C (SNMC).

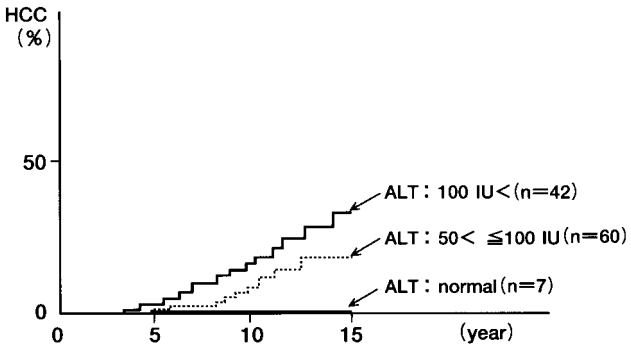

FIGURE 3. Cumulative hepatocellular carcinoma (HCC) appearance rate based on the average alanine aminotransferase (ALT) level of Group B without Stronger Neo-Minophagen C (SNMC) administration.

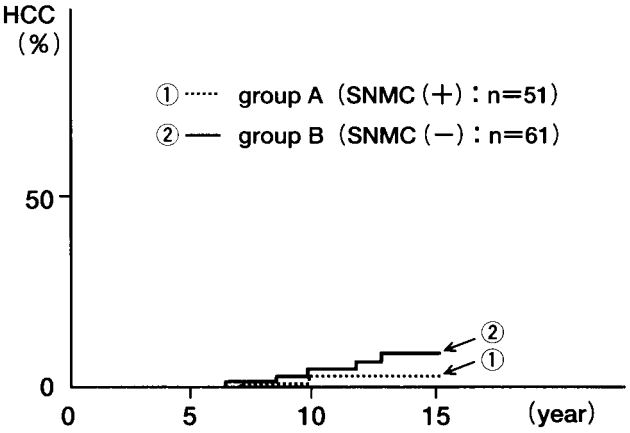

FIGURE 4. Cumulative hepatocellular carcinoma (HCC) appearance rate of patients with histologic Stage I.

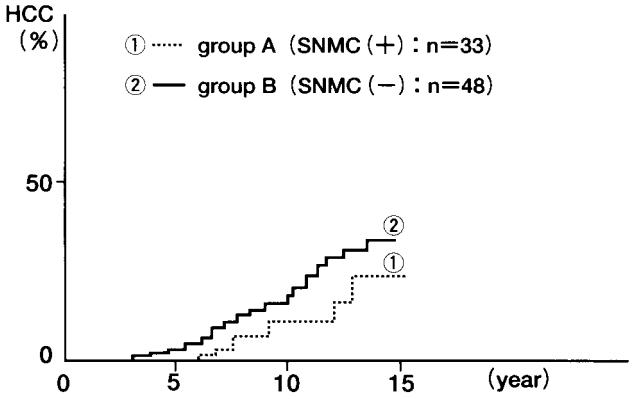

FIGURE 5. Cumulative hepatocellular carcinoma (HCC) appearance rate of patients with histologic Stages II and III.

slightly lower than that in the 48 patients who were not treated with SNMC (Group B) ($P = 0.075$).

Risk Factors for HCC

Cox regression analysis was performed with several variables, including age, gender, history of blood transfusion, histologic severity (staging), HCV genotype, ICG R15, and whether or not SNMC was administered.

Univariate analysis showed that the following four factors significantly affected the cumulative HCC appearance rate in all patients: liver histology Stage I, II, and III ($P = 0.0000$), age ($P = 0.0052$), SNMC administration ($P = 0.0319$), and average ALT level ($P = 0.0437$) (Table 3). Because the variables were mutually correlated, multivariate Cox regression analysis was performed with the four statistically significant variables in the model (Table 4). The relative risk for HCC appearance rate in patients with Stage II or III liver histology was 13.82 compared with Stage I patients, the relative risk in patients who did not receive SNMC was 2.49 compared with patients treated with SNMC.

Side Effects of SNMC Therapy

Hypokalemia (serum potassium < 3.7 mEq/mL) was observed in 9 of the 84 patients (10.7%) in Group A. No patients had a decrease <3 mEq/mL, and the values returned to the normal range (3.7–4.7 mEq/mL) after administration of 150 mg/day of spironolactone. In addition, an increase in blood pressure of $>160/90$ mm Hg was observed in 3 of the 84 patients (3.6%), but blood pressure returned to $<160/90$ mm Hg after the administration of 150–300 mg/day of spironolactone. Discontinuation of SNMC due to side effects did not occur in any of the patients.

Causes of Death

During the observation period, 8 patients in Group A (95%) died from causes including progression of HCC

TABLE 3
Factors Associated with HCC Appearance by Univariate Cox Regression Analysis

Factors	Category	Odds ratio	95% CI	*P* value
Liver histology (staging)	Stage I	1	6.30–36.60	0.0000
	Stage II, III	15.18		
Average ALT	≦50 (normal)	1	1.04~59.74	0.0437
	>50 (abnormal)	7.85		
Age	<45	1	1.46~8.85	0.0052
	≥45	3.60		
SNMC	(+)	1	1.08~5.87	0.0319
	(−)	2.52		
Gender	Female	1		0.185
	Male	2.03		
ICG R15 (%)	<15	1		0.175
	≥15	1.75		
HCV genotype	2a or 2b	1		0.354
	1b	1.64		
Transfusion	(+)	1		0.918
	(−)	1.04		

HCC: hepatocellular carcinoma; CI: confidence interval; ALT: alanine aminotransferase; SNMC: Stronger Neo-Minophagen C; ICG RIS: indocyanine green retention rate at 15 minutes; HCV: hepatitis C virus.

TABLE 4
Factors Associated with HCC Appearance by Multivariate Cox Regression Analysis

Factors	Category	Odds ratio	95% CI	*P* value
Liver histology (staging)	Stage I	1	5.4~34.5	0.0000
	Stage II, III	13.82		
SNMC	(+)	1	1.01~6.12	0.044
	(−)	2.49		

HCC: hepatocellular carcinoma; CI: confidence interval; SNMC: Stronger Neo-Minophagen C.

in four patients, liver failure caused by cirrhosis in one patient, and other diseases in three patients. In Group B, 19 patients (17.4%) died from causes including progression of HCC in 13 patients, liver failure caused by cirrhosis in 1 patient, and other disease in 5 patients.

DISCUSSION

Recently many authors have confirmed the therapeutic effect of IFN in patients with chronic HCV.[19–23] However, the HCV clearance rates were more or less constantly <40–50%; the remaining cases had abnormal ALT levels after IFN therapy. In the study hospital 442 patients with positive HCV-RNA (serum samples were stored at −80 °C until analysis by a reverse transcription nested PCR) were treated with human lymphoblastoid IFN-α between January 1988 and April 1993.[24] The total dose of IFN ranged from 336 MU to 624 MU. The HCC appearance rates in histologic Stages I, II, and III were 0.1%, 0.6%, and 1.5% per year, respectively.[24] Conversely, in patients treated with SNMC the 10-year HCC appearance rates in histologic Stages I, II, and III were 3%, and 13%, respectively. Therefore, HCC appearance rates in patients treated with IFN might be slightly lower than that of patients with SNMC. However, many side effects occur during therapy with IFN and IFN is expensive. Therefore, when HCV-RNA positive patients are treated, whether or not to use IFN therapy is an issue. In patients with IFN-resistant HCV, treatments other than IFN are necessary to protect against histologic aggravation.

Finney and Somers[25] reported on the antiinflammatory effects of glycyrrhizin, and SNMC has been widely and effectively prescribed in Japan as therapy for hepatitis. However, its pharmacologic actions have not been fully clarified. Watari[7] reported that SNMC had a protective effect on the hepatic cellular membrane. Ouchi et al.[26] reported that glycyrrhizin inhibited the production of prostaglandin E_2 by activation of macrophages and the activity of phospholipase A_2 in arachinoic acid cascades in vitro.

In a randomized, double blind controlled trial, Suzuki et al.[11] reported that SNMC was a useful agent for improving serum ALT levels in patients with chronic hepatitis, 133 patients were studied, with 67 receiving 40 mL SNMC daily and 66 a placebo for 4 weeks. A significant improvement in liver function was noted in the SNMC group ($P < 0.001$). However, ALT were slightly elevated again in the second week after the medication was stopped. Hino et al.[12] reported on the effects of a large dose of SNMC (100 mL daily for 8 weeks) in 49 hospitalized patients with chronic hepatitis. All biopsy specimens obtained before and after therapy were examined blindly by the same pathologist. Histologic improvement was observed in 35 patients (71%). However, these patients were examined for only a few months. The long term effect of SNMC administration for more than several years has not been reported. In this study, the authors retrospectively investigated the long term effect of SNMC on HCC appearance rates in patients with chronic HCV. In the current study, the HCC appearance rate in 84 Group A patients who underwent long term treatment with SNMC was significantly lower than that in the 109 Group B patients who were not treated with SNMC. Nonrandomized retrospective studies may be not appropriate for the study of the efficacy of SNMC and randomized prospective studies are best suited for this purpose. However, in recent years, it is apparent that the IFN has been widely employed for patients with chronic HCV and has stabilized liver function in most patients. Conversely, SNMC can be used in general for patients with IFN-resistant HCV and in those with side effects due to the IFN therapy. Moreover, patients with chronic HCV cannot be treated with IFN or SNMC for more than 10 years. Therefore, it will be important that a randomized prospective study is begun to evaluate the long term preventive effect of SNMC on HCC development.

Based on the results of the current study, the long term administration of SNMC for chronic HCV was considered to be effective in the reduction of liver carcinogenesis. The reason why SNMC administration reduces hepatocarcinogenesis is believed to be related to the suppression of the necroinflammatory reaction in the liver and the protection against histologic aggravation. In this study, the authors examined the cumulative cirrhosis appearance rates as well. The diagnosis of cirrhosis was based on laboratory and clinical examinations, including ultrasonography, CT, and histologic examination. Seventy-one patients in Group A and 81 patients in Group B were examined by repeated liver biopsy. The 10-year rates of cumulative cirrhosis in Groups A and B were 12% and 20%, respectively, and the 15-year rates were 21% and 37%, respectively. As a result, the cirrhosis appearance rate in Group A was borderline, significantly lower than that in Group B ($P = 0.07$). Therefore, SNMC therapy could inhibit the histologic aggravation of the liver in some chronic HCV patients. Moreover, in patients treated with SNMC, HCC appearance rates in patients with normal ALT levels was lower than that of patients with abnormal ALT levels. The reduction in risk of HCC in the SNMC group may be partly due to improvement in the ALT level. Therefore, SNMC therapy, is believed to help maintain ALT levels within normal limits.

The authors conclude that normalization of ALT levels by long term administration of SNMC will assist in protecting against hepatocarcinogenesis.

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Glycyrrhizic acid: A phytochemical with a protective role against cisplatin-induced genotoxicity and nephrotoxicity

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ABSTRACT

Aims: Glycyrrhizic acid (GA) is a main sweetening component of licorice roots and has been found to be associated with multiple therapeutic properties. In this study, we used GA as a protective agent against the clastogenic and nephrotoxic effects of cisplatin (CP).
Main methods: Mice were given a prophylactic treatment of GA orally at doses of 75 and 150 mg/kg body weight for seven consecutive days before the administration of a single intraperitoneal dose of CP at 7 mg/kg body weight. The modulatory effects of GA on CP-induced nephrotoxicity and genotoxicity were investigated by assaying oxidative stress biomarkers, lipid peroxidation, serum kidney toxicity markers, DNA fragmentation, alkaline unwinding, and micronuclei and by histopathological examination of the kidneys.
Key findings: A single intraperitoneal dose of cisplatin in mice enhanced renal lipid peroxidation, xanthine oxidase, and H_2O_2 generation; depleted glutathione content, activities of the anti-oxidant enzymes glutathione peroxidase, glutathione reductase, catalase, glutathione-S-transferase and quinone reductase; induced DNA strand breaks and micronucleus formation ($p<0.001$); and majorly disrupted normal kidney architecture. Pretreatment with GA prevented oxidative stress by restoring the levels of antioxidant enzymes at both doses. A significant dose-dependent decrease in DNA fragmentation, micronucleus formation ($p<0.05$), and the kidney toxicity markers BUN ($p<0.001$), creatinine ($p<0.01$), and LDH ($p<0.001$) and restoration of normal kidney histology was observed.
Significance: Our study supports the claim that the phytochemical GA has the potential to attenuate the side effects of anticancer drug overdose.

Introduction

Since ancient times, herbal products have been used to cure human diseases. These products are receiving more attention due to their low toxicity and high efficacy. Glycyrrhizin and its aglycone glycyrrhetic acid (GA) have been useful for various therapeutic purposes (Rahman and Sultana, 2007). GA is a hydrophilic part of glycyrrhizin, the active compound in licorice, which is a conjugate of two molecules of glucuronic acid and GA (Obolentseva et al., 1999). It is used as a flavoring agent in some candies, pharmaceuticals, and tobacco products and is known for its anti-inflammatory, anti-ulcer, anti-allergic, anti-oxidant, anti-viral and anti-tumor activities (Baltina, 2003). In many parts of the world, GA is used to treat patients with acute and chronic hepatitis (Rossum et al., 1999), and it has been demonstrated to induce IFN production and NK cell activity and modulate the growth response of lymphocytes by augmenting IL-2 production (Zhang et al., 1993). In addition, GA has been described as an anti-viral agent against human cytomegalovirus, herpes simplex virus type 1 and influenza virus (Pompei et al., 1979). GA has also been reported to prevent the progression of murine acquired immune deficiency syndrome (AIDS) in mice and improve the clinical symptoms in HIV-infected patients (Ito et al., 1988). Glycyrrhizin inhibits the generation of reactive oxygen species (ROS) by neutrophils at the site of inflammation and lowers lipid peroxidation in animal models of liver injury caused by ischemia reperfusion (Akamatsu et al., 1991). Glycyrrhizin inhibits abnormal cell proliferation, tumor formation and growth of many types of cancers (Nishino et al., 1984). Recently, GA was reported to reverse cisplatin resistance in hepatocellular carcinoma cells through inhibiting multidrug resistance-associated proteins (Wakamatsu et al., 2007). Glycyrrhizin has also been reported to ameliorate renal function defects in the early phase of ischemia and gentamicin-induced acute renal failure (Kang et al., 2003; Sohn et al., 2003). Zinc glycyrrhizate has a protective effect against the toxicity and anticancer activity of cisplatin in mice (Xu et al., 1993). Thus, GA can be considered as a potential protective agent against the nephrotoxicity associated with chemotherapeutics.

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Exposure to genotoxins causes carcinogenesis and teratogenicity (Kligerman et al., 1984). Damage to the structure of the affected DNA is unambiguously the main cause of mutagenesis (Khan et al., 2005). In addition to their generic growth properties, the majority of antineoplastic drugs have genotoxic effects that contribute to growth inhibition. These genotoxic effects may lead to the initiation of unrelated tumors years after the termination of chemotherapy (Manda et al., 2009). Cisplatin (CP) is a major antineoplastic drug used for the treatment of various solid tumors such as ovarian cancer, non-small-cell lung carcinoma (NSCLC) and head and neck cancer (HNC), both as a single agent and in combination with other agents (Muggia, 1991). CP is generally considered to exert its cytotoxic effect by binding to DNA, resulting in mutagenesis (Fichtinger-Schepman, 1984). The major target site of CP toxicity is the kidney: 20% of patients receiving high-dose CP develop severe renal dysfunction. Crosslinks between CP and DNA cause cytotoxic lesions in tumors and other dividing cells. DNA damaging agents are usually less toxic in nonproliferating cells, but the CP concentration in proximal tubular epithelial cells is approximately 5 times higher than the serum concentration (Kuhlmann et al., 1997). This disproportionate accumulation of CP in kidney tissue contributes to its nephrotoxicity (Arany and Safirstein, 2003).

GA has been used to treat organ toxicity caused by drug overdoses in animal models, as mentioned previously. Xu et al. have reported the protective character of GA against CP-induced organ toxicity, including nephrotoxicity. The present study is the first to examine the efficacy of GA to alleviate both genotoxicity and nephrotoxicity induced by CP by studying the status of antioxidant enzymes and markers of genotoxicity, including the alkaline unwinding assay, the DNA fragmentation assay and micronucleus induction in Swiss albino mice.

Materials and methods

Chemicals

Glycyrrhizin, EDTA, Tris, reduced glutathione (GSH), oxidized glutathione (GSSG), reduced nicotinamide adenine dinucleotide phosphate (NADPH), bovine serum albumin (BSA), bisbenzamide, proteinase K, EDTA, SDS, phenol, chloroform, isoamyl alcohol and RNase were obtained from Sigma, St. Louis, USA. Cisplatin under the brand name "Cytoplatin-50" was purchased from Cipla Ltd., India. All other reagents and solvents were of high analytical grade.

Animals

Eight-week-old adult male Swiss albino mice (20–25 g) were obtained from The Central Animal House Facility of Hamdard University, New Delhi, and were housed in a ventilated room at 25 ± 5 °C under a 12 h light/dark cycle. The animals were acclimatized for one week before the study and had access to standard laboratory feed (Hindustan Lever Ltd., Bombay, India) and water *ad libitum*. The study was approved by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) under the registration number and date 509/CPCSEA, May 28th, 2009. CPCSEA guidelines were followed for animal handling and treatment.

Experimental design

For the study of biochemical parameters and the micronucleus assay, 25 male Swiss albino mice were divided into five groups. Group I served as a saline-treated control. Group II served as a treatment control and was given single intraperitoneal injection of cisplatin (7 mg/kg b wt) freshly dissolved in saline (0.85% NaCl). GA was orally administered at two doses, 75 (dose I) and 150 (dose II) mg/kg b wt, to groups III and IV, respectively, for seven consecutive days. On the seventh day of pretreatment, a single intraperitoneal dose of CP (7 mg/kg b wt) was given to the animals in groups II, III, and IV. Group V only received dose II of GA for seven consecutive days. All the animals were sacrificed after 24 h of CP treatment. Kidneys and femur bones were removed and processed for enzyme estimation and micronucleus assays. The above-mentioned doses of compound were selected based on preliminary studies.

Micronucleus assay

For this test, mice were sacrificed 24 h after treatment with a single intraperitoneal dose of CP (7 mg/kg b wt). Bone marrow from femur bones was collected for the micronucleus assay. The time of peak response of micronucleus induction was selected as the sacrifice time based on the preliminary assays that were done to select an appropriate dose of CP that did not suppress cell proliferation in combination with the highest GA dose. The mouse bone marrow micronucleus test was carried out according to the method of Schmid (Schmid, 1975). Air-dried slides were stained with May–Grunwald and Giemsa as described by Schmid, made permanent, and coded. A total of 1500–2000 polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) were scored per animal by the same observer to determine the frequencies of micronucleated polychromatic erythrocytes (MnPCEs). To detect possible cytotoxic effects, the PCE:NCE ratio in 200 erythrocytes per animal was calculated according to Gollapudi and McFadden (Gollapudi, 1995). Coded slides were scored with a light microscope at a 1000× magnification.

DNA isolation

DNA extraction was done by the standard chloroform-isoamyl alcohol method. DNA was extracted from approximately 500 mg kidney tissue by homogenizing the tissue in 5 ml TNE buffer (50 mM Trisma, 100 mM EDTA, 0.5% SDS, pH 8.0) in a 2-ml ground glass homogenizer. Each sample was homogenized with 10 standardized pestle strokes to minimize any potential effect on DNA integrity introduced by the homogenization procedure. An equal volume of buffered phenol/chloroform/isoamyl alcohol (PCI; 25:24:1, v/v/v, pH 8.0) was then added to the sample. The sample was gently mixed, allowed to settle for 5 min and centrifuged for 5 min at 13,000 rpm at 4 °C. The aqueous layer was transferred to a new microcentrifuge tube, and the PCI extraction was repeated. The aqueous layer was then digested by 5 ml RNase (10 mg/ml) for 30 min at 37 °C, and the digest was extracted once by PCI and once by 500 ml chloroform. DNA was precipitated from the resulting aqueous layer by adding 2 volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate, pH 5.2. The sample was then centrifuged (13,000 rpm, 15 min), and the resulting pellet was rinsed with 500 ml of 70% ethanol and air-dried. The amount of DNA was quantified spectrophotometrically at 260 and 280 nm. A 2 mg/ml DNA sample was dissolved in 1 ml TE buffer (10 mM Trisma, 1 mM EDTA) and subsequently used in the DNA alkaline unwinding assay.

Alkaline unwinding assay

In the alkaline unwinding assay, the rate of the transition of double stranded DNA (dsDNA) to single stranded DNA (ssDNA) under predefined alkaline denaturing conditions was proportional to the number of breaks in the phosphodiester backbone and thus was used as a measure of DNA integrity. Bisbenzamide was used as a fluorescent DNA-binding dye to allow the quantification of various types of DNA. To determine the fluorescence of dsDNA, ssDNA and partially unwound DNA (auDNA), three equal portions of each diluted DNA sample were prepared. The amount of dsDNA was obtained from the fluorescence of a sample without any treatment, while the amount of ssDNA was determined from a sample that had been boiled for

30 min. The fluorescence of a DNA sample subjected to alkaline treatment (pH 12.2) on ice for 30 min provided an estimate of the amount of auDNA. The fluorescence of initial or dsDNA was determined by placing 100 mmol DNA, 100 ml NaCl (25 mM) and 2 ml SDS (0.5%) in a prechilled test tube, followed by the addition of 3 ml 0.2 M potassium phosphate, pH 9, and 3 ml bisbenzamide (1 mg/ml). The contents were mixed and allowed to react in the dark for 15 min to allow the fluorescence to stabilize. The fluorescence of the sample was measured using a spectrofluorimeter (excitation: 360 nm; emission: 450 nm). The fluorescence of ssDNA was determined as above, but using the DNA sample that had been boiled for 30 min to completely unwind the DNA. Fifty microliters of NaOH (0.05 N) was rapidly mixed with 100 ml DNA in a pre-chilled test tube (Shugart, 1988). The mixture was incubated on ice in the dark for 30 min, followed by rapid addition and mixing of 50 ml HCl (0.05 N). This was followed immediately by the addition of 2 ml SDS (0.5%), and the mixture was forcefully passed through a 21-gauge needle six times. The fluorescence of auDNA was measured as described above in triplicate, and the average was reported. The ratio between dsDNA to total DNA (F value) was determined as follows:

$$\text{F value} = (\text{auDNA} - \text{ssDNA}) / (\text{dsDNA} - \text{ssDNA}),$$

where auDNA, ssDNA and dsDNA were the degrees of fluorescence from the partially unwound, single stranded and double stranded samples, respectively. The F-value was inversely proportional to the number of strand breaks present and thus could be used as an indicator of DNA integrity.

Gel electrophoresis and DNA fragmentation

DNA samples were mixed with 10 ml loading solution (10 mM EDTA (pH 8.0), 1% (w/v) bromophenol blue and 40% (w/v) sucrose) preheated to 70 °C. Samples were loaded onto a 1.8% (w/v) agarose gel and sealed with 0.8% (w/v) low melting point agarose. DNA fragments were separated by electrophoresis at 25 V for 12 h at 4 °C in TBE buffer. DNA was visualized using ethidium bromide and photographed by a digital camera.

Post-mitochondrial supernatant preparation

Kidneys were removed quickly, cleaned of extraneous material and immediately perfused with ice-cold saline (0.85% NaCl). The kidneys were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%) using a Potter–Elvehjen homogenizer. The homogenate was filtered through muslin cloth and centrifuged at 800×*g* for 5 min at 4 °C by a REMI cooling centrifuge to separate the nuclear debris. The aliquot obtained was centrifuged at 12,000 rpm for 20 min at 4 °C to obtain PMS, which was used as a source of enzymes. All biochemical estimations were completed within 24 h of animal sacrifice (Khan and Sultana, 2005).

Reduced glutathione estimation

One-milliliter samples of PMS were precipitated with 1.0 ml sulfosalicylic acid (4%), kept at 4 °C for 1 h and then centrifuged at 1200×*g* for 20 min at 4 °C. The assay mixture contained 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml 1, 2-dithiobisnitrobenzoic acid (DTNB; 100 mM) in a total volume of 3.0 ml. The yellow color that developed was quantified at 412 nm on a spectrophotometer (Jollow et al., 1974).

Glutathione reductase activity

GR activity was determined by the method of Carlberg and Mannervik. The reaction mixture consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml GSH (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. Enzyme activity was quantified at 25 °C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized per min per mg protein using a molar extinction coefficient of $6.22\times10^3\ M^{-1}\ cm^{-1}$ (Carlberg and Mannervik, 1975).

Glutathione peroxidase activity

The reaction mixture consisted of 1.49 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 0.05 ml glutathione reductase (1 IU/ml), 0.05 ml GSH (1 mM), 0.1 ml NADPH (0.2 mM), 0.01 ml H_2O_2 (0.25 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. The disappearance of NADPH at 340 nm was recorded at 25 °C. Enzyme activity was calculated as nmol NADPH oxidized per min per mg protein using a molar extinction coefficient of $6.22\times10^3\ M^{-1}\ cm^{-1}$ (Mohandas et al., 1984).

Glutathione S-transferase activity

The reaction mixture consisted of 2.5 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml GSH (1 mM), 0.2 ml CDNB (1 mM), and 0.1 ml of the cytosolic fraction (10%) in a total volume of 3.0 ml. Changes in absorbance were recorded at 340 nm, and enzymatic activity was calculated as nmol CDNB conjugate formed per min per mg protein using a molar extinction coefficient of $9.6\times10^3\ M^{-1}\ cm^{-1}$ (Habig et al., 1974).

Malondialdehyde formation

The reaction mixture contained 0.60 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsomes and 0.2 ml ascorbic acid (100 mM) in a total volume of 1.0 ml. The reaction mixture was incubated at 37 °C in a shaking water bath for 1 h and stopped by adding 1.0 ml 10% trichloroacetic acid (TCA). Following the addition of 1.0 ml 0.67% thiobarbituric acid (TBA), all tubes were placed in a boiling water bath for 20 min and then shifted to a crushed ice-bath before centrifuging at 2500×*g* for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm against a reagent blank using a spectrophotometer (Perkin Elmer, Lamda EZ 201). The results were expressed as nmol MDA formed per hour per gram tissue at 37 °C using a molar extinction coefficient of $1.56\times10^5\ M^{-1}\ cm^{-1}$ (Wright et al., 1981).

Xanthine oxidase activity

The reaction mixture consisted of 0.2 ml PMS incubated for 5 min at 37 °C with 0.8 ml phosphate buffer (0.1 M, pH 7.4). The reaction was started by adding 0.1 ml xanthine (9 mM) and was kept at 37 °C for 20 min. The reaction was terminated by adding 0.5 ml ice-cold perchloric acid (PCA; 10% v/v). After 10 min, 2.4 ml distilled water was added, and the samples were centrifuged at 4000 rpm for 10 min. The amount in mg of uric acid formed per minute per mg protein was recorded at 290 nm (Stirpe and Della Corte, 1969).

Catalase activity

The reaction mixture consisted of 1.95 ml phosphate buffer (0.1 M, pH 7.4), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml 10% PMS in a final volume of 3 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated as nmol H_2O_2 consumed per min per mg protein (Claiborne, 1985).

Quinone reductase activity

The reaction mixture consisted of 2.13 ml Tris–HCl buffer (25 mM, pH 7.4), 0.7 ml BSA, 0.1 ml FAD, 0.02 ml NADPH (0.1 mM), and 50 μL (10%) PMS in a final volume of 3 ml. The reduction of dichlorophenolindophenol (DCPIP) was recorded calorimetrically at 600 nm, and enzyme activity was calculated as nmol of DCPIP reduced per min per mg protein using a molar extinction coefficient of $2.1 \times 10^4\ M^{-1}\ cm^{-1}$ (Benson et al., 1980).

Estimation of blood urea nitrogen

Protein-free filtrate was prepared by adding serum and an equal amount of 10% TCA. Then the mixture was centrifuged at 2000 rpm, and supernatant was taken. To 0.5 ml protein-free filtrate, 3.5 ml of distilled water, 0.8 ml diacetylmonoxime (2%) and 3.2 ml sulfuric acid–phosphoric acid reagent (prepared by mixing 150 ml 85% phosphoric acid with 140 ml water and 50 ml concentrated sulfuric acid) were added. The reaction mixture was placed in a boiling water bath for 30 min and then cooled to room temperature. The absorbance was read at 480 nm (Kanter, 1975).

Quantification of creatinine

Protein-free filtrate was prepared. To 1.0 ml serum, 1.0 ml sodium tungstate (5%), 1.0 ml sulfuric acid (0.6 N) and 1.0 ml distilled water were added. After mixing thoroughly, the sample was centrifuged at 800 ×*g* for 5 min. The supernatant was added to a mixture containing 1.0 ml picric acid (1.05%) and 1.0 ml sodium hydroxide (0.75 N). The absorbance at 520 nm was read after exactly 20 min (Hare, 1950).

Assay for hydrogen peroxide

Microsomes (2.0 ml) were suspended in a 1.0-ml solution containing phenol red (0.28 nM), horseradish peroxidase (8.5 units), dextrose (5.5 nM) and phosphate buffer (0.05 M, pH 7.0) and were incubated at 37°C for 60 min. The reaction was stopped by the addition of 0.01 ml NaOH (10 N) and then centrifuged at 800 ×*g* for 5 min. The absorbance of the supernatant was recorded at 610 nm against a reagent blank. The quantity of H_2O_2 produced was expressed as nmol H_2O_2 per hour per gram tissue based on the standard curve of H_2O_2 oxidized phenol red (Pick and Keisari, 1981).

Lactate dehydrogenase activity

The assay mixture consisted of serum, NADH (0.02 M), sodium pyruvate (0.01 M), phosphate buffer (0.1 M, pH 7.4) and distilled water in a total volume of 3 ml. Enzyme activity was recorded at 340 nm, and activity was calculated as nmol NADH oxidized per min per mg protein (Kornberg, 1955).

Estimation of protein concentration

The protein concentration in all samples was determined by the method of Lowry et al.(1951).

Histopathological examination

The kidneys were quickly removed after sacrifice of mice and preserved in 10% neutral buffered formalin for histopathological processing. The kidneys were embedded in paraffin wax and longitudinally sectioned with a microtome. Hematoxylin and eosin staining of the sections was observed under an Olympus microscope.

Statistical analysis

Differences between groups were analyzed using analysis of variance (ANOVA), followed by Dunnet's multiple comparisons test. All data points are presented as the treatment group mean ± standard error of the mean (S.E.).

Results

Effects of GA pretreatment on renal glutathione, its dependent enzymes and the antioxidant enzyme system

Table 1 shows that CP administration leads to a 60% reduction in renal GSH. In addition, there was a marked inhibition of GR by 69%, GST by 60% and GPx by 52% compared to the control (Table 1). Pretreatment with GA restored renal GSH content by 64% at dose I and 83% at dose II, GR by 66% at dose I and 200% at dose II, GST by 89% at dose I and 103% at dose II and GPx by 45% at dose I and 92% at dose II. Similarly, CP treatment inhibited catalase by 53%, as shown in Table 2, which was restored by prophylactic GA treatment by 56% at dose I and 90% at dose II.

Table 2 shows that CP administration enhances XO levels by 183%, MDA formation by 72% and H_2O_2 by 137% in comparison to the control. In addition, there was marked inhibition of QR by 55% following CP administration as compared to the control (Table 2). Pretreatment with GA restored renal XO by 41% at dose I and 53% at dose II, MDA formation by 18% at dose I and 29% at dose II and H_2O_2 by 35% at dose I and 43% at dose II (Table 2). Quinone reductase (QR) was restored by 91% at dose I and 114% at dose II (Table 2).

Effects of GA pretreatment on renal toxicity marker enzymes

The effects of the prophylactic administration of GA on cisplatin-mediated leakage of kidney toxicity marker enzymes and on the cytotoxicity marker enzyme LDH in serum are shown in Table 3. Only the CP-treated group induced BUN by 80%, creatinine by 84% and LDH by 133% compared with the control. Marked inhibition of BUN (30% at dose I and 38% at dose II) and creatinine levels (10% at dose I and 32%

Table 1
Results of pre-treatment of GA on antioxidant enzymes like GSH, GST, GR and GPX on Cisplatin administration in kidney of Swiss Albino mice.

Treatment regimen per group	GSH (n mol CDNB Conjugate formed/g tissue)	GST (n mol CDNB conjugate formed/min/mg protein)	GR (n mol NADPH Oxidized/min/ mg protein)	GPX (n mol NADPH Oxidized/min/ mg protein)
Group I (control)	0.92 ± 0.001	115.9 ± 1.853	282.7 ± 1.044	281.1 ± 2.041
Group II (only CP)	0.42 ± 0.001***	46.88 ± 2.124***	87.8 ± 0.453***	133.6 ± 0.523***
Group III (CP + GA D1)	0.69 ± 0.0004$^{\#\#\#}$	88.50 ± 3.759$^{\#\#}$	145.8 ± 0.802^{NS}	193.9 ± 12.105$^{\#\#}$
Group IV (CP + GA D2)	0.77 ± 0.001$^{\#\#\#}$	95.03 ± 3.243$^{\#\#\#}$	263.5 ± 7.137$^{\#\#\#}$	256.7 ± 0.704$^{\#\#\#}$
Group V (only GA D2)	0.86 ± 0.003	102.7 ± 5.961	280.4 ± 2.632	265.2 ± 5.063

Results represent mean ± SE of five animals per group. Cisplatin administration resulted in significant decrease in the level of GSH, GST, GR and GPx. Prophylactic treatment with GA at both doses significantly modulated the alterations induced by CP in mice kidney. Results obtained are significantly different from Control group ($^{***}p<0.001$). Results obtained are significantly different from CP treated group ($^{\#\#}p<0.01$) and ($^{\#\#\#}p<0.001$). Whereas, Results obtained are not significantly different from CP treated group (NS). Cisplatin—CP, Glycyrrhizic acid—GA; D1 = 75 mg/kg b wt; D2 = 150 mg/kg b wt.

Table 2
Results of pre-treatment of GA on antioxidant enzymes like G6PD, XO, LPO, QR and catalase on Cisplatin administration in kidney of Swiss Albino mice.

Treatment regimen per group	H_2O_2 (n moles H_2O_2/g tissue)	XO (μg uric acid formed/min/mg protein)	LPO (n mol MDA formed/hr/g tissue)	QR (nmol NADPH oxidized/min/mg protein)	Catalase (nmol H_2O_2 consumed/min/mg protein)
Group I (control)	59.8 ± 6.586	3.64 ± 0.338	5.16 ± 0.259	164.1 ± 7.823	130.3 ± 0.224
Group II (only CP)	141.6 ± 3.293***	10.30 ± 0.720***	8.89 ± 0.520***	73.7 ± 4.249***	61.57 ± 0.32***
Group III (CP + GA D1)	91.6 ± 2.813##	6.03 ± 0.024##	7.25 ± 0.105##	140.6 ± 8.452###	95.85 ± 0.029#
Group IV (CP + GA D2)	80.1 ± 6.257###	4.80 ± 0.059###	6.35 ± 0.369###	158.0 ± 4.679###	116.9 ± 0.388##
Group V (only GA D2)	61.9 ± 1.742	4.66 ± 0.309	5.96 ± 0.016	160.7 ± 17.840	121.0 ± 0.330

Results represent mean ± SE of five animals per group. There was marked increase in the levels of H_2O_2, XO and LPO, which was significantly restored to normal level by the pre treatment of GA. Cisplatin administration resulted in significant decrease in the activity of QR and catalase. Prophylactic treatment with GA at both doses significantly increased the activity of these enzymes in mice kidney. Results obtained are significantly different from Control group (***$p<0.001$). Results obtained are significantly different from CP treated group (##$p<0.01$) and (###$p<0.001$). Cisplatin—CP, Glycyrrhizic acid—GA; D1 = 75 mg/kg b wt; D2 = 150 mg/kg b wt.

at dose II) was observed in the GA-treated groups. LDH was inhibited by 34% at dose I and 46% at dose II.

Effects of GA pretreatment on genotoxicity

In the DNA alkaline unwinding assay (Fig. 1C), a 53% decrease in the F-value was noted compared to the control group, whereas the F-value increased by 71% at dose I and 101% at dose II of GA. Similarly, Fig. 1A shows that there was a 140% higher induction of micronuclei, and this level decreased with prophylactic treatment of GA by 36% at dose I and 24% at dose II. The estimated PCE:NCE ratio in bone marrow preparations shows a statistical decrease in hematopoiesis by 34% as a result of CP treatment compared to the control (Fig. 1B). GA pretreatment at dose I increased the PCE:NCE ratio by 28%; dose II increased the ratio by 46%, indicating a reversal of the cytotoxic effects caused by CP administration to mice. DNA damage was estimated by smearing and lack of an intact control band; only the GA-treated groups had less smearing and an intact band (Fig. 1D). Fig. 1D also shows that there was significant DNA fragmentation in the treated group compared to the control group, while there was less fragmentation in the GA-pretreated groups. GA treatment restored DNA integrity.

Histopathological examination

Normal glomerular and tubular histology was seen both in cortical and medullar regions of kidneys in control rats (Fig. 2A). However, CP caused extremely severe glomerular and peritubular congestion in group II, as shown in Fig. 2B. There was also severe invasion of inflammatory cells into the interstitium and the perivascular and subvascular areas in both cortical and medullar sections from the CP-treated group. In addition to these features of CP nephrotoxicity, necrosis of tubular structures was seen in proximal and distal regions. The inner cortical and outer medullar regions exhibited more extreme architectural damage than the inner medullar regions of the kidney. The nephrosis was characterized by widespread degeneration of tubular architecture, tubular congestion, swelling and necrosis. In contrast, renal sections obtained from animals that were pretreated with GA at dose I had a partial reduction of the histological features of renal injury (Fig. 2C). GA pretreatment at dose II was associated with more significant reductions in injury, similar to control rat kidneys (Fig. 2D). A higher dose of GA alone did not cause any adverse effects in the overall normal architecture of kidneys, as shown in Fig. 2E.

Table 3
Results of pre-treatment of GA on serum kidney toxicity markers BUN, Creatinine and LDH on Cisplatin administration in kidney of Swiss Albino mice.

Treatment regimen per group	BUN (mg/dl) IU/L	Creatinine (mg/dl) IU/L	LDH (n mol NADH oxidized/min/mg protein)
Group (I control)	17.27 ± 2.071	1.94 ± 0.023	349.1 ± 10.44
Group II (only CP)	31.07 ± 1.229***	3.57 ± 0.142***	814.6 ± 11.71***
Group III (CP + GA D1)	21.72 ± 0.142###	3.22 ± 0.128NS	539.5 ± 2.89###
Group IV (CP + GA D2)	19.27 ± 0.444###	2.44 ± 0.057##	439.0 ± 15.95###
Group V (only GA D2)	17.68 ± 2.069	2.26 ± 0.059	404.6 ± 10.44

Results represent mean ± SE of five animals per group. Results obtained are significantly different from Control group (***$p<0.001$). As evident from the table there was substantial increase in the serum levels of BUN, Creatinine and LDH. The levels of these toxicity markers was abrogated by the prophylactic treatment of GA. Results obtained are significantly different from CP treated group (##$p<0.01$) and (###$p<0.001$). Results obtained are not significantly different from CP treated group (NS). Cisplatin—CP, Glycyrrhizic acid—GA; D1 = 75 mg/kg b wt; D2 = 150 mg/kg b wt.

Discussion

Cisplatin is a potent chemotherapeutic agent that has gained widespread use against various malignant tumors in different experimental animals (Kociba et al., 1970). Its use, however, is limited by its nephrotoxicity and genotoxicity. The highest concentration of CP is found in cytosol, mitochondria, nuclei, and microsomes. Genomic instability is often considered to be the hallmark of cancer, and it has been shown previously that CP treatment leads to genotoxicity and DNA strand breaks, as measured by the alkaline unwinding assay. These breaks are believed to result from the interaction of CP with DNA through the binding with N^7 of purine bases, forming CP–DNA adducts (Wozniak et al., 2004). The high mutagenic potency of CP raises the concern that its use in cancer chemotherapy may be responsible for secondary malignancies, which have been observed in animals and some cured patients treated with CP (Kempf and Ivankovic, 1986; Greene, 1992; Pillaire et al., 1994).

In the present study, the development of these mutagenic parameters was seen after CP treatment *in vivo*, supporting earlier observations of its genotoxic properties (Pillaire et al., 1994; Overbeck et al., 1996). There was a concurrent decline in the F-value in the DNA alkaline unwinding assay and differences observed with agarose gel electrophoresis, which are markers for DNA integrity (Fig. 1D). Our results show that there was significant DNA fragmentation in the CP-treated group compared to the control group, whereas there was less fragmentation in the GA-pretreated group. During DNA fragmentation, DNA damage is estimated by smearing and lack of an intact band on an agarose gel. An intact band was observed in the group pretreated with GA at dose II and in the control group only.

Injury due to oxidative stress is actively involved in the pathogenesis of CP-induced acute kidney injury. Many authors have found that genotoxicity and chromosomal instability induced by many agents are strongly correlated with the parameters of oxidative stress. Reactive oxygen species (ROS) directly act on cell components, including lipids, proteins, and DNA, and destroy their structure. ROS are produced in cells via the xanthine–xanthine oxidase system, mitochondria, and NADPH oxidase. In the presence of CP, ROS are produced by all these pathways and are implicated in the pathogenesis of acute CP-induced renal injury

(Kawai et al., 2006). CP has been shown to inhibit the activity of antioxidant enzymes (glutathione-S-transferase, catalase, and glutathione reductase) in rat kidneys (Afifi, 2010). Consistent with previous studies, CP administration leads to a depletion in renal glutathione content and inhibits the activities of the renal anti-oxidant enzymes glutathione peroxidase, glutathione reductase, catalase and glutathione S-transferase. Glutathione and dependent enzymes, namely, GR, GPx and GST, were significantly restored to normal levels in GA-pretreated groups. Simultaneously, GA pretreatment restored the depleted levels of H_2O_2, QR and catalase. Earlier reports suggested that CP induces nephrotoxicity by initiating lipid peroxidation and depleting cellular thiols (Jin-Gang and Lindup, 1993). In the present study, we have observed a noticeable increase of LPO activity in the CP-treated group; further, it was found that LPO is significantly attenuated by GA pretreatment.

Chemoprotective agents are capable of exerting their antigenotoxic effects by one or a combination of mechanisms, such as inhibiting the formation of reactive carcinogenic metabolites, inducing enzymes that detoxify carcinogens, scavenging reactive oxygen species, influencing apoptosis and inhibiting cell proliferation. GA is known to possess anti-inflammatory, anticarcinogenic, nephroprotective, and immunomodulatory effects and was proposed to be a possible chemopreventive drug (Akamatsu et al., 1991; Wakamatsu et al., 2007; Xu et al., 1993; Kelloff et al., 1994). In the present work, it was found that GA pretreatment of CP-treated mice significantly decreased MDA formation and XO levels. Concomitant modulation of antioxidant status was also observed. Serum kidney toxicity markers were significantly altered, as observed in earlier studies (Ibrahim et al., 2010). Pretreatment with GA appreciably depleted serum toxicity markers, thus revealing a role for GA in modulating kidney toxicity. Mice treated with GA had significantly lower BUN, creatinine and LDH levels than those receiving only CP; thus, GA ameliorates nephrotoxicity. It is evident from this study that GA was not only able to reduce cellular damage but also suppressed DNA fragmentation and the formation of MnPCEs *in vivo*, which are the hallmarks of CP-induced genotoxicity (Mora et al., 2002). The decrease in the PCE:NCE ratio, a mark of cytotoxicity in the bone marrow of mice

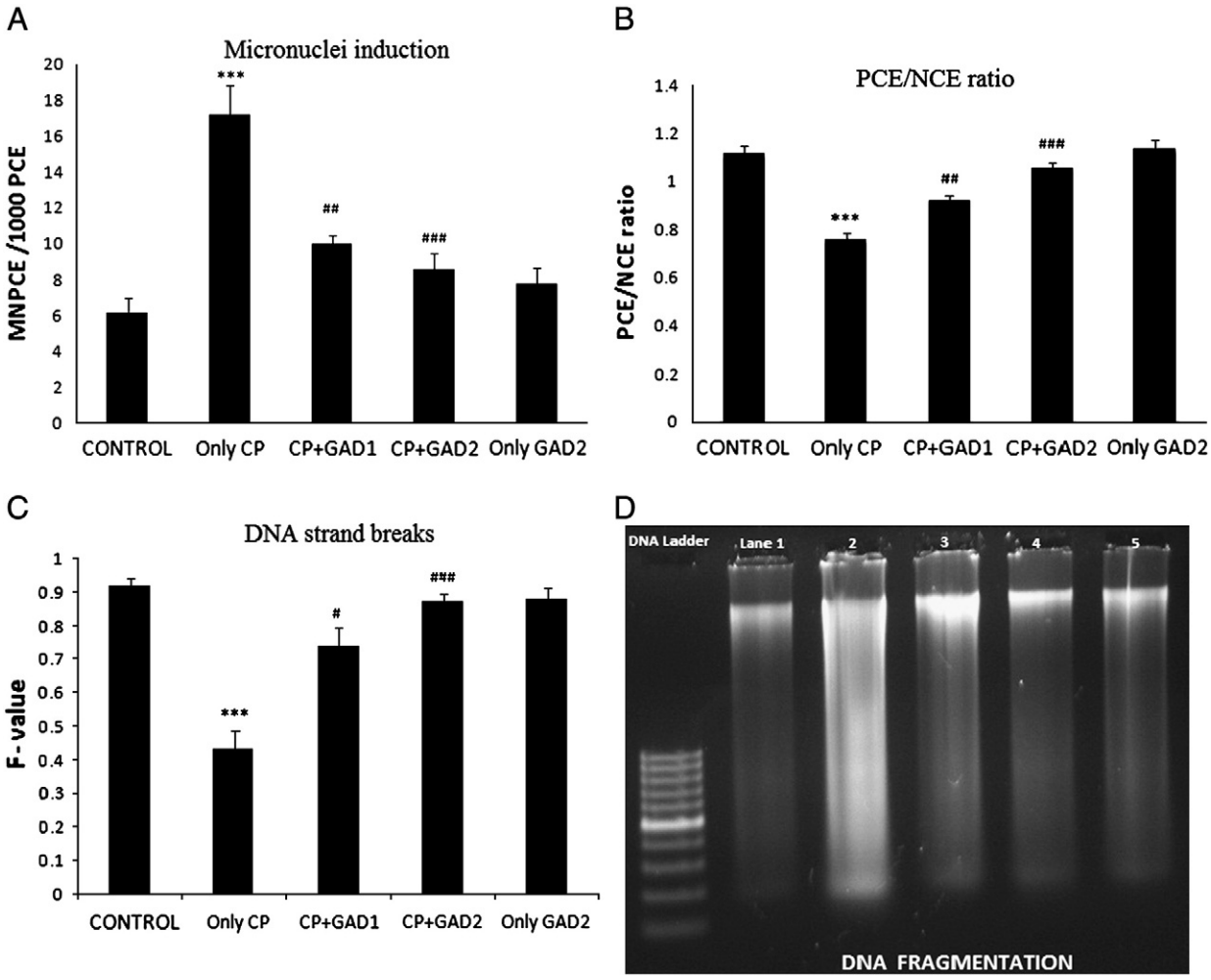

Fig. 1. Effect of GA on CP-altered markers of genotoxicity. 1 (A) Micronucleus induction in the bone marrow of mice: there was a marked increase ($p<0.001$) in the micronucleus content of the polychromatic erythrocytes (PCE) in the bone marrow of mice due to CP treatment compared to untreated controls. However, pretreatment with GA suppressed micronucleus formation significantly at dose I ($p<0.01$) and dose II ($p<0.001$). 1 (B) PCE:NCE ratios in the bone marrow of mice: There was a significant decrease in the PCE:NCE ratio in the CP-treated group, displaying induction of cytotoxicity in the bone marrow cells of Swiss albino mice. Prophylactic treatment with GA at both doses significantly reversed the cytotoxic effects of CP. Dose I of GA did not cause any toxicity. 1 (C) DNA strand breaks: results from the alkaline unwinding assay revealed a significant increase in DNA strand breaks resulting from CP injection in mice. GA treatment protected DNA from CP-induced breaks significantly at D1 ($p<0.05$) and D2 ($p<0.001$). 1 (D) Agarose (1.5%) gel electrophoresis of DNA obtained from mouse kidney: lane 1—control; lane 2—CP alone; lane 3—CP + GA D1; lane 4—CP + GA D2; lane 5—GA D2 alone; lane 6—mid-range DNA ladder. From the gel images, it is evident that CP treatment introduced DNA fragmentation, marked by the smearing of DNA in lane 2 compared to the untreated control in lane 1. There is a decrease in DNA smearing as a result of GA pretreatment at both doses in the mouse kidney. The results represent the mean ± SE of five animals per group. The results obtained are significantly different from those of the control group ($^{***}p<0.001$) and those of the CP-treated group ($^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$). CP = Cisplatin; GA = Glycyrrhizic acid; D1 = 75 mg/kg b wt; D2 = 150 mg/kg b wt.

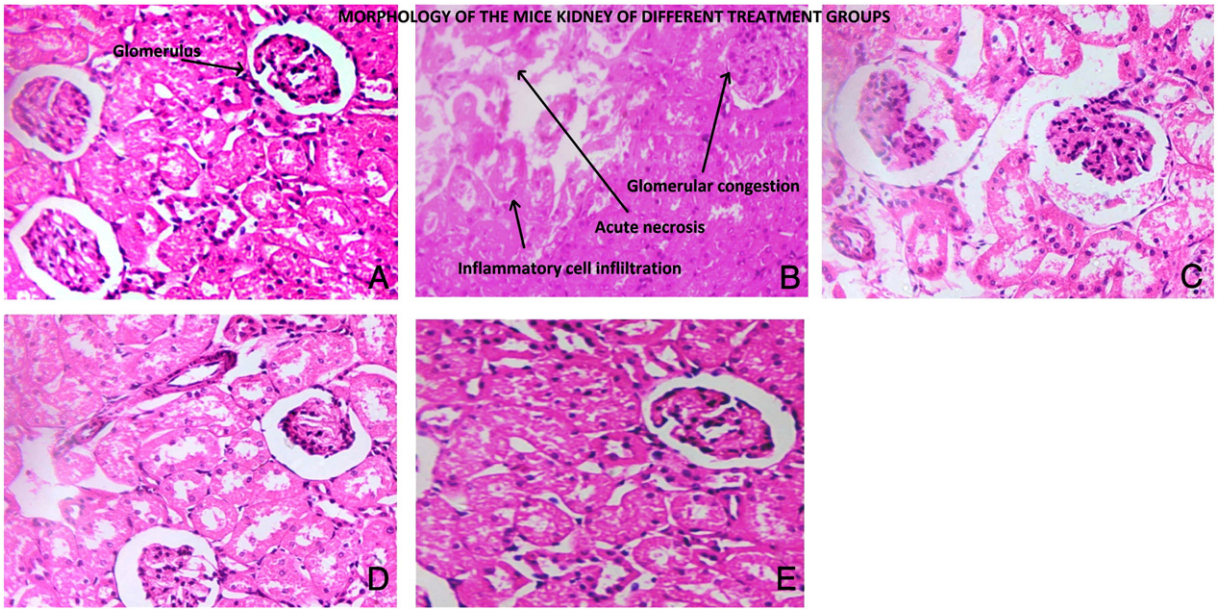

Fig. 2. Mouse kidney histology (400× magnification): Representative histological abnormalities in the regions including the inner cortical and outer medullar areas of the kidney, which are the main target sites of cisplatin nephrotoxicity, following a single intraperitoneal injection of CP and the effects of GA prophylactic treatment. (A) Normal kidney histology from a control rat. (B) After CP administration, many cortical convoluted tubules were invaded by necrotic epithelial cells or vacuolated swell cells, glomeruli were swollen, and the Bowman's capsular space was reduced. Shown are numerous inflammatory cells in the glomerular and tubular structures in the outer medulla. (C) Administration of dose I of GA (75 mg/kg b wt) partially prevented the cytotoxic damage induced by CP, as indicated by the slight cellular vacuolization of cortical convoluted tubules and few inflammatory cells. (D) Dose II of GA (150 mg/kg b wt) almost fully protected the kidney tissue from destruction induced by CP, as evident from the normal histology of the inner cortical region. (E) Dose II of GA alone did not produce abnormalities in kidney histology.

due to CP treatment, as observed in previous studies (Attia et al., 2008), was also suppressed in the GA-pretreated groups.

Our results suggest that one of the major therapeutic effects of GA in CP-induced nephrotoxicity and genotoxicity might result from its ability to directly scavenge free radicals and potentiate the antioxidant defense system. Earlier studies in rats have demonstrated that CP induces acute tubular necrosis of the straight portion of the proximal tubule, located at the cortico-medullary junction (Vickers et al., 2004). In accordance with earlier studies, CP-treated mice were found to have glomerular and peritubular congestion, with inflammatory cells invading the cortical and medullar regions of the kidneys. The main histological finding of this study was that pretreatment with GA influenced the recovery of abnormal kidney architecture induced by CP. The findings of our study are consistent with those of a previous study that revealed a protective nature of GA against CP-induced acute nephrotoxicity (Xu et al., 1993).

In conclusion, a plausible mechanism of the protective action of GA might be at least partly due to its free radical scavenging activity. Overall, these studies suggest the protective potential of GA against CP-induced genotoxicity and nephropathy; however, future studies need to be conducted at the molecular level to determine if GA can effectively inhibit the ability of CP to induce genetic damage in normal cells without interfering with its capacity to reduce tumor growth in cells treated with CP. Experiments at the molecular level will reveal the actual mechanism of action of GA, and its use may thus increase the therapeutic window of CP in cancer patients.

Conflict of interest statement

The authors of the present research work do not have any conflict of interest to present this research work.

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REVIEW ARTICLE

Review of Pharmacological Effects of *Glycyrrhiza* sp. and its Bioactive Compounds

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The roots and rhizomes of licorice (*Glycyrrhiza*) species have long been used worldwide as a herbal medicine and natural sweetener. Licorice root is a traditional medicine used mainly for the treatment of peptic ulcer, hepatitis C, and pulmonary and skin diseases, although clinical and experimental studies suggest that it has several other useful pharmacological properties such as antiinflammatory, antiviral, antimicrobial, antioxidative, anticancer activities, immunomodulatory, hepatoprotective and cardioprotective effects. A large number of components have been isolated from licorice, including triterpene saponins, flavonoids, isoflavonoids and chalcones, with glycyrrhizic acid normally being considered to be the main biologically active component. This review summarizes the phytochemical, pharmacological and pharmacokinetics data, together with the clinical and adverse effects of licorice and its bioactive components. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: licorice; *Glycyrrhiza glabra*; glycyrrhizin; glabridin; glycyrrhitinic acid; isoliquiritigenin.

INTRODUCTION

Licorice species are perennial herbs native to the Mediterranean region, central to southern Russia, and Asia Minor to Iran, now widely cultivated throughout Europe, the Middle East and Asia (Blumenthal *et al.*, 2000). They have been used medically since at least 500 BC and licorice has been described as 'the grandfather of herbs' (Ody, 2000). The genus *Glycyrrhiza* (Leguminosae) consists of about 30 species including *G. glabra*, *G. uralensis*, *G. inflata*, *G. aspera*, *G. korshinskyi* and *G. eurycarpa*, *G. glabra* also includes three varieties: Persian and Turkish licorices are assigned to *G. glabra* var. *violacea*, Russian licorice is *G. glabra* var *gladulifera*, and Spanish and Italian licorices are *G. glabra* var. *typica* (Nomura *et al.*, 2002). It is also known as liquorice, kanzoh, gancao, sweet root and yasti-madhu (Blumenthal *et al.*, 2000; Nomura *et al.*, 2002).

ACTIVE CONSTITUENTS

Saponins

Licorice root contains triterpenoid saponins (4–20%), mostly glycyrrhizin, a mixture of potassium and calcium salts of glycyrrhizic acid (also known as glycyrrhizic or glycyrrhizinic acid, and a glycoside of glycyrrhetinic acid) which is 50 times as sweet as sugar (Blumenthal *et al.*, 2000). Other triterpenes present are liquiritic acid, glycyrretol, glabrolide, isoglaborlide and licorice acid (Williamson, 2003). Recently, it was shown that high concentration glycyrrhizin production is possible within a very short production period under controlled environments (Afreen *et al.*, 2005).

Flavonoids

Other constituents include flavonoids and chalcones (which are responsible for the yellow color of licorice) such as liquiritin, liquiritigenin, rhamnoliquiritin, neoliquiritin, chalcones isoliquiritin, isoliquiritigenin, neoisoliquiritin, licuraside, glabrolide and licoflavonol (Williamson, 2003). Recently 5,8-dihydroxy-flavone-7-O-beta-D-glucuronide, glychionide A, and 5-hydroxy-8-methoxyl-flavone-7-O-beta-D-glucuronide, glychionide B were isolated from the roots of *G. glabra* (Li *et al.*, 2005). The retrochalcones, licochalcone A, B, C, D and echinatin, were recently isolated from the roots of *G. inflata* (Haraguchi, 2001) (Fig. 1), and the minor flavonoids, isotrifoliol and glisoflavanone, from the underground part of *G. uralensis* (Hatano *et al.*, 2000a).

Isoflavones

Isoflavonoid derivatives present in licorice include glabridin, galbrene, glabrone, shinpterocarpin, licoisoflavones A and B, formononetin, glyzarin, kumatakenin (Williamson, 2003). More recently, hispaglabridin A, hispaglabridin B, 4′-O-methylglabridin and 3′-hydroxy-4′-O-methylglabridin (De Simone *et al.*, 2001; Haraguchi, 2001) and glabroisoflavanone A and B glabroiso-flavanone B (Kinoshita *et al.*, 2005) have been found.

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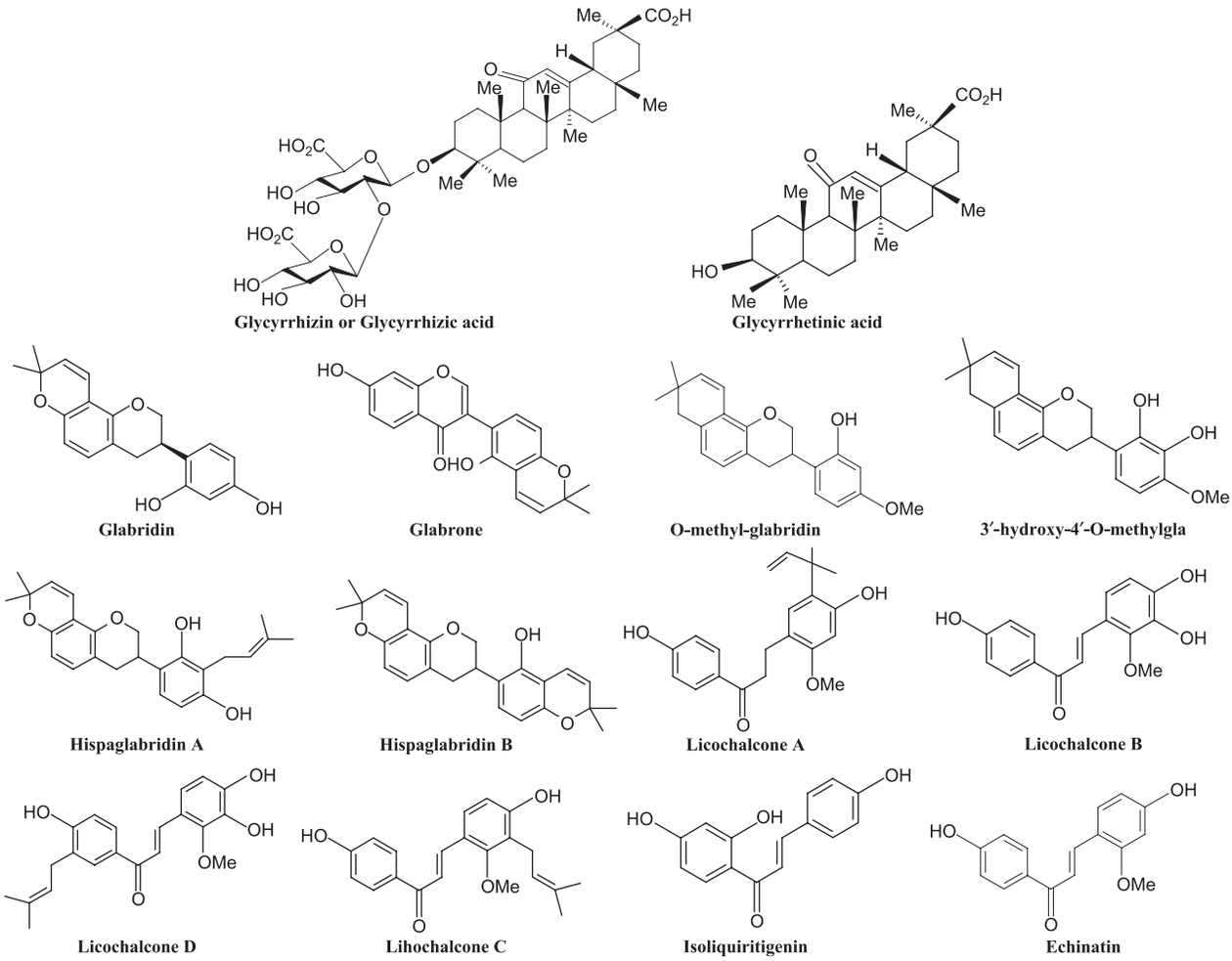

Figure 1. Chemical structure of some active components of Licochalcone C.

Coumarins

Coumarins present in *G. glabra* include liqcoumarin, glabrocoumarone A and B, herniarin, umbelliferone, glycyrin (Williamson, 2003), glycocoumarin, licofuranocoumarin, licopyranocoumarin (De Simone *et al.*, 2001; Haraguchi, 2001) and glabrocoumarin (Kinoshita *et al.*, 2005).

Stilbenoids

Four new dihydrostilbenes, dihydro-3,5-dihydroxy-4′-acetoxy-5′-isopentenylstilbene, dihydro-3,3′,4′-trihydroxy-5-O-isopentenyl-6-isopentenylstilbene, dihydro-3,5,3′-trihydroxy-4′-methoxystilbene and dihydro-3,3′-dihydroxy-5beta-d-O-glucopyranosyloxy-4′-methoxystilbene were isolated from the leaves of *G. glabra* grown in Sicily (Biondi *et al.*, 2005).

Miscellaneous compounds

G. glabra extract also contains fatty acids (C_2–C_{16}) and phenols (phenol, guaiacol), together with common saturated linear γ-lactones (C_6–C_{14}). A series of new 4-methyl-γ-lactones and 4-ethyl-γ-lactones in trace amounts has also been found (Näf and Jaquier, 2006). Asparagines, glucose, sucrose, starch, polysaccharides (arabinogalactants), sterols (β-sitosterol, dihydrostigmasterol) are also present (Hayashi *et al.*, 1998; Blumenthal *et al.*, 2000).

TRADITIONAL USES

Licorice has a long history of medicinal use in Europe and Asia. It is felt to be effective in the treatment of peptic ulcer disease, constipation, cough and other diseases which have been summarized in Table 1. As the table shows, it seems different parts of this herbs may be useful to treat some diseases.

PHARMACOLOGICAL EFFECTS

This part of review will deal with the pharmacological effects of the licorice and its bioactive components and their effects in treatment of diseases in different models of *in vivo* and *in vitro* studies. The pharmacology effects were divided into experimental and clinical studies in this review.

Table 1. Traditional use of different part of *G. glabra*

Extract	Traditional use
Fresh leaf (external)	Used for wounds (Dafni *et al.*, 1984)
Rhizome + root (infusion, oral)	Used to treat cystitis (Yarnell, 1997)
Root (oral)	Used to treat diabetes (Gray and Flatt, 1997)
Root (decoction, oral)	Used for cough, stomachache (Fujita *et al.*, 1995)
Aqueous extract of stem (oral)	Used for tuberculosis (Arseculeratne *et al.*, 1985)
Stem (oral)	Used for diabetes and as a diuretic (Rajurkar and Pardeshi, 1997)
Root (decoction, oral)	Used for kidney stones, lung ailment, ulcers (Dafni *et al.*, 1984)
Aqueous extract (oral)	Use in Addison's disease, gastric ulcers (Varshney *et al.*, 1983)
Aqueous extract (oral)	Used as anabolic and to improve the voice (Sircar, 1984)
Aqueous extract of root (oral)	Mild laxative (Armanini *et al.*, 2002)
Aqueous extract of rhizome (oral)	Contraceptive (Lee *et al.*, 1977)
Aqueous extract of rhizome + roots (oral)	Improve male sexual function (Nisteswar and Murthy, 1989)

EXPERIMENTAL STUDIES

Antiinflammatory activities

β-glycyhrritinic acid has shown antiinflammatory properties in different animal models (Capasso *et al.*, 1983; Amagaya *et al.*, 1984; Inoue *et al.*, 1989). β-Glycyhrritinic acid is the major metabolite of glycyrrhizin (Gumpricht *et al.*, 2005).

Two mechanisms have been suggested for the antiinflammatory effects of β-glycyhrritinic acid: First, it inhibits glucocorticoid metabolism and potentiates their effects. This potentiation was reported in skin and lung after coadministration of them with β-glycyhrritinic acid (Teelucksingh *et al.*, 1990; Schleimer, 1991). Since, β-glycyhrritinic acid is a potent inhibitor of 11β-hydroxysteroid hydroxygenase (Walker and Edwards, 1991), it causes an accumulation of glucocorticoids with antiinflammatory properties. Oral administration of β-glycyhrritinic acid or glycyrrhizin confirmed this result (MacKenzie *et al.*, 1990). Second, it inhibits classical complement pathway activation and its activity is dependent on its conformation (Kroes *et al.*, 1997). Thus, it is suggested that co-medication of it with hydrocortisone in the treatment of inflammatory lung disease will be useful (Schleimer, 1991).

Glycyrrhizin inhibited reactive oxygen species (ROS) generation by neutrophils which are the potent mediator of tissue inflammation in the *in vitro* study. It was thought that one of its antiinflammatory effect was due to this inhibitory effect (Akamatsu *et al.*, 1991; Wang and Nixon, 2001). Also, the generation of reactive oxygen species was also suppressed by glabridin treatment in RAW 264.7 cells (Jong *et al.*, 2005).

G. glabra and glyderinine, a derivative of glycyrrhizic acid, showed an antiinflammatory effect (Azimov *et al.*, 1988; Tokiwa *et al.*, 2004). It also reduced myocardial inflammatory edema in experimental myocardial damage (Zakirov *et al.*, 1999). In addition, glabridin and lichochalocone A have shown an antiinflammatory effect in *in vivo* studies (Furuhashi *et al.*, 2005; Jong *et al.*, 2005).

Glycyrrhetinic acid did not inhibit either cyclooxygenase 1 or 2 catalysed prostaglandin biosynthesis with an IC_{50} value of 425 μM in an *in vitro* study (Perera *et al.*, 2001). However, in another study *G. radix* was believed to be involved in COX-2 inhibition (Kase *et al.*, 1998). Furthermore, in this paper *G. radix* increased corticosterone levels in rats. Also, glycyrrhizin and glycyrrhetinic acid are known to inhibit phospholipase A_2 (Kase *et al.*, 1998). Recently, some derivatives of glycyrrhetinic acid have shown their inhibitory activity against interleukin-1b (IL-1b)-induced prostaglandin E2 (PGE2) production in normal human dermal fibroblasts (NHDF) (Tsukahara *et al.*, 2005).

Antimicrobial and antiviral activities

The methanol extract of aerial parts of *G. glabra* showed antibacterial activity against several kinds of bacteria (Sabahi *et al.*, 1987). Several flavonoids with C5 aliphatic residues were isolated as the effective constituents of licorice against methicillin-resistant *Staphylococcus aureus* (MRSA) and restored the effects of oxacillin and β-lactam antibiotic against MRSA (Hatano *et al.*, 2000b, 2005). Glabridin, glabrene and licochalcone A exhibited antimicrobial activity against *Helicobacter pylori in vitro* (Fukai *et al.*, 2002a, 2002b). The ether–water extracts of *G. glabra* were found to have effective antibacterial activity against all the five bacteria, *E. coli*, *B. subtilis*, *E. aerogenes*, *K. pneumoniae* and *S. aureus* (Onkarappa *et al.*, 2005). Glycyrrhizol A and 6, 8-diisoprenyl-5, 7, 4′-trihydroxyisoflavone from the root of *G. uralensis* exhibited potent antibacterial activity against *Streptococcus mutans* with minimum inhibitory concentrations of 1 and 2 μg/mL, respectively (He *et al.*, 2006).

Glycyrrhizic acid inhibits the replication of several viruses *in vitro* (Table 2) and some mechanisms have been found for the antiviral effects of glycyrrhizin (Van Rossum *et al.*, 1998; Cohen, 2005). In another study glycyrrhizic acid induced apoptosis of primary effusion lymphoma (PEL) cells that were transformed by Kaposi sarcoma-associated herpesvirus (KSHV) and terminated latent infection in B lymphocytes (Curreli *et al.*, 2005).

Two coumarins of *G. glabra*, glycocoumarin and licopyranocoumarin, were able to inhibit giant cell formation in HIV-infected cell cultures without any cytotoxicity (Hatano *et al.*, 1988; De Simone *et al.*, 2001) (Fig. 2). Also, Hatano *et al.* (1988) showed that lichochalcone A had anti-HIV activity (Hatano *et al.*, 1988).

Table 2. Antiviral effects of glycyrrhizin in *in vitro* study

Virus	Reference
Epstein-Barr virus (EBV)	Lin, 2003
Herpes simplex virus	Pompei *et al.*, 1979
Hepatitis A virus (HAV)	Crance *et al.*, 1990
Hepatitis B virus (HBV)	Takahara *et al.*, 1994; Sato *et al.*,1996
Hepatitis C virus (HCV)	Van Rossum *et al.*, 1998
Human cytomegalovirus (CMV)	Numazaki *et al.*, 1994
Human immunodeficiency virus (HIV)	Ito *et al.*, 1988
Influenza virus	Utsunomiya *et al.*, 1997
SARS coronavirus	Cinatl *et al.*, 2003
Varicella zoster virus (VZV)	Baba and Shigeta, 1987

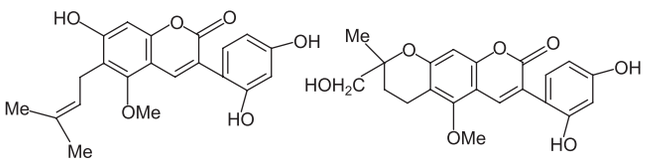

Figure 2. Anti-HIV coumarins isolated from *G. glabra.*

Antiprotozoal activities

Chinese licorice roots which can be obtained from the three species of *Glycyrrhiza* genus, *G. glabra*, *G. uralensis* or *G. inflata*, were found to potentially inhibit the growth of *Plasmodium. falciparum* and *Leishmania donovani* in *in vitro* studies (Christensen *et al.*, 1994; Christensen and Kharazmi, 2001). Chalcones such as licochalcone A from Chinese licorice roots are known to possess antiplasmodial activity with IC_{50} values between 4.5 and 0.6 mg/mL (Chen *et al.*, 1994b; Jenett-Siems *et al.*, 1999). Also, chalcones have a potent antileishmanial activity and might be developed into a new class of antileishmanial drugs (Chen *et al.*, 1993; Chen, 1994a). It was found that chalcones, such as lichochalcone A, alter the ultrastructure of the parasite mitochondria and inhibit their function by selectively inhibiting fumarate reductase (FRD) in the respiratory chain of the parasite (Zhai *et al.*, 1995; Chen *et al.*, 2001).

Antioxidative activities

The constituents of *G. inflata*, licochalcone A, B, C, D and echinatin, were effective in preventing microsomal lipid peroxidation induced by Fe (III)-ADP/NADPH and licochalcone B, D showed potent antioxidative and superoxide scavenging activities (Haraguchi *et al.*, 1998). Furthermore, the isoflavone derivatives of *G. glabra* such as glabridin inhibited lipid peroxidation in rat liver microsomes and protected mitochondrial functions from oxidative stresses (Haraguchi *et al.*, 2000). Hispaglabridin A, especially, showed a potent antioxidative activity against peroxidation induced by Fe-ascorbate (Haraguchi, 2001).

Moreover, glabridin, an isoflavan of *G. glabra*, was a potent antioxidant toward LDL oxidation in *in vitro* and *in vivo* studies (Fuhrman *et al.*, 1997; Vaya *et al.*, 1997; Belinky *et al.*, 1998a). The consumption of licorice or glabridin by atherosclerotic apolipoprotein E-deficient (E^0) mice caused a significant reduction not only in their LDL oxidation but also in the development of atherosclerotic lesions (Fuhrman *et al.*, 1997; Rosenblat *et al.*, 1999). It seems that glabridin may possess this property by two mechanisms: first it binds to the LDL and substantially protects its oxidation (Fuhrman *et al.*, 1997; Belinky *et al.*, 1998a). The hydroxyl groups on the B ring of glabridin were found to be most important for its antioxidative properties (Belinky *et al.*, 1998b). Second it accumulates in cells such as macrophages, causing a reduction of cellular oxidative stress by reducing NADPH oxidase activation and increasing cellular glutathione (GSH) (Rosenblat *et al.*, 1999, 2002). In addition, other constituents of *G. glabra* such as isoflavones hispaglabridin A, hispaglabridin B and 4′-O-methylglabridin, the two chalcones, isoprenylchalcone derivative and isoliquiritigenin were antioxidants against LDL oxidation (Vaya *et al.*, 1997).

Hepatoprotective studies

In an *in vitro* study, glycyrrhizin was hepatoprotective, probably by preventing changes in cell membrane permeability (Nakamura *et al.*, 1985). Nevertheless, it was suggested that glycyrrheitinic acid is a better hepatoprotective drug than glycyrrhizin in *in vitro* study (Nose *et al.*, 1994). This observation is in keeping with the protective effects of glycyrrhetinic acid against the carbon tetrachloride-induced hepatotoxicity and retrorsine-induced liver damage, respectively, in mice and rats (Lin *et al.*, 1999; Jeong *et al.*, 2002). Furthermore, in a hepatocyte model of cholestatic liver injury, glycyrrhizin exhibited pro-apoptotic properties, whereas glycyrrheitinic acid is a potent inhibitor of bile acid-induced apoptosis and necrosis (Gumpricht *et al.*, 2005). Some hepatoprotective effects of glycyrrhizin have been summarized in Table 3.

Antitumor activities

The aqueous extract of *G. glabra* inhibits the *in vivo* and *in vitro* proliferation of Ehrlich ascites tumor cells and inhibits angiogenesis in *in vivo* assay, peritoneal and chorioallantoic membrane assays (Sheela *et al.*, 2006). Also, the ethanol extract of *G. uralensis* root induced apoptosis and G1 cell cycle arrest in MCF-7 human breast cancer cells (Jo *et al.*, 2005). On the other hand, there are many studies about the anticancer effects of several derivatives of its components both

Table 3. Cytoprotective effects of glycyrrhizin in the liver

Study	Method	Mechanism
In vitro		
Rat hepatocytes	Incubation with anti-liver cell membrane antibody + complement	Deceased release of AST and inhibition PLA_2 (Shiki *et al.*, 1992)
Rat hepatocytes	CCl_4-induced hepatotoxicity	Deceased LDH and glutamic oxaloacetic transaminase (Nakamura *et al.*, 1985)
Rat hepatocytes	Acetaminophen or D-galactosamine induced liver injury	Increased survival rate of the hepatocyte culture (Nacagiri *et al.*, 2003)
In vivo		
Rat liver	Ischemia-reperfusion damage	Suppressed the elevation lipid peroxides, AST, ALT, LDH and decreased morphological damage (Nagai *et al.*, 1991)
Rat liver	Retrorsine-induced liver damage	Normalized serum levels of transaminase Lin *et al.*, 1999)
Rat liver	Thioacethamide-induced liver damage	Normalized serum aminotransferases, alkaline phosphatase and bilirubin (Asgary *et al.*, 2005)

Aspartate aminotransferase (AST), alanine aminotrasferase (ALT), lactate dehydrogenase (LDH), phospholipase A_2 (PLA_2), carbon tetrachloride (CCl_4).

in *in vivo* and *in vitro* studies. For more detail see Table 4.

Glycyrrhetinic acid could also trigger the pro-apoptotic pathway by inducing mitochondrial permeability transition and this property may be useful for inducing apoptosis of tumor cells (Salvi *et al.*, 2003; Fiore *et al.*, 2004). Recently, licochalcone E, a new retrochalcone from the roots of *G. inflata*, exhibited the most potent cytotoxic effect compared with the known antitumor agents, licochalcone A and isoliquiritigenin (Yoon *et al.*, 2005).

Central nervous system studies

Glabridin inhibited serotonin reuptake (Ofir *et al.*, 2003). In addition, recently, the aqueous extract of *G. glabra* L. showed antidepressant activity in both the forced swim test (FST) and tail suspension test (TST) in mice (Dhingra and Sharma, 2005). The ethanol extract of *G. glabra* had an anticonvulsant effect in PTZ and lithium-pilocarpine-induced convulsion models (Ambawade *et al.*, 2002). Also, the aqueous extract of *G. glabra* showed memory enhancing effects in the plus-maze and passive avoidance paradigm (Dhingra *et al.*, 2004). Moreover, chronic administration of the extract of *G. glabra* in both low and high doses induced correction of the passive avoidance performance in ovariectomized female rats (Fedotova *et al.*, 2005). Combined treatment with licorice root and vibration resulted in increased succinate dehydrogenase (SDH) activity in different parts of the brain, improved brain energy supply and ameliorated the effect of vibration (Oganisyan *et al.*, 2005). In addition, isoliquiritigenin showed protective effects in cerebral ischemia-reperfusion injury in rats (Zhan and Yang, 2006).

Carbenoxolone has shown anticonvulsant, sedative and muscle relaxant activities in mice and in genetically epilepsy prone rats (GEPRs) (Hosseinzadeh and Nassiri Asl, 2003; Gareri *et al.*, 2004). Also, it was able to suppress the generation of superoxide anions and hydrogen peroxide in macrophages and it also showed protective effects in the skeletal muscle and hippocampus against acute ischemic-reperfusion effects in rats (Suzuki *et al.*, 1983; Hosseinzadeh *et al.*, 2005a). In addition it could decrease the learning performances of rats in a spatial memory task (Hosseinzadeh *et al.*, 2005b).

Cardiovascular studies

Licorice showed an antiplatelet aggregation effect (Tawata *et al.*, 1992; Yu *et al.*, 2005). In other experiments, glycyrrhizin has been identified as a thrombin inhibitor in *in vitro* and *in vivo* studies and it was believed that glycyrrhizin might be used as a model for searching new antithrombotic drugs (Francischetti *et al.*, 1997; Mendes-Silva *et al.*, 2003). Also, *G. glabra* accelerated the metabolism of cells in the bone marrow erythroid stem and increased the animal's resistance to stress (Adamyan *et al.*, 2005).

Isoliquiritigenin, an active component of licorice, is reported to have a vasorelaxant effect (Yu and Kuo, 1995). It could also able to decrease tube formation in vascular endothelial cells. Thus, the anti-angiogenic effect of licorice extract depended on the anti-tube formation effect of isoliquiritin (Kobayashi *et al.*, 1995). On the other hand, as for the estrogen-like activities of glabridin in *in vivo* and *in vitro* studies, it was demonstrated that it could modulate vascular injury and atherogenesis. Therefore, it is suggested for the prevention of cardiovascular diseases in post-menopausal women (Somjen *et al.*, 2004b).

Immunological studies

Several immunomodulatory activities have been attributed to glycyrrhizin and glycyrrhetinic acid (Ohuchi *et al.*, 1981; Kobayashi *et al.*, 1993; Zhang *et al.*, 1993; Kondo and Takano, 1994; Raphael and Kuttan, 2003). The same results were seen with lichochalchone A and some analogues which showed immunomodulatory effects (Barfod *et al.*, 2002).

On the other hand, glycyrrhizin selectively activated extrathymic T cells in the liver and in human T cell

Table 4. Anticancer effects of some active component of licorice

Compound	Method	Effects
Lichochalcone A	MCF-7 breast, HL-60 leukemia and PC-3 prostate cancer cell lines	Antitumor activity, induced apoptosis by modulating bcl-2 protein expression (Rafi *et al.*, 2000, 2002; Fu *et al.*, 2004)
	DMBA-initiated and TPA-promoted skin papilloma in mice	Antitumor promoting activity by preventing TPA to bind to the membrane receptors (Kitagawa *et al.*, 1986; Shibata *et al.*, 1991)
	TPA-promoted $^{32}P_i$-incorporation into phospholipids of HeLa cells	Inhibitory effect (Shibata *et al.*, 1991)
Glycyrrhetinic acid (GA)	Tumor promoted by TPA *in vivo* study	Antitumor-promoting activity (Kitagawa *et al.*, 1986)
Glycyrrhizic acid (aqueous extract of licorice root)	AFB1-induced cytotoxicity in human HepG2 cells	Protective effect and prevent chemical-induced carcinogenicity by inhibition the activation of hepatotoxic metabolites (Chan *et al.*, 2003)
Isoliquiritigenin (ILG)	AOM-treated ddY mice	Inhibited induction of ACF and colon carcinoma development (Baba *et al.*, 2002; Takahashi *et al.*, 2004)
	DMBA-induced skin carcinogenesis in mice	Inhibited epidermal ODC and suppressed DMBA effects (Yamamoto *et al.*, 1991)
	B16 melanoma 4A5 cells	Induced cell death and promotion of Bax expression (Iwashita, *et al.*, 2000)
	MGC-803 gastric cancer cells	Antiproliferative activity (Ma *et al.*, 2001)
	MCF-7 breast cancer cells	Antiproliferative activity (Maggiolini *et al.*, 2002)
	DU 145 and LNCaP prostate cancer cells	Antiproliferative activity (Kanazawa *et al.*, 2003)
	MLL(rat) and DU145 (human) prostate cancer cells	Inhibited cell growth and decreased cell number, induced apoptosis (Jung *et al.*, 2006)
	A549 lung cancer cells	Antiproliferative activity, enhanced expression of $p21^{CIP1/WAF1}$ expression (Hsu *et al.*, 2004; li *et al.*, 2004)
	Pulmonary metastasis model of murine renal cell carcinoma cell line (Renca)	Reduced pulmonary metastasis (Yamazaki *et al.*, 2002)
	Hep G2	Induced apoptotic cell death by inhibiting the NF-kappaB survival-signaling pathway (Hsu *et al.*, 2005)
Glabridin	In the human breast cell line	Antiproliferative effects (Tamir *et al.*, 2000)
Dibenzoylmethane (DBM)	DMBA-induced mammary tumor in Sencar mice	Inhibited formation, proliferation of total DMBA-DNA adducts in mammary gland (Lin *et al.*, 2001)
	LNCaP, DU145, and PC-3 prostate carcinoma cell lines	Cytostatic effect with deregulation cell cycle (Jackson *et al.*, 2002)

Dimethylbenz [*a*] anthracene (DMBA), 12-O-tetradecanoylphorbol 13-acetate (TPA), aflatoxin B1 (AFB1), hepatoma cell line (HepG2), azoxymethane (AOM), aberrant crypt foci (ACF), ornithine decarboxylase (ODC), MAT-LyLu (MLL), 7,12-dimethylbenz[a]anthracene (DMBA).

lines and glycyrrhizic acid enhanced Fas-mediated apoptosis without alteration of caspase-3-like activity (Kimura *et al.*, 1992; Ishiwata *et al.*, 1999). Glycyrrhizin also improved the impaired resistance of thermally injured mice to herpes virus infection (Utsunomiya *et al.*, 1995). Moreover, glycyrrhetinic acid was an inducer of type 2 antagonistic CD41 T cells in *in vivo* and *in vitro* studies (Kobayashi *et al.*, 1993; Utsunomiya *et al.*, 1995; Nakajima *et al.*, 1996). It improved the resistance of mice infected with LP-BM5 murine leukemia virus (MAIDS) mice to *Candida albicans* infection (Utsunomiya *et al.*, 2000). Also, it stimulated macrophage-derived NO production, and was able to up-regulate iNOS expression through nuclear factor *k*B (NF- *k*B) transactivation in murine macrophages (Jeong and Kim, 2002). Both of them could induce interferon activity and augment natural killer cell activity and in this study glycyrrhizin was superior to glycyrrhetinic acid in inducing interferon (Abe *et al.*, 1982). It also has inhibitory effects on TNF-alpha-induced IL-8 production in intestinal epithelial cells (Kang *et al.*, 2005).

In addition, there are some studies on the immunomodulatory effects of polysaccharide fractions obtained from shoots of *G. glabra* and hairy roots of *G. uralensis in vitro* (Nose *et al.*, 1998). GR-2IIa and GR-2IIb, two isolated acidic polysaccharides of *G. uralensis*, have shown anticomplementary activity. Also, GR-2IIc had both anticomplementary activity and mitogenic activity (Zhao *et al.*, 1991; Yamada *et al.*, 1992; Kiyohara *et al.*, 1996). Recently, the haemolytic activities of *G. uralensis* saponins (GLS) and its adjuvant potentials against ovalbumin (OVA) were established in mice (Sun and Pan, 2006).

Renal studies

Glabridin showed an antinephritis effect in the mouse glomerular disease model (Fukai *et al.* 2003). Also, glycyrrhizin could ameliorate renal defects in gentamicin-induced acute renal failure in rats (Sohn *et al.*, 2003). Also, the extract of *G. radix* could protect the kidneys against peroxynitrite ($ONOO^-$)-induced oxidative stress

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in vivo through scavenging $ONOO^-$ and/or its precursor NO (Yokozawa *et al.*, 2005).

Cytotoxic activities

Sixty nine compounds of *Glycyrrhiza* phenols showed an inhibitory activity on the growth of *Bacillus subtilis* H17 and M45 and some of them, such as isoliquiritigenin, were positive in the rec-assay (Fukai *et al.*, 1998).

Respiratory studies

Recently in one study, *G. radix* produced a persistent antitussive effect in the guinea-pig, suggesting that liquiritin apioside, a main antitussive component, plays an important role in the earlier phase, while liquiritigenin and liquiritin play an important role in the late phase (Kamei *et al.*, 2005). This result is keeping with the previous antitussive effects of licorice.

Effects on gap junction channels

Glycyrrhitinic acid and its derivatives were shown to inhibit gap junction channels (Davidson and Baumgarten, 1988). The inhibitory effects of 18β-glycyrrhetinic acid on gap junction channels of arteriolar smooth muscle, endothelial cells, renal pelvis, ureter and mesenteric small arteries were studied (Yamamoto *et al.*, 1998; Santicioli and Maggi, 2000; Matchkov *et al.*, 2004).

Endocrinological studies

Some effects of licorice on the endocrine system in *in vitro* and *in vivo* studies are summarized in Table 5. It seems that this herb acts on the metabolism of steroids with different mechanisms.

Other studies

In endocrinological studies, glabridin increased the growth of mouse osteoblastic (MC3T3-E1) and human cell lines (Somjen *et al.*, 2004a; Choi, 2005). The alcohol extract of licorice reduced the glucose levels of genetically diabetic KK-A^y mice (Kuroda *et al.*, 2003).

Table 5. The effects of licorice on the function of different enzymes

Enzyme	Effects
11β-HSD Type 1	Inhibition (Jellinck *et al.*, 1993; Hult *et al.*, 1998;
11β-HSD Type 2	Inhibition (Monder *et al.*, 1989; Ferrari *et al.*, 2001; Palmero *et al.*, 2004)
3HSD	Inhibition (Latif *et al.*, 1990)
17HSD	Inhibition (Armanini *et al.*, 2003)
17-20 lyase	Inhibition (Armanini *et al.*, 2003)
Aromatase	Increase (Sakamoto and Wakabayashi, 1988)
5α-Reductase	Increase (Latif *et al.*, 1990; Fugh-Berman and Ernst, 2001)

In addition, dermatological studies showed that three flavonoids of licorice, licuraside, isoliquiritin and licochalcone A, have high potential for studying depigmenting agents by inhibiting tyrosinase (Fu *et al.*, 2005). The same results were reported for glycyrrhisoflavone and glyasperin C (Kim *et al.*, 2005).

CLINICAL STUDIES

Gastrointestinal effects

It was shown that oral licorice in a combination product could heal ulcers as effectively as an H2 blocker (Kassir, 1985; Aly *et al.*, 2005). Glycyrrhizinic acid, a major component of licorice, has antiulcer properties, it seems by raising the local concentration of prostaglandins that promote mucous secretion and cell proliferation in the stomach, leading to healing of ulcers in experimental studies (Van Marle *et al.*, 1981; Baker, 1994).

Carbenoxolone, a hemisuccinate derivative of 18β-glycyrrhetinic acid, and enoxolone are two chemical synthetic derivatives of licorice which have been used in clinical therapies (Fig. 3). Enoxolone, an analogue of carbenoxolone, has been used for the treatment of peptic ulcer disease and other GIT disorders, skin disorders, mouth and throat disorders (Sweetman, 2005). Carbenoxolone has been used for peptic ulcer disease, gastro-oesophageal reflux and also it has been used for the symptomatic management of mouth ulceration as a gel or mouthwash (Sweetman, 2005).

Anticancer effects

Licorice root has been identified by the National Cancer Institute as possessing cancer-preventive properties (Craig, 1999; Wang and Nixon, 2001). It has been used among patients with prostate cancer as an ingredient of PC-SPES, a commercially available combination of eight herbs (DiPaola *et al.*, 1998).

Antioxidative effects

G. glabra extracts showed great antioxidant and free radical scavenging activities in topical formulations and may be used in topical formulations in order to protect the skin against damage caused by free radical and reactive oxygen species (Di Mambro and Fonseca, 2005).

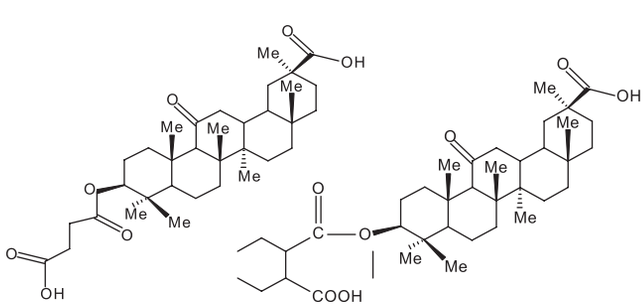

Figure 3. Chemical structures of carbenoxolone and enoxolone.

Antiviral and hepatoprotective effects

In the world, especially in Asia, glycyrrhizic acid is used intravenously for the treatment of chronic hepatitis B and C and its preparation under the name of Stronger Neo-Minophagen C (SNMC) decreased aminotransferase levels in patients with chronic hepatitis in multiple double-blind studies (Van Rossum *et al.*, 1999; Iino *et al.*, 2001; Zhang and Wang, 2002). It is suggested that glycyrrhizin has a preventive effect on the development of hepatocellular carcinoma (HCC) in patients with HCV-associated chronic hepatitis (Arase *et al.*, 1997; Miyakawa and Iino, 2001).

Licorice has been reported to have a direct hepatoprotective effect (Luper, 1999; Leung *et al.*, 2003). Glycyrrhizin, its major component, is often used to treat patients with chronic liver damage who do not receive or respond to interferon (IFN) therapy (Okuno *et al.*, 2001). Stronger Neo-Minophagen C® (SNMC), containing 2 mg/mL of glycyrrhizin, has been used clinically as an antihepatitis agent (Shibata, 2000).

Dermatological studies

G. glabra L. has been used in herbal medicine for skin eruptions, including dermatitis, eczema, pruritus and cysts (Saeedi *et al.*, 2003). In this section the various studies of licorice on the skin are summarized in Table 6.

Recently glycyrrhizin treatment has showed protective effects against UVB-irradiated human melanoma cells (Rossi *et al.*, 2005). Moreover, licorice extract and its active component, glycyrrhizic acid has been described as effective skin whitening effects (Smith, 1999). The group of Briganti classified liquiritin as a skin turnover accelerator (Briganti *et al.*, 2003). However, it was suggested that liquiritin causes depigmentation by two mechanisms: first, via melanin dispersion by means of the pyran ring of its flavonoidal nucleus; second the acceleration of epidermal renewal (Amer and Metwalli, 2000). Concerning the mechanisms of glabridin on melanogenesis and inflammation, it has been shown that it inhibits the tyrosinase activity of melanocytes and as a result, it seems that hydroquinone will be replaced by licorice extract in a new preparation for dermal melasma (Piamphongsant, 1998). However, in a few cases, allergic dermatitis can develop to oil soluble licorice extracts (Nishioka and Seguchi, 1999).

Table 6. Licorice and its components in skin therapies

Compound	Treatment
Licorice (topical gel 2%)	Atopic dermatitis (Saeedi *et al.*, 2003)
GA	Inflammatory dermatoses (Cohen and Heidary, 2004)
Deglycyrrhizinated licorice and carbenoxolone (topical)	Recurrent aphthus stomatitis (RAS) (Scully *et al.*, 2002)
Liquiritin (topical 2%)	Hyperpigmentation (in patient with bilateral and symmetrical idiopathic epidermal melasma) (Amer and Metwalli, 2000)
Glabridin	Melanogenesis, inflammation (Yokota *et al.*, 1998; Petit and Pierard, 2003; Halder and Richards, 2004)

Glycyrrhetinic acid (GA).

Endocrinological effects

Glycyrrhiza root has been shown to decrease circulating levels of testosterone in men and women (Armanini *et al.*, 1999, 2002; Rafi *et al.*, 2002; Armanini *et al.*, 2004). But it was not able to reduce salivary testosterone in men significantly (Josephs *et al.*, 2001). Moreover, it induced regular ovulation and pregnancy in infertile hyperandrogenic patients (Yaginuma *et al.*, 1982).

On the other hand, isoliquiritigenin (ILC), glabrene and glabridin are phytoestrogens. ILC and glabrene can bind to the human estrogen receptor (ER) with higher affinity than glabridin. It was suggested that isoflavenes may serve as natural estrogen agonists in preventing the symptoms and diseases associated with estrogen deficiency (Tamir *et al.*, 2000, 2001). In some traditional Chinese medicine preparations, the root of *G. glabra* is used for treatment menopause-related symptoms. But there are no clinical data regarding its safety or efficacy for treating hot flashes (Santoro *et al.*, 2004).

Moreover, the activity of 11β-HSD-2 potently is blocked *in vivo* and *in vitro* by glycyrrhetinic acid by two mechanisms, direct competitive inhibition and pretranslational inhibition (Ferrari *et al.*, 2001). It seems that this herb acts on the metabolism of steroids with different mechanisms. The consumption of licorice extract and glycyrrhetinic acid could decrease body fat mass in humans and a possible mechanism seems to be by inhibiting 11β-HSD1 at the level of fat cells (Armanini *et al.*, 2005).

Respiratory diseases

Licorice has been used as a cough-relieving medicinal herb from ancient times. It seems that mucilage present in it or secretion produced under the influence of the active substances covers the oral and throat mucosa soothing its irritability and relieving dry cough (Ody, 2000; Puodziuniene *et al.*, 2005).

Other effects

Ammonium glycyrrhizate (from licorice root) is used in toothpastes, mouth rinses and other products for the control of periodontal disease (Goldie, 2005). The extract of *G. glabra* in combination with other herbs, such as ImmunoGuard®, has been effective for the prophylactic management and treatment of patients with Familial Mediterranean Fever (FMF) (Amaryan *et al.*, 2003).

INDUSTRIAL USES

Commercially, licorice is added to chewing gum, chocolate candy, cigarettes, smoking mixtures, chewing tobacco and snuff as sweetening agents (Tyler *et al.*, 1988; De Klerk *et al.*, 1997) and as a depigmentation

Table 7. Products containing considerable amounts of glycyrrhizinic acid (De Klerk *et al.*, 1997)

Confectionery	Licorice sticks, bricks, cakes, toffee, pipes, bars, balls, tubes, Catherine wheels, pastilles and allsorts
	Sorbits chewing gum
	Stimorol chewing gum
Health products	Licorice flavored diet gum
	Throat pearls
	Licorice flavored cough mixtures
	Herbal cough mixtures
	Licorice tea
All types of licorice root	Russian, Iranian, Chinese, Turkish, Afghan and unknown origin
	Chewing tobacco
	Alcoholic drinks

agent in cosmetics (Nomura *et al.*, 2002). Also, licorice is frequently employed to mask the taste of bitter drugs such as aloe, quinine and others. The surfactant property of the saponins may also facilitate the absorption of poorly absorbed drugs, such as the anthraquinone glycosides (Tyler *et al.*, 1988). Some of the products which have glycyrrhizinic acid are summarized in Table 7 (De Klerk *et al.*, 1997).

SIDE EFFECTS AND TOXICITY

Large amounts of licorice may result in severe hypertension, hypokalemia and other signs of mineralocorticoid excess. This hypertension is caused by decreased 11β-HSD2 activity. This enzyme is responsible for the renal conversion of cortisol to cortisone. Thus, licorice leads to activation of renal mineralocorticoid receptors by cortisol, resulting in a state of apparent mineralocorticoid excess and suppression of the rennin angiotensin system (Conn *et al.*, 1968, Stewart *et al.*, 1990; Van Uum, 2005). Some side effects due to the consumption of licorice have been reported by different groups and are summarized in Table 8.

Carmines *et al.* (2005) reported that adding licorice extract to cigarette tobacco at levels of ≤5% (about 0.269% glycyrrhizic acid) did not significantly alter the toxicity of smoke. Also, in this paper, it was mentioned that licorice is not a teratogen or genotoxic (Carmines *et al.*, 2005). In another study, the toxicity of licorice extract was shown in the liver of Black molly fish (Radhakrishnan *et al.*, 2005).

Table 8. Some side effects associated with licorice extract treatment

Side effects	Reference
Neurologic	
Headache	De Groot *et al.*, 1988
Paralysis	Van Den Bosch *et al.*, 2005
Transient visual loss	Dobbins and Saul, 2000; Fraunfelder, 2004
Cardiovascular	
Torsades de points tachycardia	Eriiksson *et al.*, 1999
Cardiac arrest	Bannister *et al.*, 1977
Hypertension	Olukoga and Donaldson, 2000
Edema	De Groot *et al.*, 1988; Shibata, 2000
Endocrine	
Hypokalemia	Nielsen and Pedersen, 1984; Olukoga and Davidson, 2000
Reduction testosterone	Armanini *et al.*, 1999
Premature birth	Strandberg *et al.*, 2001
Renal	
Acute renal failure	
Musculoskeletal	
Muscle weakness	Van Den Bosch *et al.*, 2005
Myopathy	Gross *et al.*, 1966; Shintani *et al.*, 1992
Myoglobinuria	Gross *et al.*, 1966
Rhabdomyolysis	Van den Bosch *et al.*, 2005
Other	
Increase body weight	Bernardi *et al.*, 1994

PHARMACOKINETICS

After oral administration, glycyrrhizin is metabolized to glycyrrhetinic acid by intestinal bacteria which contain β-D-glucuronidase (Hattori *et al.*, 1985). Furthermore, intravenously administered glycyrrhizin is metabolized in the liver by lysosomal β-D-glucuronidase to 3-mono-glucuronide glycyrrhetinic acid. This metabolite is excreted with bile into the intestine, where it is metabolized by bacteria into glycyrrhetinic acid, which can be reabsorbed (Akao *et al.*, 1991) (Fig. 4).

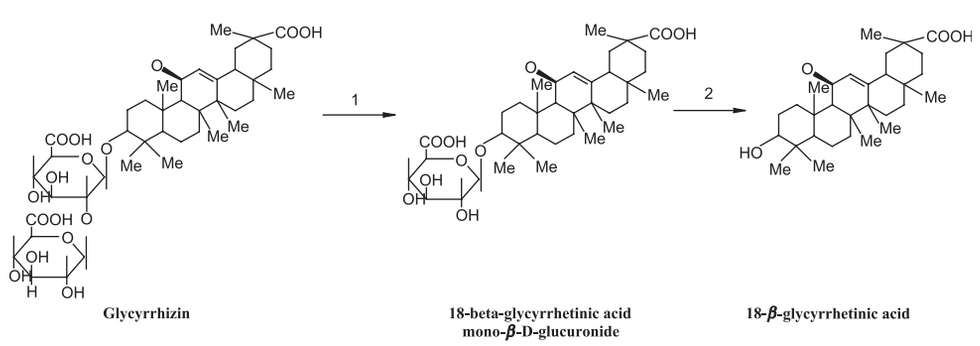

Figure 4. Metabolism of glycyhrrizin in the liver (1) by lysosomal β-D-glucuronidase to 3-mono-glucuronide glycyrrhetinic acid and then in the intestine (2) by bacteria β-D-glucuronidase after intravenous administration (Akao *et al.*, 1991).

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Other components of the extract could affect the pharmacokinetics of glycyrrhizin (G) and glycyrrhetic acid (GA), a main metabolite of G. After administration of aqueous licorice root extract (LE) to rats and humans, G and GA levels were lower compared with G alone and the pharmacokinetic curves showed significant differences in the areas under the plasma-time curve (AUC), C_{max}, and T_{max} parameters. Also, the data obtained from urine samples confirmed a reduced bioavailability of G present in LE compared with pure G. Interaction between the G constituent and other components in LE during intestinal absorption was mentioned. Thus, modified bioavailability could explain the various clinical adverse effects resulting from the chronic oral administration of G alone as opposed to LE (Cantelli-Forti *et al.*, 1994). However, it seems that the pharmacokinetics differ in other species. In another study the AUCs of G and GA after oral administration of LE were significantly higher than those after pure G in rabbits and the bioavailabilities of G and GA were significantly better from licorice than from pure G in rabbits, but the presystemic metabolism of pure G in the rabbit is rather different from that in rat, pig and human (Hou *et al.*, 2005). It was shown that the pharmacokinetics of G is nonlinear. After bolus intravenous administration at a dose of 20, 50, or 100 mg/kg in rat, the decline in the concentration of G in plasma, was generally biexponential at each dose, but the terminal disposition became much slower as the dose was increased. In addition, the apparent total body clearance decreased significantly with increases in the dose. But the apparent distribution volume after intravenous administration was unaffected by the dose (Tsai *et al.*, 1992). Administration of different oral doses of 18-beta-glycyrrhetinic acid (β-GRA) in healthy volunteers showed a biphasic decay of the plasma concentration-time curve at doses >500 mg. The peak plasma concentration and the AUC increased with increasing β-GRA doses. Urinary elimination of β-GRA and its glucuronides over 24 h was less than 1% of the dose administered. The data based on single dose kinetic analysis revealed that after multiple doses of 1.5 g β-GRA/day, 11 beta-hydroxysteroid dehydrogenase (11 beta-HSD) might be constantly inhibited, whereas at daily doses of 500 mg or less, such an inhibition might occur only transiently (Krahenbuhl *et al.*, 1994).

Administration intravenously of G to an animal model of liver disease (D-galactosamine-intoxicated (GAL) rat), significantly decreased the apparent volume of distribution (V_{dss}) and the total body clearance (CL_{total}) than those in normal rats. When G was administered orally, the AUC, the mean residence time (MRT) and the time to reach the maximum plasma concentration (T_{max}) for G were higher, but the maximum plasma concentration (C_{pmax}) in GAL rats was lower than that in normal rats. But, the bioavailability of G was not significantly changed. Also, the AUC for GA, after oral administration of G was higher in GAL rats than in normal rats, although there was no significant difference in MRT or T_{max}, C_{pmax} or the bioavailability for GA between GAL and normal rats. However, the changes in the absorption rate and reduction of the hepatic elimination rates in GAL rats could explain these differences (Wang *et al.*, 1996). GA has a large volume of distribution, a long biological half-life, and undergoes substantial enterohepatic circulation (Tyler *et al.*, 1988). Thus, large doses of KCl supplementation for weeks are necessary because of the long half-life of glycyrrhetic acid (Van Den Bosch *et al.*, 2005).

In another study, liquiritin apioside showed a peak plasma concentration 15 min after administration in guinea-pigs, which gradually decreased and was almost undetectable 4 h after administration. Liquiritigenin, an aglycone of liquiritin apioside, appeared in the plasma 2 h after the administration of liquiritin apioside and remained for more than 6 h after administration. The plasma concentration of unchanged liquiritigenin was observed 15 min after administration and then gradually increased for more than 6 h after administration (Kamei *et al.*, 2005).

Glycyrrhizin, genistein, glycyrrhisoflavone, glicoricone, licofuranone, licopyranocoumarin licocoumarone and other licorice constituents were found to inhibit monoamine oxidase (MAO) *in vitro* (Hatano *et al.*, 1991b). However, the clinical significance of this is not known and not all these compounds are found in all species.

Based on the phenolic constituent of licorice sp, they were classified into three types A, B, C:

Type A: roots and rhizomes of *G. uralensis* containing licopyranocoumarin, glycycoumarin and/or licocoumarone, which were not found in *G. glabra* and *G. inflata*. Type B: *G. glabra*, containing glabridin and glabrene, which were not found in the samples of the other two species. Type C: *G. inflata*, containing licochalcones A and B, which were not found in the other two species.

Extracts of some licorice specimens of types A, B, and C inhibited 40–56% of xanthine oxidase activity. Extracts of some licorice specimens of types A and B also showed inhibitory effects on monoamine oxidase (44–64%) (Hatano *et al.*, 1991a).

DRUG INTERACTIONS

The extract of *G. uralensis* showed potent CYP3A4 inhibitory activity (Hu *et al.*, 1999; Budzinski *et al.*, 2000; Tsukamoto *et al.*, 2005). After bioassay purification, other components such as (3*R*)-vestitol, 4-hydroxyguaiacol apioglucoside, liquiritigenin 7, 4′-diglucoside, liquiritin apioside showed potent CYP3A4 inhibitory activities among them (Tsukamoto *et al.*, 2005). Glabridin was also found to inactivate the enzymatic activities of CYP 3A4 and 2B6 and competitively inhibited 2C9 (Kent *et al.*, 2002).

In other hands, prolonged intake of high LE or G doses may result in accelerated metabolism of coadministered drugs. Daily oral doses of LE or G for 1, 4 or 10 consecutive days in mice, were able significantly to induce hepatic CYP3A- and, to a lesser extent, 2B1- and 1A2-dependent activities, as well as 6-beta- (mainly associated to CYP3A), 2-alpha-, 6-alpha- (CYP2A1, 2B1), 7-alpha-, 16-alpha- (CYP2B9) and 16-beta-testosterone hydroxylase (TH) activities. Thus, the induction of cytochrome P450-dependent activities by long-term ingestion of licorice may have clinical consequences for patients taking drugs metabolized by the same CYP enzymes (Paolini *et al.*, 1998). But, high doses of LE and G could cause significant adverse effects. Thus, it seems that routine licorice consumers under CYP3A induction might therefore be predisposed to associated

Table 9. Some drug interaction due to consumption of licorice and its bioactive components

Licorice	Drug	Results of interaction
Gan Cao (*G. uralensis*)	Warfarin	Increase metabolism of warfarin in rats (Mu *et al.*, 2006)
G. glabra	Acetaminophen	Increased the excretion of acetaminophen–glucoronide conjugate in rats (Moon and Kim, 1996)
G. glabra	Prednisolone	Decreased CL, increase AUC and Cp of prednisolone (Chen *et al.*, 1991)
GA	Hydrocortisone	Increase effect of hydrocortisone in mice (Teelucksingh *et al.*, 1990)
GA	Oral contraceptive	Hypertension, edema, hypokalemia, increase sensitivity to glycyrrhizin, sensitivity to adverse effects in women is more than in men (Bernardi *et al.*, 1994; De Klerk *et al.*, 1997)

Clearance (CL), area under curve (AUC), plasma concentration (Cp), glycyrrhetinic acid (GA).

adverse effects. Furthermore, consumption of licorice is contraindicated during pregnancy and for patients with liver disorders, hypokalemia like those who are taking cardiac glycosides. The aldosterone effects of licorice root may counteract antihypertensive action of prescribed medications (Cassileth and Barazzuol, 2001). Recently, a direct interaction of glycyrrhetinic acid absorption with sennosides and its derivatives has been studied in humans (Mizuhara *et al.*, 2005). Some drug interactions of licorice which have been reported are summarized in Table 9.

CONCLUSION

In summary, licorice is used throughout the world as a traditional herbal remedy. As for the properties of licorice and its active constituents, it is suggested that their potential roles are evaluated for their effects in the treatment of different kinds of disease such as cancer, atherosclerosis, immunodeficiency, hormone deficiency endocrine and skin diseases. However, it is necessary to carry out further studies to confirm these effects.

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Chapter 5
Implications of Acetaldehyde-Derived DNA Adducts for Understanding Alcohol-Related Carcinogenesis

Silvia Balbo and Philip J. Brooks

Abstract Among various potential mechanisms that could explain alcohol carcinogenicity, the metabolism of ethanol to acetaldehyde represents an obvious possible mechanism, at least in some tissues. The fundamental principle of genotoxic carcinogenesis is the formation of mutagenic DNA adducts in proliferating cells. If not repaired, these adducts can result in mutations during DNA replication, which are passed on to cells during mitosis. Consistent with a genotoxic mechanism, acetaldehyde does react with DNA to form a variety of different types of DNA adducts. In this chapter we will focus more specifically on N^2-ethylidene-deoxyguanosine (N^2-ethylidene-dG), the major DNA adduct formed from the reaction of acetaldehyde with DNA and specifically highlight recent data on the measurement of this DNA adduct in the human body after alcohol exposure. Because results are of particular biological relevance for alcohol-related cancer of the upper aerodigestive tract (UADT), we will also discuss the histology and cytology of the UADT, with the goal of placing the adduct data in the relevant cellular context for mechanistic interpretation. Furthermore, we will discuss the sources and concentrations of acetaldehyde and ethanol in different cell types during alcohol consumption in humans. Finally, in the last part of the chapter, we will critically evaluate the concept of carcinogenic levels of acetaldehyde, which has been raised in the literature, and discuss how data from acetaldehyde genotoxicity are and can be utilized in physiologically based models to evaluate exposure risk.

Keywords Acetaldehyde • DNA adducts • N^2-ethyldeoxyguanosine • Upper aerodigestive tract cancers

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5.1 Introduction

The designation of alcohol as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC) represented an important change in our understanding of the health effects of alcohol consumption [1]. While previous IARC working groups had classified the carcinogenicity of alcoholic beverages, they left open the possibility that the carcinogenic effects resulted from contaminants in the alcoholic beverages, rather than alcohol itself. Thus, the important question of the carcinogenicity of alcohol per se was not definitively addressed. The notable aspect of the 2007 working group meeting was that alcohol (ethanol) itself was identified as carcinogenic to humans [2]. This classification therefore allows the scientific community to focus on the mechanistic question of how alcohol in alcoholic beverages increases the risk of cancers at certain sites in the body. Given the diversity of target tissues for alcohol-related carcinogenicity (liver, female breast, colorectum, upper aerodigestive tract), it is possible, and indeed likely, that different mechanisms are involved at different target tissues.

An obvious possible mechanism for the carcinogenicity of alcohol, at least in some tissues, involves the metabolism of ethanol to acetaldehyde. Redressing an oversight from the 2007 monograph, the 2009 IARC working group concluded that "acetaldehyde associated with the consumption of alcoholic beverages is carcinogenic to humans (Group 1)" [3]. This conclusion was based in a large part on the dramatically elevated risk of esophageal cancer from alcohol drinking in individuals who are unable to metabolize acetaldehyde due to a genetic variant in ALDH2 [4–6]. Based on these and other data, the strongest evidence for a causative role for acetaldehyde is for alcohol-related cancers of the UADT. The UADT includes the oral cavity, larynx, pharynx, and esophagus.

It is worth emphasizing here that the IARC Group 1 classification specifically applies to acetaldehyde *associated with the consumption of alcoholic beverages*. Acetaldehyde alone remains classified as Group 2b, possibly carcinogenic to humans. We will return to this topic in the last part of this chapter focusing on carcinogenic levels of acetaldehyde.

Broadly speaking, there are two mechanistically different types of carcinogens: genotoxic and non-genotoxic [7]. Genotoxic carcinogens react directly to chemically modify the DNA, resulting in the increased rate of mutagenesis and therefore increased rate of carcinogenesis. Well-known examples of genotoxic carcinogens are ultraviolet light, components of cigarette smoke, and aflatoxin. In contrast, non-genotoxic carcinogens increase the risk of cancer by mechanisms that do not involve direct DNA damage. Examples of non-genotoxic carcinogenic mechanisms include inflammation, which can result in DNA damage from inflammatory mediators, and hormone-like effects. The two mechanisms are not mutually exclusive. Notably, the IARC carcinogen classifications encompass both genotoxic and non-genotoxic agents. As stated in the preamble to the IARC *Monographs: "*… an agent is termed 'carcinogenic' if it is capable of increasing the incidence of malignant neoplasms, reducing their latency, or increasing their severity or multiplicity." This broad and mechanism-independent aspect of the IARC classification system is intentional;

"The aim of the *Monographs* has been, from their inception, to evaluate evidence of carcinogenicity at any stage in the carcinogenesis process, independently of the underlying mechanisms."

From a mechanistic standpoint, genotoxic and non-genotoxic mechanisms may have different time courses and other significant differences with practical implications for risk assessment and disease prevention (e.g., linear risk extrapolation versus thresholds for exposure; see [7]). Therefore, the main focus of this chapter will be on efforts made and strategies developed to assess the role of direct genotoxicity in the carcinogenic effect of acetaldehyde. Consistent with a genotoxic mechanism, acetaldehyde can react with DNA to form a variety of different types of DNA adducts. Since the general topic of acetaldehyde-DNA adducts was covered in a recent review [8], here we will focus more specifically on the major DNA adduct formed from the reaction of acetaldehyde with DNA, N^2-ethylidene-deoxyguanosine (N^2-ethylidene-dG) and highlight recent data on the measurement of this DNA adduct in the human body after alcohol exposure. Because the results are of particular biological relevance for alcohol-related cancer of the UADT, we will also focus on the histology and cytology of these target tissues, with the goal of placing the adduct data in the relevant cellular context for mechanistic interpretation. We also discuss the sources and concentrations of acetaldehyde and ethanol in different cell types during alcohol consumption in humans. Finally, we will critically evaluate the concept of carcinogenic levels of acetaldehyde, which has been raised in the literature [9–11], and discuss how data from acetaldehyde genotoxicity are utilized to identify exposure risk.

5.2 DNA Adducts from Acetaldehyde and Alcohol

Acetaldehyde's genotoxic effect is attributable to its reactivity. The electrophilic nature of its carbonyl carbon results in reactions with DNA, generating DNA adducts [12]. The main reactions occur with deoxyguanosine (dG) followed by deoxyadenosine (dA) and then deoxycytosine (dC) [6, 13]. The binding of acetaldehyde to these nucleosides leads principally to the formation of a Schiff base on the exocyclic amino groups. The resulting imines are unstable at room temperature and neutral pH. However, these compounds can be stabilized using reducing agents, ultimately resulting in ethyl-adducts which are then easier to detect and to quantify.

The most abundant and well-studied acetaldehyde-DNA adduct is N^2-ethylidene-dG which can be stabilized by reduction to N^2-ethyl-dG. These adducts are illustrated in Fig. 5.1.

The instability of N^2-ethylidene-dG prevents direct investigation of its biological properties. In contrast, N^2-ethyl-dG is stable in aqueous solution, as well as under the conditions used for automated oligonucleotide synthesis. Therefore, most of the experimental data available for the biological effects of N^2-ethylidene-dG are inferred from experiments using N^2-ethyl-dG as a stable analog. This is a common approach in the field of DNA damage and mutagenesis. For example, abasic sites in DNA,

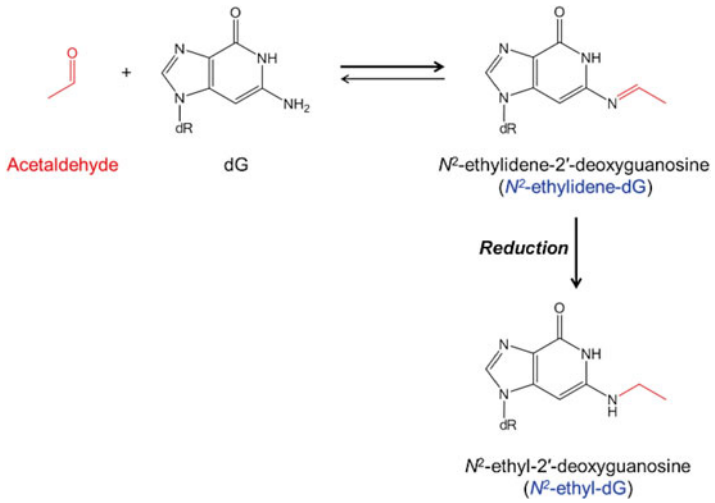

Fig. 5.1 The reaction of acetaldehyde with deoxyguanosine results in the formation of *N2-ethylidendene-dG*; this unstable Schiff base can be converted through a reduction step to the more stable form: N^2-ethyl-dG (dR = 2′-deoxyribose)

which result from depurination, are one of the most common forms of endogenous DNA damage [14]. Because authentic abasic sites are unstable, much of the information we have about their biological effects is derived from studies of tetrahydrofuran as a structural analog [15].

Studies in vitro indicate that N^2-ethyl-dG does not significantly inhibit the replicative DNA polymerase delta [16]. The effects of the lesion on the other major replicative DNA polymerase, epsilon, have not been directly assessed. However, in vivo studies in mammalian cells indicate that the lesion does block replication but is weakly mutagenic, causing primarily −1 frameshift deletion mutations [17–19]. In light of the discussion above, however, it is important to carefully evaluate the limitations of N^2-ethyl-dG as a structural analog.

Figure 5.2 shows energy-minimized models of an N^2-ethylidene-dG paired with dC, an N^2-ethyl-dG paired with dC, and an unmodified dG paired with dC. At first glance, all three models appear similar. The ethyl/ethylidene moiety can be accommodated in the minor groove, with no structural impediment to the guanosine base forming Watson–Crick type H bonds with the appropriate atoms on dC. However, one notable difference is that while dG and N^2-ethyl-dG each form three H bonds with dC, N^2-ethylidene-dG can only form two H bonds. The missing H atom is due to the presence of a double bond between the nitrogen atom and the carbon from acetaldehyde. Viewed from this perspective, the formation of N^2-ethylidene-dG essentially results in G:C base pair with the stability of an A:T base pair.

Studies of frameshift mutagenesis in experimental systems have documented that runs of A:T base pairs are prone to frameshift mutations resulting from a template dislocation mechanism [20]. This observation has generally been explained

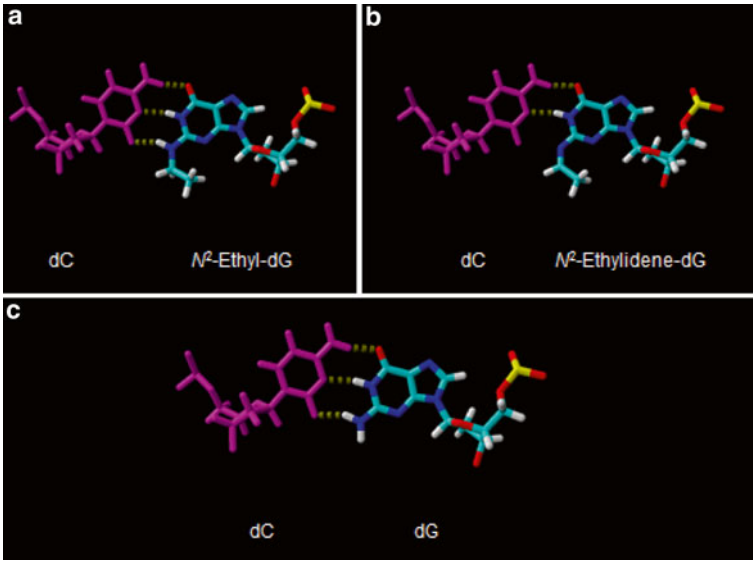

Fig. 5.2 Energy-minimized models showing the difference in base pairing of N^2-ethylidene-dG and N^2-ethyl-dG compared to deoxyguanosine. *Panel A*: the model shows N^2-ethylidene-dG paired with deoxycytosine (dC). *Panel B*: the model shows N^2-ethyl-dG paired with dC. *Panel C*: the model shows an unmodified dG paired with dC

by the reduced stability of A:T base pairs, which increases the probability of primer-template misalignment resulting in frameshift mutations. Adapting this model to N^2-ethylidene-dG in a run of G:C base pairs, an analogous mechanism for frameshift mutagenesis can be hypothesized (see Fig. 5.3). The important point is that it would not be possible to test this hypothesis using N^2-ethyl-dG as a model, because N^2-ethyl-dG forms three H bonds with dC (as shown in panel A of Fig. 5.2). However, this hypothesis could be tested in vivo, perhaps using a yeast strain with run of G:C base pairs in a mutational reporter gene. It should be pointed out, however, that runs of G:C base pairs are generally refractory to frameshift mutagenesis due to their inherent stability.

5.2.1 Other Acetaldehyde-DNA Adducts

In addition to N^2-ethylidene-dG, the most well-studied acetaldehyde-related DNA adducts are the crotonaldehyde-derived propano-dG (CrPdGs) adducts [21]. The condensation of two molecules of acetaldehyde can also produce a reactive electrophile, 3-hydroxybutanal (crotonaldehyde), which can also form a Schiff base on the same amino group of dG. These CrPdG adducts can have multiple biologic effects as a result of their ability to undergo a ring opening reaction. Ring opening yields another aldehyde moiety which can react with proteins to form DNA-protein

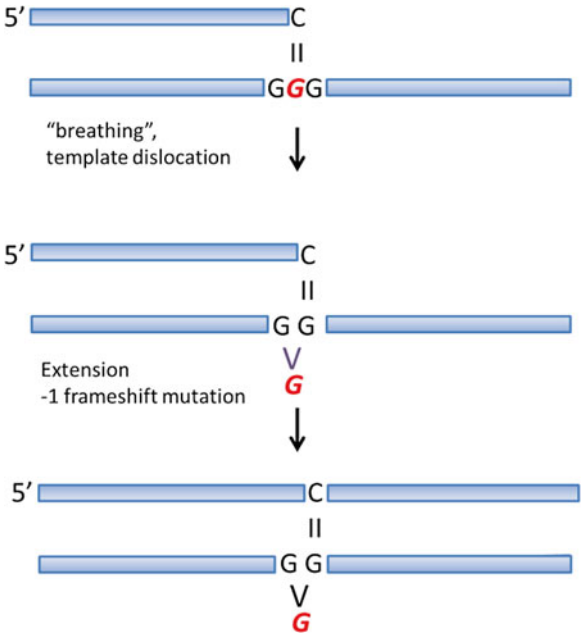

Fig. 5.3 Mechanisms of frameshift mutagenesis hypothesized for N^2-ethylidene-dG

cross-links or (in some sequence contexts) with deoxyguanosine on the opposite strand to form DNA-interstrand cross-links. The biological effects of these adducts have been reviewed recently [8].

It is also worth mentioning the early studies of Fraenkel-Conrat and colleagues, who showed that ethanol and acetaldehyde in combination could react with DNA bases to generate mixed acetal DNA adducts [22]. However, these adducts were very unstable, at least under the in vitro conditions investigated. As such, the biologic significance of these adducts, if any, is currently unclear.

In summary, acetaldehyde has been shown to form several DNA adducts, including N^2-ethylidene-dG and the CrPdG adducts. Under in vivo conditions, which are of the most direct relevance to human carcinogenesis, N^2-ethylidene-dG has been the most well studied, and therefore, we review these studies in the following paragraphs.

5.3 N^2-Ethylidene-dG as a Biomarker of DNA Damage Resulting from Acetaldehyde Derived from Ethanol

As mentioned above, the major reaction of acetaldehyde with DNA occurs on the exocyclic amino group of guanine forming N^2-ethylidene-dG. This adduct is stable in DNA but it easily breaks down when released as a nucleoside.

Fang et al. were the first to report the detection of this adduct, as its reduced form N^2-ethyl-dG, in leukocyte DNA of alcoholics. In their work a ^{32}P-postlabelling method was used for the adduct quantitation. Only samples from heavy drinkers showed detectable amounts of N^2-ethyl-dG likely resulting from the reduction of N^2-ethylidene-dG by endogenous reducing agents such as ascorbic acid and glutathione [23].

In order to avoid the degradation of N^2-ethylidene-dG during DNA hydrolysis and increase the sensitivity of the method, a new approach was developed by Wang et al. [24]. A reducing agent, $NaBH_3CN$, was introduced prior to DNA hydrolysis. Additionally, a stable isotope dilution method was used for the quantitation of the DNA adduct by liquid-chromatography-electrospray ionization-tandem mass spectrometry-selected reaction monitoring (LC-ESI-MS/MS-SRM). The use of this new method allowed a selective detection of N^2-ethyl-dG and an accurate quantitation. By reducing the degradation of the adduct through its conversion into a more stable compound, N^2-ethyl-dG was detected in DNA from all samples analyzed. This resulted in an increased sensitivity which allowed for the detection of lower levels of the DNA adduct, expanding its application beyond the quantitation in samples from heavy drinkers, and set the stage for the broader use of this adduct as a marker for acetaldehyde-induced DNA damage. Since then, N^2-ethyl-dG has been measured in DNA from various samples, for the investigation of the effects on DNA of acetaldehyde from different sources [25, 26].

N^2-Ethyl-dG has been used successfully to measure ethanol-induced DNA damage in HeLa cells expressing ADH1B, which corresponded to the activation of the Fanconi anemia-breast cancer susceptibility (FA-BRCA) DNA damage response network [27]. In a different study, the same DNA adduct was quantified to investigate ethanol-induced DNA damage in the brain of ethanol-treated mice. Higher levels of N^2-ethyl-dG were observed in brain DNA from mice exposed chronically and acutely to ethanol compared to controls [28]. These examples demonstrate that N^2-ethyl-dG is an extremely valuable tool for the investigation of DNA damage associated with acetaldehyde exposure from alcohol consumption and thus for the investigation of alcohol-related mechanisms of carcinogenesis.

The measurement of levels of N^2-ethyl-dG has indeed been crucial in studies focusing on the investigation of effects of alcohol exposure in ALDH2 deficiency. Several studies have used this adduct to detect the DNA damage induced by ethanol exposure in wild-type and *Aldh2* knockout mice, used as a model for *ALDH2* deficiency in humans. Increased levels of the adduct have been found in the liver, esophagus, tongue, and submandibular gland DNA of *Aldh2* knockout mice exposed to ethanol [29, 30]. These findings, together with the results from a study showing increased levels of N^2-ethyl-dG in peripheral blood of ALDH2-deficient alcoholics [31], contributed substantially to the evidence supporting an acetaldehyde-mediated mechanism in alcohol-related carcinogenesis. Together with the epidemiological data showing a dramatic increase of risk for esophageal and head and neck cancers in ALDH2-deficient drinkers, the results from the DNA damage studies contributed to the classification of acetaldehyde related to alcohol consumption as carcinogenic to humans (Group 1) by the IARC [3].

5.3.1 Experimental Studies of Acetaldehyde-DNA Adduct Formation from Alcohol Drinking in Humans

The studies described above clearly demonstrate that increased levels of acetaldehyde-DNA adducts can be observed in animals exposed to ethanol and in human alcohol abusers. However, the studies do not address the minimal amount of ethanol exposure necessary to increase DNA adduct levels or the time course or persistence of the adducts. Moreover, previous studies have not specifically investigated acetaldehyde-DNA adduct formation in humans in a known target tissue for alcohol-related carcinogenesis. Overall, little is known about the formation and lifetime of DNA adducts in the human body. Experimental studies have shown that with constant dosing a steady state concentration of DNA adducts will occur, where the number of new adducts formed equals the number of adducts lost due to repair or instability. However, repair processes vary depending on the cell type and remove different adducts with various efficiencies. Consequently, the lifetime of DNA adducts in vivo can be highly variable according to the tissue or cell type in which they are formed [32]. For instance, easily accessible surrogate tissues such as buccal cells or peripheral blood cells have very different lifetimes. In particular peripheral blood white cells include large cell subpopulations with major differences in lifespan. Lymphocytes are long-lived cells with a life span up to several years, while neutrophils are extremely short-lived cells with a life span of 2–3 days. The quantitation of N^2-ethylidene-dG in these cell types could potentially reflect very different exposure effects and provide very different information on the formation, accumulation, and elimination of the DNA adduct. Therefore, to address these important questions, Balbo et al. performed a biomonitoring study on human subjects before and after consumption of increasing amounts of ethanol [33, 34].

Ten healthy volunteers were recruited. Subjects were required to refrain from any alcohol consumption other than that administered for the study, starting from 1 week prior to the beginning of the experiment and throughout its entire duration. Three increasing alcohol doses were administered during the experiment, one dose a week, starting from the lowest. The alcohol dose administered was selected taking into account gender and weight in order to target specific blood alcohol levels, all below intoxication (defined as a blood alcohol level of 0.08 % [35]). The 3 doses selected for the study can roughly be described as corresponding to 1, 2, and 3 vodka drinks per subject. Overall, the alcohol doses had an ethanol content that ranged between 20 and 50 g which corresponded to an ethanol concentration in the drink ranging between 1.5 and 2.5 M. These concentrations ultimately resulted in a final blood alcohol concentration in the range of 0.01–0.02 M.

Levels of N^2-ethyl-dG were measured in DNA isolated from oral cells collected with a nonalcoholic mouthwash and from white blood cells. Granulocytes and lymphocytes were isolated from the blood to test potential effects of different cellular life span and repair mechanisms. A sample was collected before drinking to establish a baseline and then at several time points after exposure to each dose (2, 4, 6, 24, 48, and 120 h) to assess the kinetics of adduct formation and disappearance.

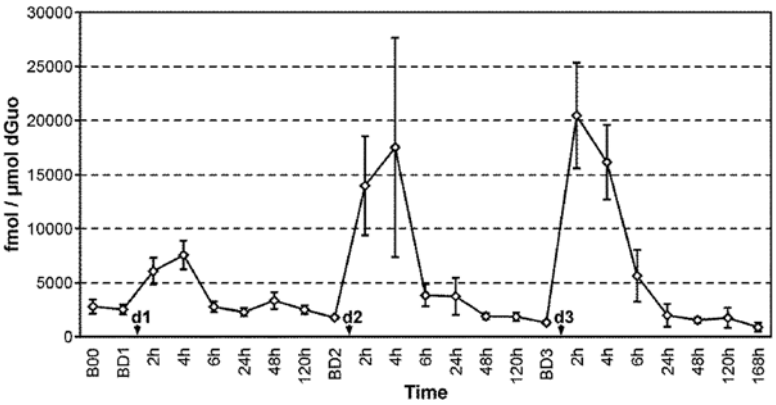

Fig. 5.4 Summary of the results obtained from a study investigating the effects on oral cell DNA of consumption of increasing doses of alcohol. The study was performed on samples collected form ten subjects who abstained from drinking any alcoholic beverage other than the doses provided over the entire duration of the study. The graph reports the mean levels of N^2-ethyl-dG (fmol/µmol dG) measured in oral cell DNA at various intervals before and after three increasing alcohol doses. The first time point reported on the *left* (B00) refers to 1 week before consumption of the first alcohol dose. Starting from this time point, participants began to abstain from consuming any alcoholic beverage. The next time point (BD1) refers to the baseline level detected 1 week later, 1 h before consumption of the first dose (d1, lowest). Subsequently, the graph shows the levels of N^2-ethyl-dG measured at the various time points considered after each dose (2–120 h). The DNA adduct levels were measured at the same time points before and after exposure to the next two doses (d2, intermediate, and d3, highest). Levels of the adduct increased 2 h after exposure even after consumption of the lowest dose and returned to baseline 24 h after exposure. A clear dose–response effect of alcohol on N^2-ethyl-dG levels was found. The baseline time points measured 1 h before the dose (BD1–BD3) are 7 days apart. Values are means and SEs. Data from reference [33, 34]

Considering the results from the oral cavity first, as shown in Fig. 5.4, increased levels of N^2-ethyl-dG were already detected at 2 h after alcohol consumption and reached a peak between 2 and 6 h. Interestingly, adduct levels had returned to baseline after 24 h, indicative of either DNA repair or cell turnover. We will return to this point in the discussion of the histology of the oral epithelia (discussed in detail below). Most importantly, peak adduct levels showed a clear dose–response relationship to the amount of alcohol consumed.

A different pattern of adduct formation was observed in white blood cells after alcohol drinking. Quantitation of the DNA adduct in granulocytes and lymphocytes did not show a major difference between the two cell types. An increase after the alcohol doses was detected, but in contrast to the oral cavity, no clear dose response was observed. Additionally, the high baseline levels and the high intra- and interindividual variability did not allow the clear identification of an effect directly attributable to the alcohol dose.

To our knowledge, this is the first study to investigate the effects of alcohol consumption on the time course of DNA adduct formation in healthy volunteers. All previous published studies on N^2-ethyl-dG levels in humans were done on heavy drinkers or alcoholics [23, 29]. Furthermore, no information on the persistence of

this specific modification was reported. These results clearly demonstrate that even a single drink of alcohol results in a significant and dose-dependent increase in acetaldehyde-DNA adducts in cells in the human oral cavity, a known target tissue for alcohol-related carcinogenesis.

While N^2-ethylidene-dG is the major adduct formed after reaction of acetaldehyde with DNA, as mentioned above, several other adducts can result from acetaldehyde reactions with DNA, although generally they are formed in lower yield. Because of its high levels, N^2-ethylidene-dG is easier to detect and measure and thus could be considered as a general indicator of exposure of DNA to acetaldehyde and a proxy for detection of other DNA modifications. Consequently, these results provide a good starting point for future studies focusing on mapping multiple acetaldehyde-derived DNA modifications and investigating their potential role in the carcinogenic process.

These findings demonstrate the utility of oral cell DNA for the investigation of the role of alcohol-related DNA adducts in head and neck carcinogenesis. These observations support the hypothesis that alcohol drinking increases the risk of oral cancer via a mechanism involving a genotoxic effect of acetaldehyde. Before exploring this question in more detail, however, it is necessary to put the results from Balbo et al. into the relevant cellular context. For this purpose, below we briefly review the anatomy and histology of the oral cavity and esophagus.

5.3.2 Anatomical Considerations

A fundamental aspect of genotoxic carcinogenicity is the formation of mutagenic DNA adducts in proliferating cells. If left unrepaired, these adducts can result in mutations during DNA replication, which are passed on to daughter cells during mitosis. DNA adducts that form in terminally differentiated cells (G0) do not directly contribute to carcinogenesis, because of the absence of DNA replication. As such, it follows that a key issue for interpreting the relationship between DNA adduct formation and carcinogenesis is the cell type in which the DNA adduct formed.

The oral cavity and esophagus are both squamous epithelial tissues, in which cells in the upper layers are continuously replaced by new cells generated in the lower layers (for review see [36]). A schematic representation of these tissues is shown in Fig. 5.5. As indicated in the figure, proliferating cells (i.e., cells that replicate their DNA) are located in the basal and suprabasal layers of the epithelium. After differentiation, cells move up toward the surface of the epithelial layer. During transit, these cells flatten out (desquamate) and are ultimately sloughed off into the saliva or lumen of the esophagus. The time for newly generated cells in the oral cavity to transit from the basal layer through the surface of the (non-cornified) epithelial layer has been estimated at roughly 4 days (in rabbits) [22, 37]. As the DNA labeling index of cells in the basal layers of humans and rabbits is similar, the 4-day transit time is likely to be a reasonable estimate for humans as well.

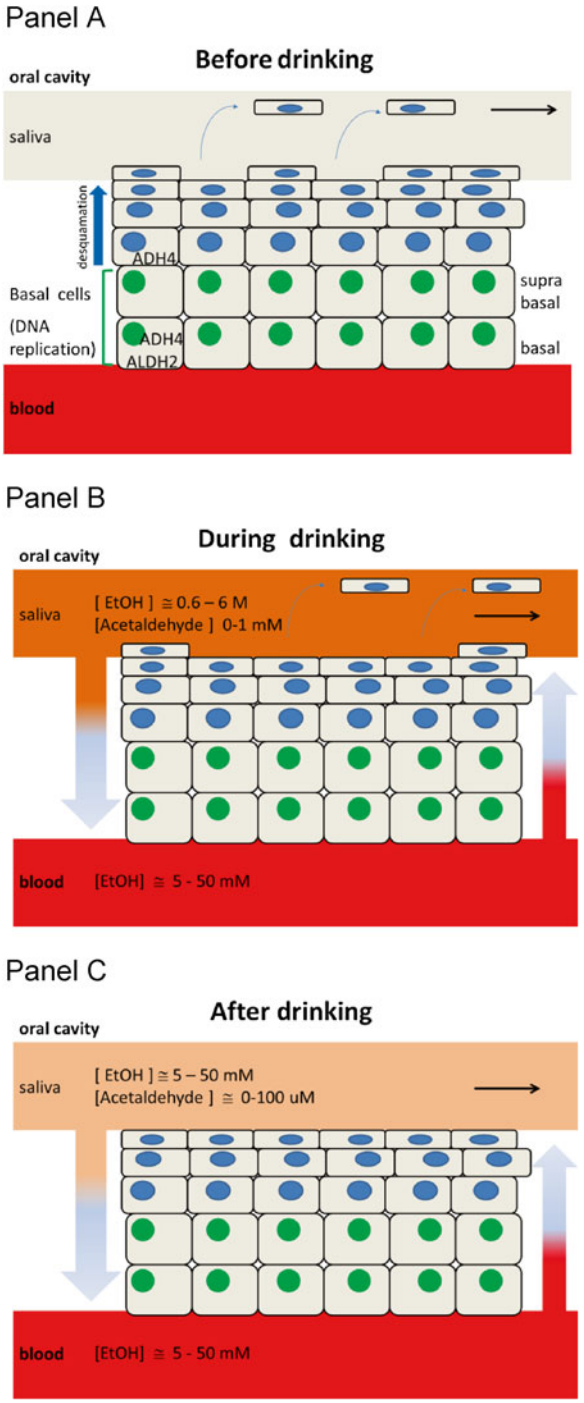

Fig. 5.5 Schematic representation of the squamous epithelial tissue of the oral cavity before, during, and after alcohol drinking. *Panel A* shows the various cell layers: cells in the upper layers are continuously replaced by new cells generated in the lower layers, the basal and suprabasal layers of the epithelium, where proliferating cells (i.e., cells that replicate their DNA) are located. After differentiation, cells move up toward the surface of the epithelial layer. During transit, these cells flatten out (desquamate) and are ultimately sloughed off into the saliva. *Panel B* shows the levels of exposure to alcohol and to acetaldehyde of the various levels of the epithelium when drinking alcohol. In addition to high concentrations of ethanol and acetaldehyde diffusing from the epithelial surface downward into the deeper cell layers (*brown arrow*), some alcohol reaches the blood stream from where it can diffuse into epithelial cells (*red arrow*), allowing metabolism to acetaldehyde in situ. Acetaldehyde levels in the blood are very low and considered negligible for this model. *Panel C* illustrates the levels of exposure to ethanol and acetaldehyde of the various layers of the epithelium after alcohol drinking when the ethanol concentration levels between the saliva and the blood stream reach the equilibrium

When drinking alcohol, the oral cavity and esophagus are transiently exposed to alcohol and acetaldehyde concentrations that are essentially the same as those in the beverage itself. While the time of exposure is only on the order of seconds, the alcohol and acetaldehyde concentrations can be very high. For example, the concentration of ethanol in hard liquor (100 proof) is roughly 7–8 M, in wine approximately 2 M, and in beer between 500 and 700 mM. Acetaldehyde concentrations in alcoholic beverages can vary between undetectable levels (vodka) to roughly 200 mM or more, depending on the beverage [38, 39]. By 30 min after alcohol drinking, salivary ethanol levels have largely equilibrated with blood levels [40, 41].

In addition to the saliva and alcoholic beverage, acetaldehyde is generated within esophageal epithelial cells as a result of ethanol metabolism. Cells of the upper GI tract including the esophagus express ADH7, as opposed to ADH1 proteins that are expressed in the liver. Compared to ADH1, which has a low Km for ethanol oxidation (on the order of 1 mM), the Km of ADH7 is substantially higher (around 25 mM) [42]. However, as noted above, the concentration of ethanol in alcoholic beverages can be in the molar range, which would clearly saturate the enzyme. Moreover, blood alcohol levels during alcohol intoxication could reach 25 mM, or even higher concentrations with heavy drinking, which would result in substantial metabolism to acetaldehyde by ADH7 in epithelial cells. Notably, the catalytic activity of ADH7 (K_{cat}) for acetaldehyde production is 1–2 orders of magnitude higher than ADH1 [42].

Returning to the study of Balbo et al., the use of a mouthwash to obtain cells for analysis would primarily collect those terminally differentiated epithelial cells that were at the surface of the epithelial cell layer, in contact with the contents of the oral cavity, including those cells in the process of sloughing off (see Fig. 5.5 panel A). Therefore, acetaldehyde-DNA adduct levels in these cells does not directly monitor adduct levels in the proliferating cell layers that are of greatest relevance to carcinogenesis.

An important aspect of the Balbo et al. findings is the reduction in adduct levels over time, reaching baseline by 24 h after alcohol drinking. Since the half-life of N^2-ethylidene-dG in DNA at 37 °C is 24 h, the return of adduct levels cannot be completely explained by spontaneous adduct loss. If so, there are at least two possible explanations for the return of adduct levels to baseline. One possibility is that the adducts were repaired via DNA repair. While neither base excision repair nor direct repair have been shown to be able to remove N^2-ethyl-dG (used as a surrogate for N^2-ethylidene-dG), it is possible that the nucleotide excision repair mechanism could remove the lesion. Another possibility is that the decline in adduct levels over time reflects changes in the cell population being sampled. As shown in Fig. 5.5, the epithelium is not a static cell population, but one in which cells are born, differentiate, and depart over time, in a directional manner. As such, the cells collected at the 24 h time point would have been in a different physical location relative to the epithelial surface during the alcohol drinking and immediately afterwards, when salivary acetaldehyde levels would be highest. Therefore, to the extent that N^2-ethylidene-dG adducts were the result of salivary acetaldehyde formation, the cells collected at the 24 h time point may have been at least partially protected from DNA damage by the overlying cells.

The two possibilities are not mutually exclusive, and both could be assessed experimentally. The role of NER could be readily tested by exposing normal and NER-deficient human cells to acetaldehyde, then assaying the disappearance of N^2-ethylidene-dG adducts over time in the two cell types. Assessing the kinetics of adduct formation in different cell layers of the oral epithelium in vivo is more technically challenging. Theoretically, antibodies against acetaldehyde-DNA adducts could be developed, which might be useful for a semiquantitative assay of adduct levels in different cell types with human biopsies. However, the sensitivity and specificity of such antibodies are difficult to ensure. Alternatively, the proliferating cell layer could be dissected out for adduct analysis (e.g., by laser capture microdissection), but the amount of DNA would be insufficient for analysis by mass spectrometry given the sensitivity of the technique currently. Conceivably, cell types of interest could be separated in bulk using cell-sorting techniques (e.g., [43]). However, this would require amounts of tissue that could not be obtained from humans.

Regarding animal models, it is important to note here that there is a major species difference in the K_m of ethanol oxidation by ADH7 between humans, mice, and rats. Specifically, while as noted above the Km of the human ADH7 for ethanol is approximately 25 mM [42], that of the mouse ADH7 homolog is roughly 200 mM [44], and for the rat is >2 M [45]. Therefore, to the extent that ethanol metabolism in oral or esophageal epithelial cells plays an important role in alcohol-related acetaldehyde-DNA adduct formation, the major difference in ADH7 for ethanol metabolism is a significant limitation of either rodent species as an animal model of humans.

5.4 Why Are ALDH2-Deficient Individuals at Such Elevated Risk of Esophageal Cancer from Drinking Alcohol?

The dramatically elevated risk of esophageal cancer in individuals with deficient ALDH2 is well known [46, 47]. However, the question of why these individuals are at such elevated risk of esophageal cancer, as opposed to other types of cancers, is not well understood. It has been shown that ALDH2-deficient individuals experience higher levels of salivary acetaldehyde from ethanol [48, 49] Importantly, this difference is only detectable when ethanol is actually ingested [50]. However, it is not clear that the difference in acetaldehyde concentrations in the saliva during alcohol drinking can fully explain the dramatically elevated esophageal cancer risk. In this context, a crucially important question concerns the localization of the ALDH2 enzyme in cells of the esophageal epithelium. Yin et al. [51] reported that ALDH2 activity in homogenates of the human esophageal mucosa, as measured on agarose gels, was barely detectable. Using standard enzymes assays, "low K_m ALDH" (assayed at 200 μM acetaldehyde) was reported to be less than 10 % of the high K_m form. However, these studies did not completely exclude the possibility of ALDH2 expression in a population of cells in the esophagus.

In contrast to biochemical studies, immunohistochemical studies of ALDH2 in the human esophagus [50, 52] did in fact detect ALDH2 staining in a subset of cells,

specifically cells localized to the basal cell layer where proliferating cells are found. The ALDH2 antibody used in this work was developed by Weiner and colleagues [53] and had been validated using tissues from knockout mice lacking Aldh2. In support of these findings, data available on a public database shows that ALDH2 mRNA can be detected in the human esophagus (http://www.ncbi.nlm.nih.gov/geoprofiles/83899354) [54]. Interestingly, the staining intensity varied depending upon the drinking history of the tissue donors: the strongest staining was more often observed in samples from individuals with a history of alcohol drinking. These observations raise the intriguing possibility that ALDH2 expression may be inducible by heavy alcohol drinking in the human esophagus. Consistent with these observations and the idea that ALDH2 can be inducible, ALDH2 expression can be increased by low pH in a human esophageal cell line (http://www.ncbi.nlm.nih.gov/geoprofiles/11619953) [55].

Taken together, these data indicate that ALDH2 is expressed in proliferating cells of the human esophagus, where it could play a role in protecting genomic DNA against acetaldehyde generated from ethanol metabolism by ADH7 in situ. If so, then the absence of this activity in proliferating esophageal cells of ALDH2-deficient alcoholics would provide a compelling explanation for the dramatically elevated risk of esophageal cancer from high levels of alcohol drinking in this population.

One final point to be made here is that the proliferating cell layer of the oral and esophageal epithelium is not static. Under normal conditions, cell proliferation and differentiation are balanced to maintain the structure and function of the epithelium. However, in response to wounding or damage, the balance between proliferation and differentiation can shift to regenerate the damaged tissue [56]. An important study by Salaspuro and colleagues [57] in fact demonstrated that chronic exposure of rats to acetaldehyde in the drinking water does increase the size of the proliferating cell compartment in oral and gastrointestinal epithelia, as measured by the thickness of the epithelial layer and number and depth of cells staining for the proliferation maker Ki67. While the concentration of acetaldehyde in the drinking water used in that work (120 mM) is far in excess of what would be considered clinically relevant during alcohol drinking in humans, as noted by the authors, acetaldehyde is highly volatile (boiling point 24 °C) and would therefore likely diffuse from the bottles, reducing the actual concentration. Also, the bottles were only changed every three days. Therefore, the actual acetaldehyde concentration that tissues were exposed to from the drinking water was likely to be far less than 120 mM, especially by the third day after bottle change. Also, as shown in Fig. 5.5b, during alcohol drinking in humans, acetaldehyde can be generated intracellularly from ethanol metabolism at high blood ethanol concentrations, and these intracellular levels could be quite high, especially in ALDH2-deficient individuals. This issue could be addressed in the laboratory using cells expressing human ADH7, exposed to different ethanol concentrations spanning the range that could be generated in the blood during heavy alcohol drinking in humans.

The expansion of the proliferative cell compartment and hyperregeneration as a result of the toxic or damaging effects of acetaldehyde derived from ethanol metabolism (see [58, 59]) would be examples of non-genotoxic mechanisms for

acetaldehyde-related carcinogenesis. However, these mechanisms could synergize with genotoxicity, in that more proliferating cells with mutagenic DNA adducts essentially expand the target tissue for carcinogenesis. It is therefore likely that acetaldehyde acts as both a genotoxic and non-genotoxic carcinogens in the human UADT.

5.5 Is There a "Carcinogenic" Level of Acetaldehyde?

As noted in the Introduction, the 2009 IARC classification specifically identifies acetaldehyde associated with the consumption of alcoholic beverages as a Group 1 carcinogen. Acetaldehyde alone remains classified in Group 2B (possibly carcinogenic to humans). However, the concept of a "carcinogenic level of acetaldehyde" (variously described as between 50 and 100 μM) has entered the literature [11, 39, 60]. As this term is based upon studies of DNA adduct formation, and genotoxic end points, we would like to briefly discuss its derivation and implications.

One of the studies cited in support of this concept was the work of Theruvathu et al. [61] (one of us, P.J.B., was an author on that paper). This work was intended to address a specific mechanistic question, which was whether polyamines could stimulate the formation of CrPdG adducts in DNA during exposure to acetaldehyde. In that study, Theruvathu et al. incubated purified genomic DNA with increasing concentrations of acetaldehyde, with or without a physiologically relevant concentration of polyamine, at 37 °C for 24 h [61]. While the acetaldehyde concentrations used were within the range that could plausibly occur in the human body during alcohol drinking, the work was not intended to be a basis for establishing a mutagenic or carcinogenic level of acetaldehyde. DNA adduct formation in living cells exposed to acetaldehyde is likely to be much lower than when pure DNA is exposed, due to reactions of acetaldehyde with other cellular molecules, as well as ongoing DNA repair. In fact, it would be important and relevant to assess CrPdG adduct levels in human cells exposed to different acetaldehyde concentrations for different periods of time. Highly sensitive and specific assays for CrPdG adducts in cellular DNA have been developed and could be used utilized for this purpose [31].

Another study that has been cited to support the carcinogenic level of acetaldehyde is the work of Obe and Ristow [62], who investigated the effect of acetaldehyde on sister chromatid exchanges (SCE) in mammalian cells. The lowest concentration that was shown to increase SCE levels in that study was roughly 90 μM. While an increase in SCEs is evidence of genotoxicity, SCEs are not mutations, and their relevance for predicting cancer risk is controversial [63].

A more important issue that is not explicitly considered when discussing carcinogenic levels of acetaldehyde is the histology of the oral cavity. As we have illustrated in Fig. 5.5, the proliferating cells in the oral cavity that are of most relevance to oral cancer lie several layers below the epithelial surface. For this reason, the concentration of acetaldehyde that proliferating cells in the oral epithelium are actually exposed to will be much lower than the levels in saliva, due to the reaction of acetaldehyde with components of the overlying, terminally differentiated cells.

Estimating a carcinogenic acetaldehyde concentration in the oral cavity based upon an acetaldehyde concentration that increases SCEs in monolayer cells in tissue culture fails to take this protective cell layer effect into account.

It is in fact possible to develop valid models of the effect of acetaldehyde on different epithelial tissues, which could be relevant to understand carcinogenic mechanisms, and to provide a basis for risk assessment. Templates for such an approach are physiologically based pharmacokinetic models for vinyl acetate toxicity and carcinogenesis in the oral and nasal epithelium [63]. Importantly, vinyl acetate is metabolized to acetaldehyde and acetic acid in epithelial cells, and therefore, some of the information that was used in developing the vinyl acetate models may be directly applicable to acetaldehyde carcinogenicity as well. As in the case of vinyl acetate, different models should be developed for different tissues, taking into account the expected concentrations of ethanol and acetaldehyde, as well as the time course of exposure, and considering the unique biology of different tissues (e.g., oral cavity versus esophagus versus colorectum).

5.6 Summary and Conclusions

The ability of acetaldehyde to form covalent adducts with DNA is consistent with a genotoxic mechanism for alcohol-related carcinogenicity, but does not prove such a mechanism, nor does it rule out non-genotoxic effects. More data on the kinetics of acetaldehyde-DNA adduct formation and repair in specific cell types in target tissues for alcohol-related carcinogenicity will be important to fully understand carcinogenic mechanisms. Data from humans is of the most direct relevance to human carcinogenesis, and whole genome sequencing of human tumors may provide evidence of genetic signatures corresponding to specific DNA adducts (see [8]). However, given the ethical and practical limitations of collecting relevant tissues from human, data from well-designed animal studies, taking into account relevant species differences as noted above, will also provide important mechanistic insights of relevance not only for alcohol-related carcinogenesis but also to the broader question of risk assessment for acetaldehyde alone.

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ADVANCES IN HEPATOLOGY

Current Developments in the Treatment of Hepatitis and Hepatobiliary Disease

Section Editor: Eugene R. Schiff, MD

Alcoholic Liver Disease

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G&H What is alcoholic liver disease, and how prevalent is it in the United States?

LB Alcohol abuse is the third leading preventable cause of death in the United States, according to the Centers for Disease Control and Prevention. Alcoholic liver disease is one of many conditions associated with alcohol misuse. It has a broad spectrum, ranging from mild fatty changes in the liver to frank cirrhosis of the liver. Nearly all individuals who consume a significant amount of alcohol (ie, >40 grams a day) will develop early fatty changes that can be seen on liver biopsy, and approximately 10% to 20% will eventually go on to develop cirrhosis.

In addition, there is another distinct form of alcoholic liver disease, acute alcoholic hepatitis, which is a lethal and feared complication of alcohol abuse. This condition occurs in approximately 30% to 45% of alcoholics at some point in their lives. This is a dangerous disease in which the 28-day mortality in severe cases can be as high as 50%.

G&H Have there been any recent trends in the epidemiology of alcoholic liver disease?

LB In the United States, the prevalence of alcoholic liver disease is essentially flat, with perhaps a very slow downward trend in the past 5 years or so. In the general population, alcohol has been observed to play a role approximately half of the time in hospital discharges related to cirrhosis, so it is estimated that approximately 50% of cirrhosis cases are alcohol-related.

I work in the Veterans Affairs (VA) health care system, where the incidence, or rate of new cases, of alcohol-related liver disease has been starting to trend downward since approximately 2010, and the overall prevalence (ie, the total number of cases per 100,000 in the general population) has essentially been stable. In the VA health care system, alcohol accounts for approximately 30% of cirrhosis cases, and there is another 30% of patients who have both alcoholic liver disease and another liver disease, such as viral hepatitis. Therefore, in the VA health care system, alcohol is a factor in approximately 60% of all cases of cirrhosis. Other data suggest that this is similar in the general US population as well.

G&H What other consequences and complications are associated with alcoholic liver disease?

LB Alcohol abuse is very costly and causes significant morbidity and mortality. All of the health consequences of alcohol, not just those limited to the liver, were estimated to account for 11% of the total US health care cost in 2006. The annual inpatient cost for alcohol-related cirrhosis, not including outpatient treatment, was approximately $850 million in 2014, according to the National Institute on Alcohol Abuse and Alcoholism. Up to 50% of severe cases of acute alcoholic hepatitis will lead to death, even with medical support.

G&H Are there any ways to counteract these consequences of alcoholic liver disease?

LB Of course, the best way to counteract alcoholic liver disease is prevention. Unfortunately, for US patients who already engage in problematic alcohol use, treatments are

very often limited in terms of access and efficacy. Once cirrhosis or acute alcoholic hepatitis develops, quitting drinking becomes a matter of life and death.

G&H Could you expand on the risk of mortality in these patients?

LB Alcoholic liver disease is the second most common cause of disease in patients who are listed for liver transplant, but listed patients represent a highly selected group, given that patients with active substance use disorders are generally ineligible for transplant listing. In terms of mortality rates, an estimated 18,000 people in the United States died of alcoholic liver disease in 2013, and the death rate is highest in younger and middle-aged individuals. However, these numbers are likely underestimates because alcohol is a cofactor in other liver diseases and therefore may not be accurately counted as a cause of death. For example, in people who have viral hepatitis and alcoholic liver disease, alcohol is often not listed as one of the main causes of death.

G&H Should patients with alcoholic liver disease be screened for other conditions?

LB The American Association for the Study of Liver Diseases recommends that all patients with cirrhosis, including those with alcoholic cirrhosis, be screened regularly for hepatocellular carcinoma. Patients with alcoholic cirrhosis should also receive routine screening for gastroesophageal varices.

In addition, all individuals who are known or suspected to misuse alcohol should undergo additional screening for mental health and psychosocial concerns. This would include asking about comorbid conditions such as nonalcohol substance use, mental illness, and housing insecurity. Management of these comorbidities is very complex and requires multidisciplinary support from other services, such as primary care, mental health, and social work.

G&H What symptoms are associated with this condition? Are any patients asymptomatic?

LB Presentations of patients with alcoholic liver disease can vary. Alcoholic liver disease is frequently asymptomatic in its early stages, which is why health care providers should be vigilant and should screen patients for alcohol use as part of routine practice.

Patients with acute alcoholic hepatitis are often extremely ill, and the condition can manifest as liver failure. Clinically, the classic presentation for acute alcoholic hepatitis is a patient with a history of alcohol abuse who presents with a fever, enlarged liver, and jaundice. Often, the patient also has leukocytosis and moderately elevated transaminase levels.

Finally, there is a subset of patients who have alcohol-related cirrhosis. These patients can be asymptomatic in the early stages, but when they develop decompensated liver disease, they become highly symptomatic.

G&H How is alcoholic liver disease usually diagnosed?

LB Usually, alcoholic liver disease can be diagnosed based on patient history and laboratory test results, especially if the patient has examination findings that suggest chronic liver disease. Sometimes, a liver biopsy is performed if the diagnosis is not clear or if there are multiple disease etiologies suspected. Liver biopsy is still the gold standard for diagnosis. However, because liver biopsy is so invasive and carries a risk of complications, clinical practice is moving toward noninvasive methods of ascertaining liver fibrosis in lieu of liver biopsy.

G&H Other than alcohol abuse, are there any risk factors for alcoholic liver disease?

LB With research on this issue starting to enter the literature, we are developing a better understanding of all of the risk factors for alcoholic liver disease beyond just the amount and duration of alcohol intake. For example, alcoholic liver disease appears to be more likely to occur in women, younger people, and those with poor nutritional status. There are also multiple genetic factors that we are only starting to understand related to how the body breaks down ethanol and how it handles inflammation.

There is an emerging new body of research on the relationship between the gut microbiome, the liver, and alcoholic liver disease. It turns out, interestingly, that alcohol disrupts the mucosal barrier of the gut, which leads inflammatory bacterial products called lipopolysaccharides to enter the blood supply. This causes liver inflammation to accelerate and, if the process spirals out of control, it produces the clinical syndrome of alcoholic hepatitis.

G&H How are these patients treated? Is cessation of alcoholic intake sufficient?

LB Once someone develops acute alcoholic hepatitis, a number of interventions have traditionally been used. An exciting recent trial on this subject was STOPAH (Steroids or Pentoxifylline for Alcoholic Hepatitis), which was a randomized trial of 2 commonly used drugs for alcoholic hepatitis, pentoxifylline and prednisolone. The trial found that neither of these drugs has a sustained benefit

on mortality after 28 days, and only prednisolone reduces mortality before 28 days. Thus, the researchers concluded that pentoxifylline should no longer be used for treatment of acute alcoholic hepatitis.

My personal opinion is that after 28 days, survival really depends on the patient quitting drinking. There is no drug that will beat alcohol abstinence in terms of the long-term impact on patient prognosis. Even so, approximately one-third of patients with alcoholic cirrhosis who quit drinking will be dead in 5 years. Thus, even with alcohol cessation, there is still a significant risk of mortality.

Dr Beste has no relevant conflicts of interest to disclose.

Suggested Reading

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Genotoxicity of visible light (400–800 nm) and photoprotection assessment of ectoin, L-ergothioneine and mannitol and four sunscreens

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Abstract

This study was designed to determine the genotoxic effects of visible (400–800 nm) and ultraviolet A (UVA)/visible (315–800 nm) lights on human keratinocytes and CHO cells. The alkaline comet assay was used to quantify DNA-damage. In addition, photo-dependent cytogenetic lesions were assessed in CHO cells by the micronucleus test. Three protective compounds [ectoin, L-ergothioneine (ERT) and mannitol] were tested with the comet assay for their effectiveness to reduce DNA single-strand breaks (SSB). Finally, the genomic photoprotections of two broad-band sunscreens and their tinted analogues were assessed by the comet assay. The WST-1 cytotoxicity assay revealed a decrease of the keratinocyte viability of 30% and 13% for the highest UVA/visible and visible irradiations (15 and 13.8 J/cm^2, respectively). Visible as well as UVA/visible lights induced DNA SSB and micronuclei, in a dose-dependent manner. The level of DNA breakage induced by visible light was 50% of the one generated by UVA/visible irradiation. However, UVA radiations were 10 times more effective than visible radiations to produce SSB. The DNA lesions induced by visible and UVA/visible lights were reduced after a 1-h preincubation period with the three tested compounds. The maximal protective effects were 92.7%, 97.9% and 52.0% for ectoin (0.1 mM), ERT (0.5 mM) and mannitol (1.5 mM), respectively, against visible light and 68.9%, 59.8% and 62.7% for ectoin (0.1 mM), ERT (0.5 mM) and mannitol (1.5 mM), respectively, against UVA/visible light. Thus, visible light was genotoxic on human keratinocytes and CHO cells through oxidative stress mechanisms similar to the ones induced by UVA radiations. The four tested sunscreens efficiently prevented DNA lesions that were induced by both visible and UVA/visible irradiations. The tinted sunscreens were slightly more effective that their colorless analogues. There is a need to complement sunscreen formulations with additional molecules to obtain a complete internal and external photoprotection against both UVA and visible lights.

Keywords: Visible light; Comet assay; Oxidative damage; In vitro micronucleus; Protective compounds; Sunscreens

1. Introduction

Over the years, changes in lifestyle have led to a significant increase in the human exposition to solar radiations, leading to a dramatic rise in the incidence of skin cancers [1]. Photo-induced cancers are due to a complex multistage phenomenon, mediated via alterations in various cellular mechanisms [2]. Cell initiation is usually considered as the first step in photo-carcinogenesis processes. It essentially consists in irreversible genetic alterations, which ultimately lead to DNA mutations [2]. Ultraviolet (UV) radiations are known to induce DNA lesions, and the implication of ultraviolet A radiations (UVA, 315–400 nm) in these genotoxic damages has been recently proven, although their accurate biological effects are still unclear [3]. UVA radiations represent 95% of natural ultraviolet reaching the Earth's surface. These radiations are

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less energetic than ultraviolet B (UVB, 280–315 nm) but, because of longer wavelengths, can pass through standard glass like window and automotive glasses [4]. They penetrate deeply into the basal layer of the human skin, and may affect highly proliferative cells like keratinocytes and melanocytes [5]. At a molecular level, the biological effects of UVB and UVA are different: UVB are responsible of direct photolesions on DNA, i.e. cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts [6,7], leading, when they are not repaired, to mutagenesis [8]. In contrast, UVA radiations are only weakly absorbed by DNA, thus UVA genotoxicity is mainly mediated by indirect mechanisms: UVA can interact with endogenous chromophores like porphyrins, bilirubin, flavins, melanin and melanin precursors [9] which act as photosensitizers. These molecules may ultimately generate reactive oxygen species (ROS). Moreover, high UVA doses (50–200 J/cm^2), have been shown to generate CPDs, through a triplet photosensitization mechanism that involves unidentified chromophore(s) [10–12]. Cells possess natural antioxidant defense mechanisms to counteract oxidative aggressions but, when these systems become overwhelmed, the reactive species can cause cell injuries and particularly direct damage to DNA [9]. The genotoxic effects of ROS include oxidized bases like 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) [13] and DNA single-strand breaks (SSB) [14]. A long-term exposure to UVA could lead to photoageing, photodermatoses [15,16], immunosuppression [17], and photo-carcinogenesis [18].

Visible light (400–800 nm) has been used for a long time for its therapeutic effects particularly in dermatological diseases [19] and in some localized cancers [20]. However, the photogenotoxic outcomes of visible radiations on cellular DNA are probably underestimated, since UVA and visible radiations are often studied together [21,22]. Some studies have already revealed the genotoxic effects of blue light often used in therapeutic: blue light has been shown to induce DNA SSB, sister chromatid exchanges [23] and intracellular ROS [24]. Visible light could therefore exert an indirect genotoxic effect via oxidative DNA lesions [25].

Chemical protection against photodamage includes (i) the use of protective compounds (internal photoprotection), such as ectoin, L-ergothioneine (ERT) [26] or mannitol [27] to reinforce the antioxidant potential of the cells (ii) the application of broad-spectrum sunscreens (external photoprotection) [28] to reduce the amount of UV radiations reaching the skin. Efficacy of these two approaches can be assessed by using the single cell gel electrophoresis assay, also called the comet assay, which is a simple, reliable and sensitive technique for the evaluation of DNA strand breaks and alkali-labile sites in individual eukaryotic cells [29]. It has been efficiently used to quantify DNA-damage induced by ultraviolet radiations, to assess the genomic photoprotection of sunscreens against UVA radiations [30,31] and to evaluate the photoprotective effectiveness of various antioxidants [32]. Photo-dependent cytogenetic lesions may be evaluated by the micronucleus (MN) test [33,34]. This assay is considered as a sensitive tool to evaluate chromosomal damage induced by chemical or physical agents, leading to clastogenic (breaking) or aneugenic (abnormal segregation) events [35].

In this study, we first evaluated the genotoxic effects of visible light (400–800 nm) by the alkaline comet assay on human normal keratinocytes, and the in vitro micronucleus test on Chinese Hamster Ovary (CHO) cells. Then, the photoprotective capacities of three exogenous compounds (ectoin, ERT and mannitol) and four broad-spectrum sunscreens against UVA/visible and visible irradiations were measured by the comet assay.

2. Materials and methods

2.1. Reagents

Most of the chemicals used for cell cultures, comet assay and micronucleus test were provided by Sigma–Aldrich (Saint Quentin Fallavier, France). Fetal calf serum, Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS), trypsin–ethylenediamine-tetra-acetic acid (trypsin/EDTA, 0.05%: 0.02%, v/v) and trypsin 2.5% (in physiological serum, v/v) were from Eurobio (Les Ulis, France). Agarose for the comet assay was from Promega (Charbonnières, France). Bovine pituitary extract (BPE), recombinant epidermal growth factor (rEGF), keratinocyte serum-free medium (K-SFM) and PBS with Ca^{2+} and Mg^{2+} were purchased from GIBCO-BRL (Cergy Pontoise, France).

Sunscreen formulations Photoderm SPF 50+® (tinted and not tinted) and Photoderm Max SPF100® (tinted and not tinted) were graciously supplied by Bioderma laboratories (Lyon, France).

2.2. Irradiation procedure

Irradiation experiments were carried out with a Suntest CPS+ solar simulator (Atlas Material Testing Technology BV, Moussy le Neuf, France) equipped with a xenon arc lamp (1100 W) and special glass filters restricting transmission of light below 290 nm and near IR-blocking filter. The irradiation intensity was fixed at 500 W/m^2 for the comet assay or 750 W/m^2 for the micronucleus assay. The temperature of the samples was kept at 4 °C using a 10% polypropylene glycol in distilled water cooling system into the irradiation chamber. UVA–visible light (315–800 nm) was obtained using the solar ID65 filter plus a window glass filter and visible light alone (400–800 nm) was obtained with a yellow GG 420 nm cut off filter (Schott AG, Clichy, France), placed inside the irradiation chamber and maintained at 3.5 cm above the slides by an appropriate support. The total irradiation dose corresponded to 8% UVA and 92% visible light. The spectral irradiance of the xenon lamp equipped with UVA and visible cut-off filters is reported in Fig. 1.

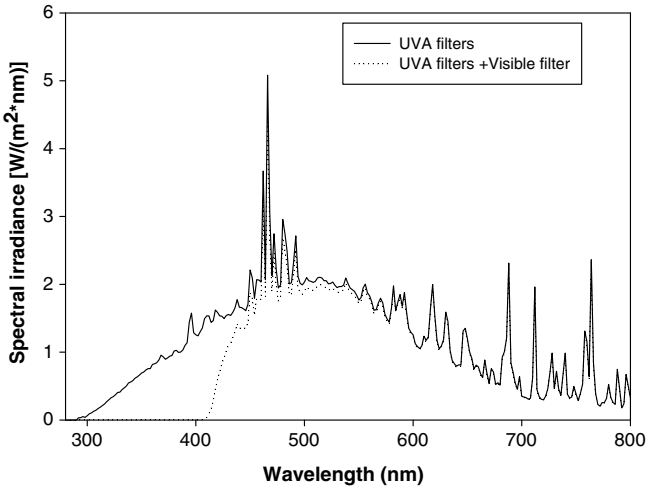

Fig. 1. Irradiation spectra of the xenon arc lamp equipped with UVA and UVA/visible filters used with the Atlas Suntest CPS+. Solid line: Irradiation spectrum of the xenon lamp equipped with the UVA/visible filter (315–800 nm). Dotted line: Irradiation spectrum of the xenon lamp equipped with the visible filter (400–800 nm).

2.3. Cell cultures

Cultures of human primary skin keratinocytes were carried out from neonatal foreskins, and isolation of keratinocytes was conducted as already described [26]. Briefly, after cutting up of the foreskin in small (5 × 5 mm) fragments, skin was incubated in 2.5% trypsin (v/v in physiological serum) during 90 min at 37 °C with gentle shaking. Trypsin was inactivated with 50 μl of fetal calf serum, dermis was eliminated and epidermis fragments were transferred into PBS. The cell pellet was resuspended by pipetting and cells were centrifuged at 1200*g* for 5 min. Finally, cells were seeded in 25 cm^2 flasks containing 7 ml complete K-SFM (with BPE 25 μg/ml and rEGF 0.1–0.2 ng/ml). Cultures were maintained at 37 °C and 5% CO_2/95% air in a humidified incubator. The culture medium was changed every 2 days and pure keratinocytes cultures were generally obtained after 2 weeks.

Chinese Hamster Ovary cells (CHO-K1, ATCC), were selected for their suitability for the MN assay [36]. They were cultured in McCoy's 5A culture medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (10 μg/ml) and 1 mM glutamine. Cultures were kept in humidified incubator at 37 °C with 5% CO_2 and sub-cultured every 2 days.

2.4. Cytotoxicity test

Keratinocyte viability was assessed after exposure to each dose of UVA and/or visible radiation implemented in the comet assay by a cell proliferation assay using WST-1 reagent. WST-1 is a tetrazolium dye that is cleaved by mitochondrial dehydrogenases in living cells to a formazan dye with an absorbance at 450 nm. Briefly, keratinocytes were plated 24 h before experiment on a 96-well multiplate, and then submitted to irradiation with UVA/visible or visible light in PBS with calcium and magnesium. After irradiation, cells were washed and WST-1 in culture medium was added to each well. Following a 30-min incubation period at 37 °C, absorbance at 450 nm was measured by a MRX® II microplate reader (Dynex Technologies, VA, USA).

2.5. Quantification by the comet assay of DNA-damage induced by UVA/visible and visible radiations on human normal keratinocytes

2.5.1. Slide preparation and irradiation procedure

The day before the experiment, keratinocytes (10^5 cells per condition) were plated in 60-mm Petri dishes and kept at 37 °C in a humidified atmosphere containing 5% CO_2. For each irradiation dose, an internal standard was incorporated: 10^5 cells were incubated separately with 60 μM of methyl-methane-sulfonate (MMS) at 37 °C for 2 h before slide preparation.

The alkaline version of the comet assay was carried out according to the procedure of Singh et al. [37] with minor modifications as described previously [38]. Two slides were prepared per condition. Twenty-four hours before the experiment, the slides were covered with 1.8% normal melting-point agarose (NMPA) prepared in Ca^{2+} and Mg^{2+} free PBS and were dried for 12 h. The day of the experiment, 85 μl of normal agarose (0.8%, w/v in PBS) were layered on to the slides, and a coverslip was applied. After solidification of the agarose on an ice bath, the coverslip was removed. The following steps were performed under yellow light to avoid additional DNA lesions. After trypsinization of the keratinocytes, the cells were resuspended at a density of 10^5 cells ml^{-1} and were centrifuged at 3000*g* for 5 min. The cell pellet was diluted into 75 μl of 0.5% molten low melting-point agarose (LMPA) and this suspension was added as a second layer on the slides. A coverslip was immediately applied over the agarose, and slides were maintained on ice for hardening of the agarose. Then, coverslips were removed, and a final layer of 75 μl of 0.5% LMPA was added. After coverslipping the slides, agarose was allowed to harden on ice. Finally, coverslips were removed and the slides were irradiated at 5, 10 or 15 J/cm^2, with UVA/visible with or without the visible light filter. Negative control and internal standard slides were maintained in the dark.

2.5.2. Comet assay

After the irradiation procedure, the slides were immediately placed in freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris, pH 10, 1% L-lauroyl sarcosinate, and 10% dimethylsulfoxide and 1% Triton X-100) for 90 min at 4 °C to remove cellular proteins. Then the slides were transferred in a horizontal electrophoresis unit and the DNA unwinding was carried out in a fresh electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA [pH > 13]) for 20 min at room temperature. Electrophore-

sis was next conducted in the same buffer during 20 min (25 V, 300 mA). Finally, the slides were covered three times during 5 min with 0.4 M Tris buffer (pH 7.5) and dehydrated in 100% methanol. After drying, slides were maintained in dry atmosphere until analysis. DNA was stained with 50 μl of 2 μg/ml ethidium bromide and the analysis was conducted with a fluorescent microscope (Olympus BH2-RFL). The microscope was equipped with an oil immersion Apo/Dplan 20× lens, a 515–560 nm excitation filter, a 590 nm barrier filter, and connected to a high sensitivity CDD COHU camera (0.01 lux). The final magnification was 250×. DNA lesions were quantified as Olive Tail Moment (OTM; arbitrary units) and Tail DNA using Fenestra Komet 5.5 image analysis software (Kinetics Imaging, Nottingham, UK). One hundred cells, randomly chosen, were analyzed per condition (50 cells/slide), thus a total of 100 OTM were calculated for each sample. Then, the 100 measured OTM values/sample were divided into 40 classes between the minimal and the maximal values. A non-linear regression analysis was performed on the OTM distribution frequencies with a χ^2 function model using TableCurve 2D software (version 5.0; Jandel Scientific Software, San Rafael, CA). The calculated degrees of freedom (n) for this function were a quantitative measure of the DNA-damage for a sample according to Bauer et al. [39] and Jean et al. [30]. The n, named OTM χ^2 and expressed in arbitrary units (a.u.), was used as the sole parameter for quantifying levels of DNA-damage of a sample.

2.6. Quantification by the micronucleus test of DNA-damage induced by UVA/visible and visible radiations on CHO cells

Twenty-four hours before the experiment, a total of 5×10^4 CHO cells were plated in Labtek® chamber slides and kept at 37 °C in a humidified atmosphere containing 5% CO_2. On the day of the experiment, culture medium was replaced by PBS with Ca^{2+} and Mg^{2+} and the cells were irradiated by UVA–visible or by visible light alone, as described above. The combined light doses were 15, 20, 25 and 30 J/cm^2, respectively. Assays were performed in duplicate, and a negative control maintained in the dark was included in each set of experiments. After irradiation, PBS was removed and cells were allowed to divide in the dark at 37 °C in a 5% CO_2 atmosphere in fresh culture medium for 3 h. Cytokinesis was interrupted by the addition of cytochalasin B (2 μg/ml) and the incubation was extended for 24 h. At 27 h after irradiation, cells were washed twice with PBS and submitted to a 5-min fixation step with absolute methanol. The air-dried slides were stained with 10% Giemsa in distilled water during 15 min.

The index of binuclearity (IBIN), considered as a measure of cytotoxicity, was determined by scoring the number of binucleated cells among 500 Giemsa-stained cells with well-preserved cytoplasm [40]. It was expressed as the following ratio:

$$\text{IBIN} = ((\text{mononucleated cells}) + 2 * (\text{binucleated cells}) + 3 * (\text{cells with at least 2 nuclei}))/500$$

The micronucleated cell rates were determined for concentrations inducing less than 50% toxicity; 2000 binucleated CHO were examined and micronuclei were identified according to the morphological criteria defined by Kirsch-Volders et al. [40]. Statistical differences between negative controls and irradiated samples were performed by the contingency 2 × 2 χ^2-test.

2.7. Evaluation of photoprotection by three protective compounds in human keratinocytes

Twenty-four hours prior the comet assay, the keratinocytes at approximately 80% confluence were seeded in 12-well plates at a density of 5×10^4 cells/well. On the day of the experiment, stock solutions of ectoin (4 mM), ERT (4 mM) and mannitol (12 mM) were prepared in ultrapure water. Each compound, at the required final concentrations, was added into the wells at, respectively, 0.025–0.05–0.1 mM for ectoin, 0.1–0.25–0.5 mM for ERT and 0.5–1–1.5 mM for mannitol. Culture medium alone was added for control samples. Incubation was conducted for 1 h at 37 °C. After the required incubation period, the medium was removed; the cells were washed three times with Ca^{2+} and Mg^{2+} free PBS and harvested with trypsin/EDTA for 5 min. After a centrifugation at 3000g for 5 min, cells were embedded in agarose gel and placed on microscopic slides. Immediately after the irradiation, according to the protocol described above, at a combined light dose of 15 J/cm^2, cells were submitted to the comet assay. For each experiment, controls included untreated non-irradiated cells (negative control) and untreated irradiated cells (positive control).

2.8. Evaluation of photoprotection by sunscreens in human keratinocytes

The absorption curves of the four tested sunscreens are displayed in Fig. 2. From these curves, the Sun Protection Factor (SPF) and the Protection Factor UVA (PUVA) were calculated for each sunscreen by the in vitro Diffey method [41]. SPF were 49.9 and 112.7 for Photoderm SPF 50+® and its tinted analogue, respectively, and 139.9 and 231.0 for Photoderm Max SPF100® and its tinted analogue, respectively. PUVA were 45.6 and 100 for Photoderm SPF 50+® not tinted and tinted, respectively, and 52.6 and 79.5 for Photoderm Max SPF100® not tinted and tinted, respectively. Just before irradiation, the sunscreens were uniformly spread on a quartz slide (5 × 5 cm^2) at an amount of 2 mg/cm^2 and placed above the embedded cells in the solar simulator. Slides were submitted to the alkaline comet assay immediately after the irradiation, according to the protocol described above at a combined light dose of 15 J/cm^2. Unprotected cells and non-irradiated cells were used as positive and negative controls, respectively.

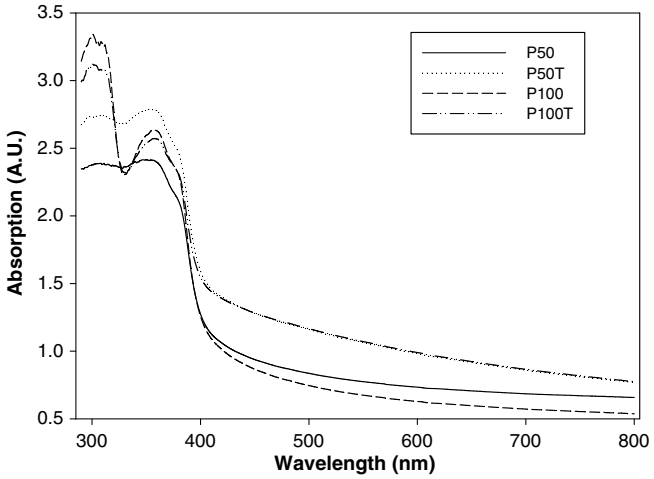

Fig. 2. Absorption spectra of Photoderm SPF 50+® (tinted and not tinted) and Photoderm Max SPF100® (tinted and not tinted) from 280 to 800 nm. Each sunscreen was spread on a quartz slide (5 × 5 cm) to reach a final amount of 2 mg/cm^2. P50: non-tinted Photoderm SPF 50+®. P50T: tinted Photoderm SPF 50+®. P100: non-tinted Photoderm Max SPF100®. P100T: tinted Photoderm Max SPF100®.

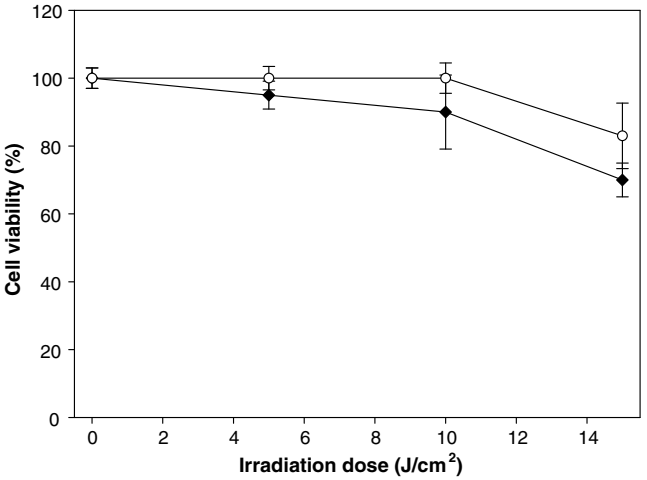

Fig. 3. Viability of human keratinocytes irradiated with increasing doses of UVA/visible light (5–15 J/cm^2) with (○) and without (♦) the visible filter. The cell viability was measured 30 min after irradiation by the WST-1 assay and the results are expressed as a percentage of the reading in control wells. Each point represents the mean ± SE of four measures.

3. Results

3.1. Cytotoxicity induced by exposure to UVA/visible and visible light in normal keratinocytes

Keratinocytes were irradiated with increasing doses of UVA/visible light (5–15 J/cm^2) with and without the visible filter. The cell viability was determined 30 min after irradiation by the WST-1 assay. Results are expressed as percentage of cell viability in reference to the control (non-irradiated keratinocytes), which corresponds to 100% cell viability. The results are included in Fig. 3. A slight decrease in cell viability was observed after irradiation by UVA/visible. For the highest irradiation dose (15 J/cm^2), the decrease of the cell viability was 30%.

The viability of cells was not affected by irradiation with visible light except for the higher tested dose (13.8 J/cm^2) which slightly reduced the cell survival by approximately 17%.

3.2. Induction of DNA-damage by UVA/visible and visible radiations on human keratinocytes measured by the alkaline comet assay

Keratinocytes embedded in agarose were irradiated at increasing doses of UVA/visible (5–15 J/cm^2) with or without the visible light filter in the solar simulator, then immediately submitted to the comet assay to avoid DNA repair processes. Because primary human keratinocytes showed variability in their response to UVA and visible light, an

Table 1
DNA-damage induced by UVA/visible and visible light in human keratinocytes

Dose (J/cm^2)	Sample	TD (%)		OTM (a.u.)		OTM χ^2 (a.u.)
		Mean ± SEM	Median	Mean ± SEM	Median	
5	NI	6.02 ± 0.42	5.49	2.27 ± 0.17	2.03	2.90 ± 1.25
	MMS	33.77 ± 1.25	32.61	15.42 ± 0.59	14.66	15.44 ± 2.06***
	Visible	11.60 ± 0.91	9.51	3.81 ± 0.34	2.72	3.78 ± 1.00***
	UVA/visible	14.05 ± 0.90	13.14	5.75 ± 0.43	4.08	4.78 ± 0.79***
10	NI	20.70 ± 1.26	18.89	11.18 ± 0.90	9.12	4.42 ± 1.02
	MMS	53.75 ± 1.39	53.22	52.17 ± 1.46	32.60	20.7 ± 3.54***
	Visible	23.19 ± 0.96	21.73	16.32 ± 0.82	14.57	6.34 ± 1.39***
	UVA/visible	26.09 ± 0.95	24.93	19.30 ± 0.82	19.00	7.76 ± 1.16***
15	NI	5.57 ± 0.60	4.67	1.84 ± 0.19	1.35	2.72 ± 0.20
	MMS	32.19 ± 1.05	32.52	15.36 ± 0.47	15.53	20.96 ± 0.59***
	Visible	12.47 ± 1.01	11.45	4.71 ± 0.36	3.68	5.91 ± 0.21***
	UVA/visible	17.94 ± 1.07	16.74	7.42 ± 0.45	6.72	9.37 ± 0.31***

NI: non-irradiated cells; MMS: cells treated with 60 μM methyl-methane-sulfonate for 2 h at 37 °C (internal standard); Visible: cells irradiated with the visible light (400–800 nm); UVA/visible: cells irradiated with UVA/visible light (315–800 nm); TD: Tail DNA; OTM: Olive tail moment; OTM χ^2: degree of liberty of the χ^2 function calculated by non-linear regression analysis.
*** $P < 0.001$ by the comparison of means (t-test).

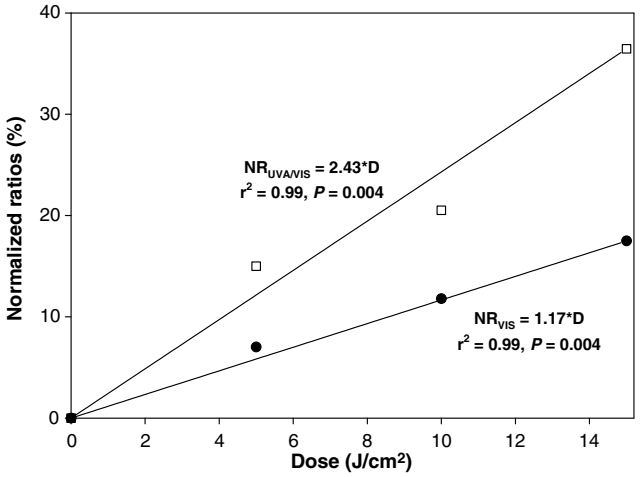

Fig. 4. Dose–response relationship of DNA-damage versus UVA/visible and visible irradiation in human keratinocytes measured by the comet assay. Cells were irradiated at increasing dose of UVA/visible 5–10 J/cm^2 with and without visible filter, then immediately submitted to the comet assay. The OTM χ^2 are expressed as normalized ratios, according to the following formula:

$$\text{ratio} = \frac{\left(\text{OTM } \chi^2_{\text{sample}} - \text{OTM } \chi^2_{\text{NI}}\right)}{(\text{OTM } \chi^2_{\text{MMS}} - \text{OTM } \chi^2_{\text{NI}})} * 100$$

where OTM χ^2_{sample} is the OTM χ^2 of the irradiated sample; OTM χ^2_{MMS} the OTM χ^2 of the treated sample with 60 μM methyl-methane-sulfonate (positive control); OTM χ^2_{NI} is the OTM χ^2 of the non-irradiated sample (negative control). White squares: UVA/visible light; black circles: visible light only.

internal standard, namely the alkylating agent methyl-methane-sulfonate, was included for each tested irradiation dose. Complete results concerning the DNA-damaging effects of UVA/visible and visible radiations on human keratinocytes are reported in Table 1. DNA strand-break induction was significant at the lowest irradiation dose of 5 J/cm^2, with OTM χ^2 of 4.78 ± 0.79 for UVA/visible light and OTM χ^2 of 3.78 ± 1.00 for visible light. It dramatically increased between 10 and 15 J/cm^2, with OTM χ^2 reaching 9.37 ± 0.31 and 5.91 ± 0.21 for UVA/visible and visible radiations, respectively, at the highest irradiation dose. Normalized ratios (%) are illustrated in Fig. 4. Significant increases in DNA strand breaks were calculated after irradiation by UVA/visible and visible light, in a linear dose–response model ($r^2 = 0.99$, $P = 0.04$ for both UVA/visible and visible light). Slope comparison of the curves showed that the genotoxic effects of visible light represented around 50% of the total effects of UVA/visible irradiation (with slope values of 1.17 and 2.43 a.u., respectively).

3.3. Induction of DNA-damage by UVA/visible and visible radiations on CHO cells measured by the micronucleus test

Micronucleus test was carried out on CHO cells irradiated by UVA/visible and visible light at four increasing doses (15–30 J/cm^2). CHO cells were selected for this series of experiments because of their high capacity to divide as compared to human normal keratinocytes. Complete results are reported in Table 2. Both UVA/visible and visible radiations produced a dose-dependent increase of micronuclei rates. Micronuclei induction was significant at the lowest irradiation dose tested (15 J/cm^2) with 24.5 ± 4.9 micronucleated cells per 1000 observed after UVA/visible irradiation and 18 ± 1.4 micronucleated cells observed after visible irradiation. This genotoxic activity was maximal after an irradiation dose of 30 J/cm^2, with micronucleated cell rates reaching $37 \pm 2.8‰$ and $26.5 \pm 2.1‰$ for UVA/visible and visible light, respectively. The micronuclei induction rates of visible light represented approximately 60% of the global effects of UVA/visible irradiation, demonstrating that visible light could induce chromosome rearrangements.

3.4. Evaluation of the protective effects of ectoin, ERT and mannitol

Keratinocytes were incubated 1 h at 37 °C in culture medium with various concentrations of each compound. Embedded cells were submitted to irradiation by UVA/visible or visible radiations. The irradiation dose was fixed at

Table 2
Micronuclei induction by UVA/visible and visible light in CHO cells

Dose (J/cm^2)	Sample	Binucleated cells	Mononocleated cells	IBIN	IBIN (%)	MNC (‰)
0	NI	486.5 ± 3.5	13.5 ± 3.5	1.95	100	7.5 ± 0.7
15	Visible	485.0 ± 5.7	15.0 ± 5.7	1.95	100	$18.0 \pm 1.4^{***}$
	UVA/visible	478.0 ± 2.8	22.0 ± 2.8	1.92	98	$24.5 \pm 4.9^{***}$
20	Visible	477.0 ± 3.5	22.5 ± 3.5	1.92	98	$21.5 \pm 2.1^{***}$
	UVA/visible	473.0 ± 4.2	27.0 ± 4.2	1.90	97	$32.5 \pm 2.1^{***}$
25	Visible	472.5 ± 10.6	27.5 ± 10.6	1.91	98	$25.0 \pm 2.8^{***}$
	UVA/visible	476.0 ± 4.2	24.0 ± 4.2	1.91	98	$35.5 \pm 3.5^{***}$
30	Visible	472.5 ± 3.5	27.5 ± 3.5	1.90	97	$26.5 \pm 2.1^{***}$
	UVA/visible	460.5 ± 6.4	39.5 ± 6.4	1.85	95	$37.0 \pm 2.8^{***}$

NI: non-irradiated cells; IBIN: percentage of binuclearity index as compared to the control culture; MNC: micronucleated cell rates per 1000 binucleated cells; Visible: cells irradiated with the visible light (400–800 nm); UVA/visible: cells irradiated with UVA/visible light (315–800 nm).
*** $P < 0.001$ by the 2×2 χ^2 contingency test.

15 J/cm^2 throughout the experiments. Immediately after the irradiation, slides were immersed in lysis solution for the continuation of the comet assay.

For the three tested molecules, the highest concentration showed neither cytotoxicity nor DNA-damaging activity as evaluated by the trypan blue staining (data not shown). Three concentrations of ectoin were tested: 0.025, 0.05 and 0.1 mM. Results are illustrated on Fig. 5. All the tested concentrations showed a highly significant level of photoprotection against UVA/visible light and visible light with a dose–response relationship. At 0.1 mM, the OTM χ^2 were 2.37 ± 0.41 and 4.04 ± 0.16 for visible and UVA/visible irradiations, respectively, corresponding to 92.7% and 68.9% levels of photoprotection.

The effects of ERT on DNA SSB induced by UVA/visible and visible radiations are reported in Fig. 6. Three concentrations of ERT were tested: 0.1, 0.25 and 0.5 mM. Two concentrations exerted a significant protection against UVA/visible light, and a maximal protection rate of 59.8% was obtained at the concentration of 0.5 mM, with an OTM χ^2 value of 5.15 ± 0.20. The protective activity of ERT against visible radiations was more efficient, with a maximal protection rate reaching 97.9% at the concentration of 0.5 mM, with an OTM χ^2 value of 2.29 ± 0.19.

Mannitol exerted a significant dose-dependent photoprotection against UVA/visible radiations (Fig. 7): the lowest concentration (0.5 mM) decreased the OTM χ^2 value at 11.93 ± 0.40 corresponding to a statistically significant protection of 16.9%. A maximal protection rate of 62.7% could be observed at 1.5 mM, with an OTM χ^2 value of 6.71 ± 0.29. The protective activity of mannitol against visible radiations was lower since the calculated protection rates were no statistically significant (2.6% and 13.8%) at the concentrations of 0.5 and 1 mM, respectively. However, a statistically significant 52.0% protection rate was obtained at the concentration of 1.5 mM, with an OTM χ^2 value of 5.18 ± 0.19. These results overall demonstrated that the presence of endogenous protective compounds during UVA/visible or visible irradiation led to a significant protective effect against DNA-damage. They also indicated that the efficacy of the protection depended on the nature of both the molecules and the radiations.

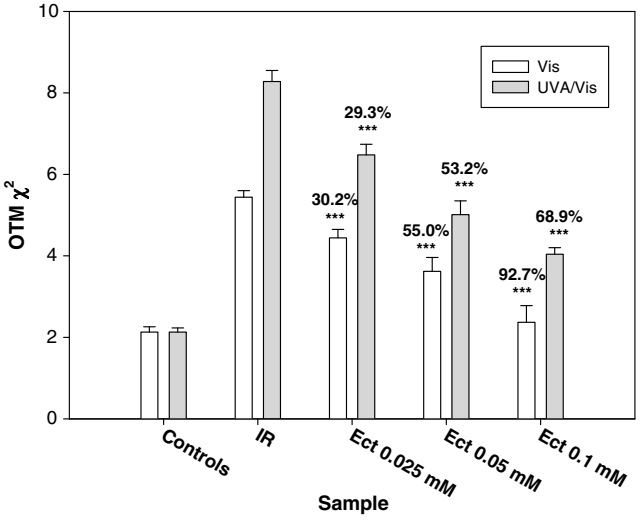

Fig. 5. Photoprotection of ectoin on human keratinocytes measured by the alkaline comet assay. Cells were incubated for 1 h at 37 °C with ectoin (0.025–0.1 mM) and were irradiated with a combined light dose of 15 J/cm^2 (corresponding to 1.2 J/cm^2 UVA and 13.8 J/cm^2 visible) with or without visible filter. After irradiation, cells were immediately submitted to the comet assay. Control: non-irradiated cells; IR: untreated irradiated cells; NS: non-significant; $^{***}P < 0.001$.

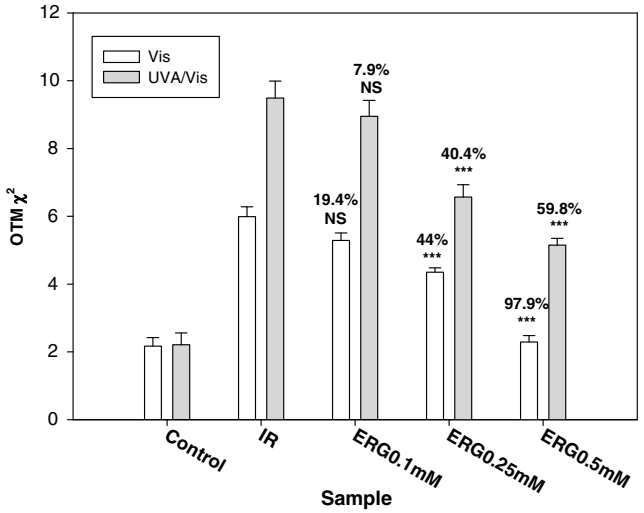

Fig. 6. Photoprotection of ERT (0.1–0.5 mM) on human keratinocytes measured by the alkaline comet assay. Experimental conditions are described in the legend of Fig. 4. Control: non-irradiated cells. IR: untreated irradiated cells. NS: Non-significant. $^{***}P < 0.001$.

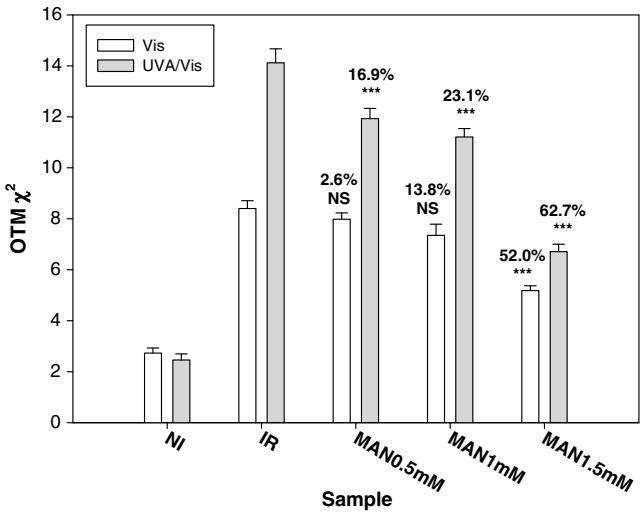

Fig. 7. Photoprotection of mannitol (0.5–1.5 mM) on human keratinocytes measured by the alkaline comet assay. Experimental conditions are described in the legend of Fig. 4. Control: non-irradiated cells. IR: untreated irradiated cells. NS: Non-significant. $^{***}P < 0.001$.

3.5. Evaluation of photoprotective effects of broad-spectrum sunscreens

Sunscreens were evenly spread on quartz slides at 2 mg/cm^2 before irradiation. The irradiation dose was fixed at 15 J/cm^2 throughout the experiments. Embedded keratinocytes were submitted to irradiation as described in the previous section, protected or not by sunscreens, and directly immersed in lysis solution.

The protective effects of sunscreens against DNA-damage induced by UVA/visible and visible light, as assessed by the comet assay on human keratinocytes, are reported in Fig. 8. The four sunscreens (Photoderm SPF 50+®, tinted Photoderm SPF 50+®, Photoderm Max SPF100® and tinted Photoderm Max SPF100®) significantly reduced the DNA-damaging activities of UVA/visible and visible radiations. Photoderm SPF 50+® and Photoderm Max SPF100® exhibited protection rates of 79.9% (OTM $\chi^2 = 3.64 \pm 0.23$) and 86.7% (OTM $\chi^2 = 3.14 \pm 0.15$), respectively, against UVA/visible light, while their tinted analogues appeared more active, with protection rates of 92.6% (OTM $\chi^2 = 2.69 \pm 0.22$) and 97.1% (OTM $\chi^2 = 2.40 \pm 0.21$), respectively. Similarly, a high photoprotective activity against visible light could be obtained: Photoderm SPF 50+® and Photoderm 100+® exerted a protection rate of 82.8% (OTM $\chi^2 = 2.77 \pm 0.24$) and 92.5% (OTM $\chi^2 = 2.47 \pm 0.24$), respectively, while their tinted analogues both displayed a 100% protective effect (OTM $\chi^2 = 2.12 \pm 0.08$ and 2.16 ± 0.12, respectively). These results indicated that sunscreens could efficiently prevent DNA-damage induced by both UVA/visible and visible lights, and implied that tinted ointments exerted a higher protective activity than their colorless counterparts.

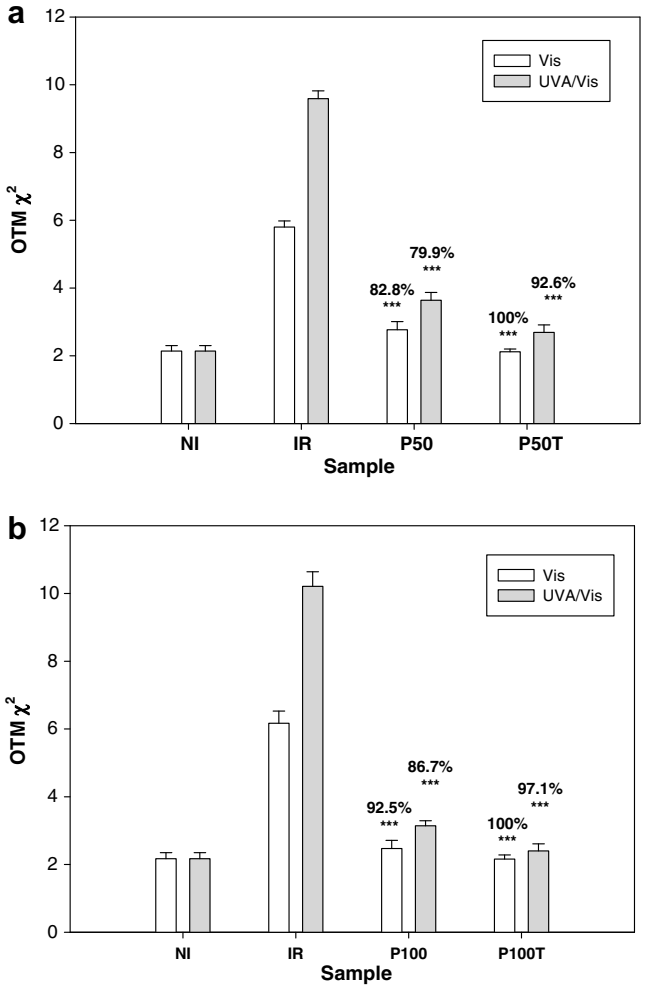

Fig. 8. Photoprotection of sunscreen Photoderm SPF 50+® (tinted and not tinted, A) and Photoderm Max SPF 100® (tinted and not tinted, B) measured by the comet assay. Keratinocytes were irradiated with a combined light dose of 15 J/cm^2 (corresponding to 1.2 J/cm^2 UVA and 13.8 J/cm^2 visible) and immediately submitted to the comet assay. NI: non-irradiated control. IR: unprotected irradiated cells. P50: irradiated cells protected by Photoderm 50+®. P50T: irradiated cells protected by tinted Photoderm 50+®. P100: irradiated cells protected by Photoderm Max 100+®. P100T: irradiated cells protected by tinted Photoderm Max 100+®.

4. Discussion

Solar radiations, especially UVA and UVB, are known to induce DNA lesions that may lead to carcinogenesis. If UVB radiations directly alter nuclear DNA, UVA are weakly absorbed by DNA and induce indirect DNA-damage by excitation of endogenous chromophores [42]. Little is known about the genotoxic effects of visible light (400–800 nm). The distinction between UVA and visible radiations is an artificial criterion. As a possible consequence, photobiological effects of the long UVA region and the near visible radiations (between 400 and 450 nm) could overlap [22].

In this study, the alkaline comet assay was performed to evaluate the genotoxic effects of visible light on human normal keratinocytes. These cells showed high variability in their responses to irradiation, because primary cells cultures were obtained from different human donors and the cells were in different growth stages at the time of the experiment. Moreover, the experimental variability inevitably induced an additional heterogeneity of the responses. This underlined the necessity of using internal standard for each irradiation dose as suggested by De Boeck et al. [43]. Keratinocytes were submitted to irradiations by UVA/visible (315–800 nm) with and without a visible light filter. Results showed that visible light induced statistically significant DNA SSB for all the tested doses of irradiation. The present results are consistent with the previous finding of Singh et al. [44]. The authors showed that visible light could induce DNA SSB and alkali-labile sites in rat lens epithelial cells and in human lymphocytes, as assessed by the comet assay. In our study, a linear dose–response relationship was observed between irradiation doses and normalized DNA lesions, for both UVA/visible and visible light. The level of DNA breakage induced by visible light was 50% of the one generated by UVA/visible irradiation. Thus, visible light is able to induce as much DNA breaks as UVA radiations. However, for a combined light dose of

15 J/cm^2, visible and UVA light represent a dose of 13.8 and 1.2 J/cm^2, respectively. Thus, UVA radiations are 10 time more effective than visible radiations to induce DNA SSB. Moreover, most of the energy delivered by the xenon lamp (Fig. 1) is concentrated between 450 and 500 nm, suggesting a possible implication of near-visible radiations in genotoxic lesions.

Our comet assay methodology involved the detection of direct DNA breakage (cell lysis performed immediately after the irradiation) [45]. Thus, the SSB detected by the comet assay reflected direct UVA-induced DNA lesions that were mainly produced by ROS [45,46]. Our results are consistent with this mechanism. It could be hypothesized that DNA-damage observed after irradiation of keratinocytes by visible light may also be generated by the same process than UVA rays. This has been previously demonstrated by Kielbassa et al. [47]: after irradiation of CHO cells by UV/visible light, the maximal rate of formamidopyrimidine–DNA glycosylase (Fpg)-sensitive lesions (i.e. 8-oxodG and formamidopyrimidines) was observed between 400 and 450 nm. These oxidative lesions could be due to the excitation of endogenous photosensitizers. These results have been confirmed in 1998 by Pflaum et al. [25] in HaCaT and primary keratinocytes: most of the oxidative DNA base modifications were generated in the visible range of sunlight. Moreover, these lesions could be the consequence of photoactivation of cellular porphyrins, because their maximal excitation wavelength is 406 nm. Other potential intracellular chromophores, such as flavins, that possess peaks absorption between 334 and 434 nm, could be involved in photosensitization mechanisms [48].

The chromosomal mutagenicity of visible light was investigated by the micronucleus test on the rapid growing cell line CHO. We found a non-linear dose-related induction of MNs after irradiation by both UVA/visible and visible light. UVA radiations have been shown to induce a dose-dependent formation of MNs in human fibroblasts at a dose range of 10–30 J/cm^2 [49]. Likewise, an increase of MNs and a persistent genomic instability have been found in HaCaT cells after UVA irradiation (10 J/cm^2) [50]. Visible light has also been shown to induce MNs formation in melanoma cell line and primary human skin fibroblasts [51]. Several mechanisms have been suggested for the induction of MNs by UVA and visible light: (i) oxidative damage through photosensitization processes [25] or redox status alteration [50]; (ii) generation of double strand breaks through the propagation of clustered single-strand breaks [52]; (iii) epigenetic mechanisms, such as DNA–protein crosslinks or lesions of the mitotic spindle elements [51]. Further studies would be necessary to apprehend most clearly these mechanisms.

To evaluate the importance of oxidative damage in UVA/visible and visible irradiations, we assessed the photoprotective effects of three exogenous compounds (ectoin, ERT and mannitol) on human keratinocytes by the comet assay. All the tested molecules exerted an effective dose-dependent DNA protection against both UVA/visible and visible light. These results strongly suggested the implication of ROS in the visible-induced genotoxicity, as for UVA-induced DNA lesions. Ectoin is a cyclic tetrahydropyrimidine, produced by halophilic eubacteria [53]. This natural substance has been shown to prevent UVA-induced cell damage, by the reduction of pro-inflammatory gene expression such as ICAM-1 and by the expression of heat shock proteins 70 (Hsp70s) that protect cells against heat stress and toxic chemicals [53,54]. ERT (2-mercaptohistidine trimethylbetaine) is a low-molecular-mass thiol that is synthesized in microorganisms, and can be found in some human tissues after dietary intake [55]. This molecule has been shown to possess intrinsic anti-hydroxyl radical (•OH) activity [56] and to exert protective effect against UVA radiations [26]. Similar properties have been demonstrated for mannitol, which appeared to be an effective hydroxyl radical quencher [57].

In the last part of this study, we evaluated the ability of four broad-spectrum sunscreens to protect human keratinocytes against UVA/visible and visible light genotoxicity by the alkaline comet assay. The tested sunscreens revealed an effective photoprotection against UVA/visible and visible radiations by reducing the extent of DNA breakage. For the two sunscreens, the tinted formulations demonstrated higher effective genomic photoprotection against both UVA/visible and visible light. Most commercialized sunscreens afford effectiveness against UVB and UVA, but do not give significant protection against visible radiations [58]. A small number of tinted sunscreens are already used by photosensitive patients: these formulations contain large-sized particles of titanium dioxide (pigment grade) and zinc oxide that scatter visible radiations [58]. Our study underlies the necessity of an extended protection in the visible range. It focuses on the development of new formulations that would produce an improved protection against visible light and an enhanced cosmetic acceptability.

In conclusion, this study revealed the genotoxic effects of visible light (400–800 nm) as assessed by the comet assay and the micronucleus test. These effects appeared to be mediated by oxidative stress mechanisms similar to the ones produced by UVA radiations. Moreover, our results emphasize the need to complement sunscreen formulations with internal protective compounds to afford internal photoprotection and with additional molecules that remain to be characterized, to obtain a complete external photoprotection against UVA and visible light.

5. Abbreviations

8-oxo-dG 8-oxo-7,8-dihydro-2′-deoxyguanosine
BPE bovine pituitary extract
CHO Chinese hamster ovary
CPD cyclobutane pyrimidine dimer
ERT L-ergothioneine
Fpg formamidopyrimidine–DNA glycosylase
IBIN index of binuclearity
K-SFM keratinocytes serum free medium

LMPA low melting-point agarose
MMS methyl-methane-sulfonate
MN micronucleus
NMPA normal melting-point agarose
OH hydroxyl radical
OTM Olive tail moment
PBS phosphate-buffered saline
PUVA protection factor UVA
rEGF recombinant epidermal growth factor
ROS reactive oxygen species
SPF sun protection factor
SSB single-strand breaks
UV ultraviolet

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TOPIC HIGHLIGHT

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Alcoholic liver disease

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Abstract

Alcohol use disorders affect millions of individuals worldwide. Alcohol consumption is directly associated with liver disease mortality and accounts for elevated social and economic costs. Alcoholic liver disease (ALD) may take the form of acute involvement (alcoholic hepatitis) or chronic liver disease (steatosis, steatohepatitis, fibrosis and cirrhosis). The severity and prognosis of alcohol-induced liver disease depends on the amount, pattern and duration of alcohol consumption, as well as on the presence of liver inflammation, diet, nutritional status and genetic predisposition of an individual. While steatosis is an almost completely benign disease, liver cirrhosis is associated with marked morbidity, mortality and life expectancy shortening. The median survival of patients with advanced cirrhosis is 1-2 years. Severe acute alcoholic hepatitis (AH) is associated with mortality as high as 50%. It has been managed with corticoids, pentoxifylline and enteral nutrition, although evidence based data are still conflicting. Some author suggest that pentoxifylline could be a better first-line treatment in patients with severe AH. Absolute abstinence is a basic condition for any treatment of acute or chronic ALD, the other therapeutical procedure being of a supportive nature and questionable significance. Acamprosate appears to be an effective treatment strategy for supporting continuous abstinence in alcohol dependent patients. Patients with advanced liver cirrhosis who demonstrably abstain can be considered for liver transplantation, which leads to a markedly prolonged life expectancy. The crucial step in ALD prevention is in the prevention of alcohol abuse, whereas the prevention of liver injury in active alcohol abusers is not clinically applicable.

Key words: Alcohol; Alcoholic liver disease; Liver cirrhosis; Liver fibrosis; Steatohepatitis; Steatosis

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INTRODUCTION

Alcohol is a most frequent cause of liver disease in western countries[1]. Mortality due to liver cirrhosis in those countries is in direct proportion to absolute alcohol consumption per capita-the highest in France and Spain (over

30 deaths per a population of 100 000 per year), the lowest in the northern European countries (up to 5 deaths per 100 000 inhabitants per year). In Central Europe, the figure is 15 deaths due to cirrhosis per 100 000. The highest mortality is in men aged 35-64 years, lower in women (Figure 1)[2]. The past two to three decades have seen a stabilization if not a drop in the intake of alcohol in western countries, while a very adverse trend is reported from Eastern Europe and developing countries[3].

In what is an alarming development, alcohol abuse also afflicts societies and nations without any "drinking tradition", such as in Asia. For example, in a cross-sectional study of two rural communities in China (in which almost 10 000 inhabitants were interviewed for current and lifetime alcohol use)[4], the age-standardized prevalence of lifetime alcohol dependence ranged from 4.8% to 11.8% in different regions. Unlike most western reports, alcohol dependence shows a higher prevalence than the abuse itself.

Coincidence with HIV infection is another attribute of alcohol abuse. This was described in India for example, where the recent increase in alcohol consumption in many sectors of the general population is coupled with strong evidence of the role of alcohol in the spread of HIV infection and other health risks[5]. An even more critical situation appears to have developed in Africa. Pithey *et al*[6] performed a systematic review of sub-Saharan African studies concerning the association between alcohol abuse and HIV infection. Their findings strongly support an association between the two factors. A Fisher *et al*[7] study of high-risk African women showed, even after adjustment for demographic and employment variables, that drinkers were more likely to be HIV positive than non-drinkers (relative risk 2.1). Problem drinkers were also more likely to have engaged in several types of high-risk sexual behavior and to have other sexually transmitted infections, including HSV-2.

Many studies have shown that the amount of undiluted ("pure") alcohol consumed and the duration of that consumption are closely related to cirrhosis. According to some reports, cirrhosis does not develop below a lifetime alcohol consumption of 100 kg of undiluted alcohol[8]. This amount corresponds to an average daily intake of 30 grams of undiluted alcohol for 10 years. Heavy alcoholics consuming at least 80 g of alcohol per day for more than 10 years will develop liver disease at a rate of nearly 100%. A detailed study of 256 heavy drinkers admitted to hospital not because of liver complaints, found steatosis at a rate of 45%, steatohepatitis at 34%, steatohepatitis with cirrhosis at 10% and cirrhosis alone at 10% in their liver biopsies[9]. Formerly, 40-60 g of undiluted alcohol (i.e., 2-3 beers) per day used to be reported as a safe limit for men, less (20 g/d) for women. Data from the "Dionysos" study show, however, that consumption of more than 30 g of pure alcohol daily, regardless of sex, already increases the risk of liver disease[10].

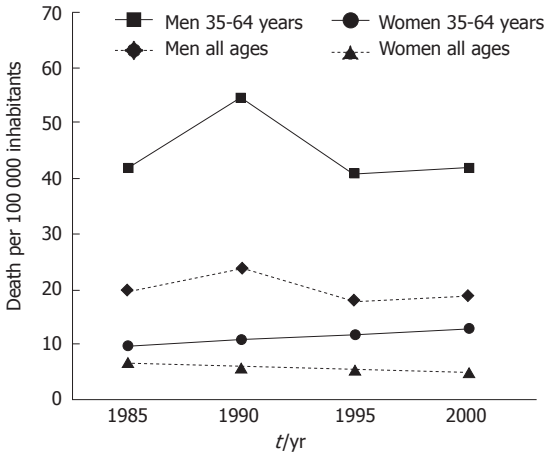

Figure 1 Mortality from cirrhosis in Czech Republic[2].

For practical purposes, alcohol intake is rated by the count of "drinks". The National Institute on Alcohol Abuse and Alcoholism defines a standard drink as 11-14 g of alcohol, which corresponds to approximately one drink of 40% spirit, one glass of wine or one 0.33 l (12-oz) beer. Hence, a "safe" daily intake of alcohol should not be more than two "drinks". On the contrary, moderate ethanol consumption (mainly wine) may mean a reduced cardiovascular risk[11], especially in women[12].

Much the same applies to Asians. For example, in the Chinese population, the ethanol risk threshold for developing alcoholic liver disease (ALD) is 20 g per day with the risk increasing in proportion to the daily intake[13]. Those drinking 20 g of ethanol per day and for less than 5 years are safe from ALD. In this study of 1270 alcohol drinkers, obesity also increases the risk. Abstinence and weight reduction will directly improve the prognosis of ALD.

As for liver injury, it has been postulated for many years that the type of alcoholic beverage makes little, if any difference. Nevertheless, some authors have proposed that mortality from cirrhosis is associated with the consumption of spirits more strongly than with other alcoholic beverages[14]. It is not clear whether this effect can be put down to the drinkers' socio-behavioral characteristics or to increased toxicity of alcoholic beverages[15].

ALD may take the form of acute involvement (alcoholic hepatitis) or chronic liver disease (steatosis, steatohepatitis, fibrosis and cirrhosis). Their progression also depends on the pattern of alcohol intake-drinking alcohol at mealtimes results in a lower risk of liver disease than consumption at other times; fitful, intermittent drinking is more sparing for the liver than a continuous supply of alcohol[16].

Although ALD is a disease that displays an absolute requirement for a voluntary environmental exposure (the consumption of alcohol), many other factors, including genetic host system attributes, are involved in the ALD evolution and progression.

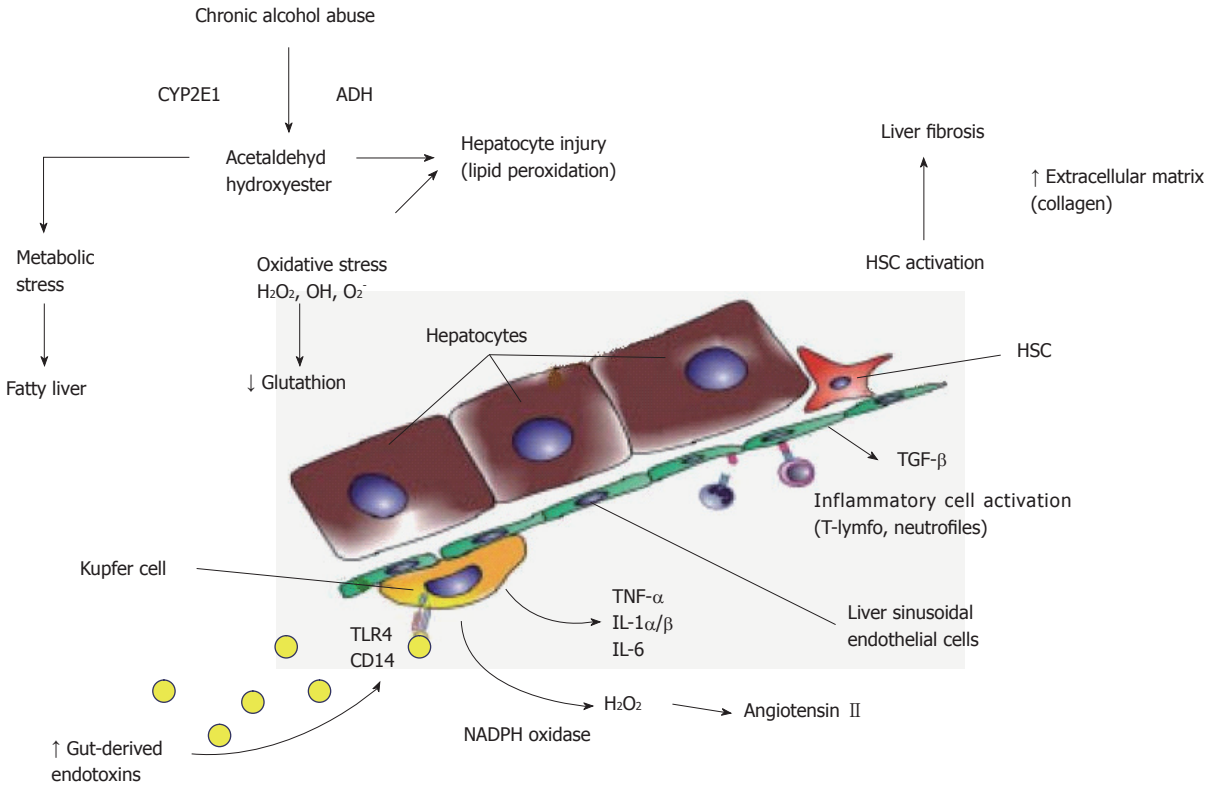

Figure 2 Pathogenesis of inflammatory changes in alcoholic liver disease[56]. ADH: Alcohol dehydrogenase; HSC: Hepatic stellate cell.

ETIOLOGY, PATHOGENESIS, NATURAL COURSE AND PROGNOSIS OF ALCOHOLIC LIVER DISEASE

The liver is the main organ of alcohol metabolism. Alcohol is metabolized in the liver in three ways: (1) by the enzyme alcohol dehydrogenase (ADH); (2) by cytochrome P-4502E1 (CYP2E1); and (3) by mitochondrial catalase. Only the first two pathways are of practical significance-ADH finds use in the degradation of limited quantities of alcohol, while alcohol-induced CYP2E1 takes place in excessive alcohol intake. Apart from the liver, ADH is also present in the gastric mucosa and the assumption is that individuals with low gastric ADH activity are more susceptible to alcoholic liver disease. This may also help to explain why women who have decreased gastric ADH activity[17] are more susceptible to developing alcoholic liver disease.

Both enzymes convert alcohol to acetaldehyde, which is in part responsible for the liver injury too. However, the process of liver injury is much more complex (Figure 2)-resulting from biochemical, genetic, cellular, immunological and humoral disorders in connection with the intake and metabolism of excessive quantities of alcohol. A major role is played there by oxidative stress (which is mainly due to alcohol-induced CYP2E1), by simultaneous shortage of antioxidants in the hepatocytes and, last but not least, by acetaldehyde alone and altered balance of many cytokines-mainly tumor necrosis factor (TNF)-α[18]. Changes in lipid metabolism and in adipose tissue also enhance the process of liver injury[19]. All above mentioned changes result in the injury of cell membranes and organelles (especially mitochondria). The mechanisms of hepatocytic damage due to excessive intake of alcohol show some similarity to changes seen in non-alcoholic steatohepatitis, except that the primary insult is different[20].

Individual susceptibility is another factor to take into account; moreover, any other liver involvement such as viral hepatitis[21] or metabolic disease adds to the risks of alcoholism, as does obesity and metabolic syndrome[22].

In fact, alcoholics were clearly shown to have an increased prevalence of HCV when compared with non-alcoholics and this combination synergistically accelerates liver injury[23]. As for alcohol influence on the liver, the caloric intake should also be taken in account. Increased caloric intake leads to excessive fat deposition and obesity in some patients and can aggravate the liver injury[24].

Of late, there has been an influx of information on correlations between genetic polymorphisms of alcohol-metabolizing enzymes and alcoholic liver disease[25]. The genetics of ALD development involves an inherited predisposition to alcohol dependence, as well as the resulting development of liver injury[26]. Family studies have established an important role of genetics in alcohol dependence. To date, only two genes, which are involved in alcohol metabolism, have shown significant involvement. The alcohol dehydrogenase ADH1B*1 allele was found to be associated with an approximately threefold increase in alcohol dependence and the aldehyde dehy-

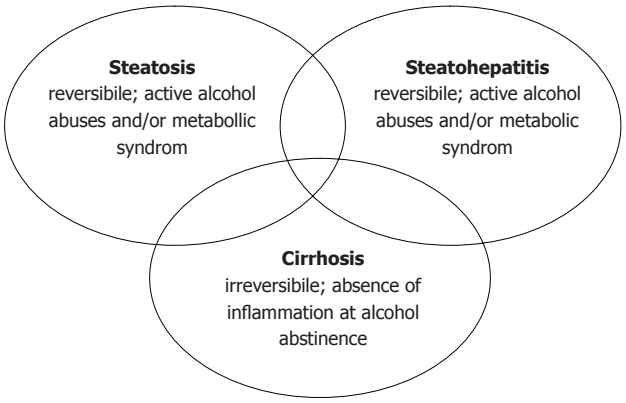

Figure 3 Spectrum of alcoholic liver disease.

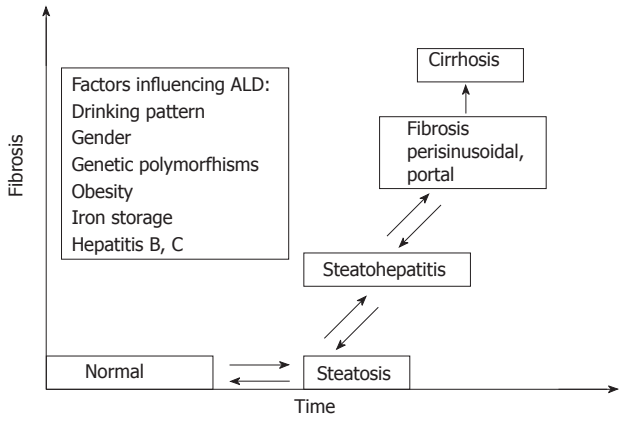

Figure 4 Dynamic process of alcoholic liver disease. ALD: alcoholic liver disease.

drogenase ALDH2*2 allele was found to be instrumental in a 10-fold reduction of the alcohol dependence risk[27]. This association was described in Asian populations[28]. Also reported have been links between alcohol dependence and certain genetic polymorphisms of genes for the GABA receptor or some other neuropeptides[29].

Although most heavy drinkers do develop fatty liver, only a minority progress to liver cirrhosis, suggesting that some other genetic or environmental factors are important for the disease progression. Evidence of genetic involvement in the progression of alcoholic fatty liver to advanced ALD comes from a twin study. The rate of alcoholic cirrhosis was described to be significantly higher in monozygotic twins than in dizygotic twins (16.9% *vs* 5.3%, respectively)[30]. A study of genes involved in alcohol metabolism (e.g., alcohol and aldehyde dehydrogenase and cytochrome P450 2E1) and genes associated with inflammation (e.g., TNF-α and interleukin-10) proved to be inconclusive, with several allelic associations detected but not verified in follow-up studies[31]. The Asian population's hypersensitivity to alcohol could be put down to polymorphisms of genes for the enzymes ADH and CYP2E1. Perhaps the most compelling genetic finding for advanced ALD risk involves the immune regulatory cytotoxic T lymphocyte antigen-4 gene, in which homozygosity for the A49G polymorphism was found to confer a significant risk of alcoholic cirrhosis (odds ratio 3.5) in Italians[32]. However, this finding has yet to be confirmed in follow-up studies.

Polymorphisms for TNF-α co-responsible for an increased risk of liver disease have been discovered in a similar way[33]. For the time being, though, we do not know how to make use of this new knowledge in routine practice.

Malnutrition is another clinical situation with an impact on the evolution of ALD. Heavy alcohol drinkers often lack proper diets or consume diets which are compromised in various nutrients, such as proteins, polyunsaturated fatty acids and vitamins[34].

Liver steatosis is the most frequent primary change in chronic alcohol abuse. Changes associated with alcohol metabolism may subsequently trigger an inflammatory reaction, resulting in alcoholic hepatitis or chronic liver disease (Figure 3).

Liver disease in alcohol abusers is more likely to take the form of chronic changes (steato-hepatitis and fibrosis), leading to cirrhosis later in life. The spectrum of histological findings can be described as a dynamic process[35] (Figure 4). Simple steatosis is reversible after a number of weeks of abstinence; steatohepatitis, a condition seen in only some alcoholics, is a fibrogenic process which can induce changes leading to cirrhosis. Steatohepatitis is also reversible, although a certain degree of fibrosis may persist. The reversibility of steatohepatitis or even fibrosis in humans is well documented by trials on the treatment of chronic hepatitis C[36] and experimentally on NASH models[37]. Steatohepatitis, in particular, often coincides with liver cirrhosis in active alcoholics and is a frequent cause of decompensation of cirrhosis[38].

Simple steatosis is regarded as a benign condition; nevertheless, given continued abuse, it too, can induce fibrogenesis[39]; in any case, up to 20% of the patients with simple steatosis are likely to develop fibrosis or cirrhosis within a period of ten years[40]. The prognosis of a patient with cirrhosis depends mainly on the presence of complications because of portal hypertension and continued abuse of alcohol. Abstainers with decompensated cirrhosis have a five year survival at a rate of 60% against the 30% survival rate in those who continue in the abuse[41].

Severe alcoholic hepatitis, although relatively rare, has a death rate of up to 50%. Identifying individuals with a high mortality risk is crucial in the management of acute alcoholic hepatitis. Multiple prognostic factors were studied over the last decade, including Child-Pugh classification (CTP), Maddrey score (bilirubin mg/dL + 4.6 × prothrombin time)[42] and others. The MELD score was found a more valuable model than CTP or the Maddrey score in the detection of high risk patients admitted with alcoholic hepatitis[43]. Alternatively, the more recent Glasgow alcoholic hepatitis score could be used[44]. A Glasgow score exceeding 9 points is associated with poor prognosis (Table 1).

Table 1 Glasgow alcoholic hepatitis score[44]

Parameter/score	1	2	3
Age (yr)	< 50	⩾ 50	-
Leucocytes (10^9/L)	< 15	⩾ 15	-
Urea (mmol/L)	< 5	⩾ 5	-
INR	< 1.5	1.5-2	> 2
Bilirubin (μmol/L)	< 125	125-250	> 250

The score is to be added to each parameter, the sum total being between 5 and 12 points. The value of 9 and higher implies poor prognosis in alcoholic hepatitis. **INR: International normalised ratio.**

CLINICAL MANIFESTATION AND LABORATORY FINDINGS

Patients with steatosis are usually symptom-free; they may have slightly elevated liver function tests and enlarged liver (both are often discovered accidentally during examination for other reasons).

In the stage of acute alcoholic hepatitis, there may be nausea, loss of appetite, gradual loss of weight, icterus and other symptoms of liver dysfunction (prolonged prothrombin time, hypoalbuminemia, ascites, and hepatic encephalopathy). Patients with alcoholic hepatitis usually show increased liver test results, including gamma-glutamyl transferase (GGT), hypergammaglobulinemia and enlarged liver.

Sonography is the basic imaging technique for liver examination. Liver biopsy, while not always necessary, can help to differentiate simple steatosis from steatohepatitis, fibrosis or incipient cirrhosis. Precise definition of the liver fibrosis stage is essential for management and prognosis in clinical practice. Recently, blood markers and instrumental methods have been proposed for non-invasive assessment of liver fibrosis[45]. However, there are still some doubts as to their implementation in clinical use. Non-invasive examination with transient elastography takes advantage of the fibrotic liver tissue ability to change the velocity of ultrasound propagation. The results of this method correlate well with the bioptically proved degree of fibrosis[46]. Similar results could be obtained from a combination of biochemical and clinical parameters of fibrosis. As for the clinical picture, the state of alcoholic liver cirrhosis shows no difference from cirrhosis of other etiology[38].

ASSESSMENT OF ACTIVE ALCOHOL ABUSE

Assessment of continued alcohol abuse in patients with alcoholic liver disease is essential for their treatment as well as prognosis. Those with alcoholic cirrhosis also make up a significant part of patients indicated for liver transplantation (30%-50%), bearing in mind that abstinence is an essential condition for considering this treatment. Continued alcohol abuse is evaluated on the basis of clinical history, psychological examination and laboratory testing. Thorough clinical and psychological examination is the crucial condition for alcohol abuse diagnosis. Regarding the clinical history, the diagnosis of alcohol abuse and dependence was substantially improved by implementation of simple methods such as a single question inquiring how often the maximum daily alcohol limit has been exceeded[47]. Other clinical screening tools such as the need to cut down, annoyed by criticism, guilty about drinking need for an eye-opener in the morning (CAGE), and the alcohol use disorders identification test (AUDIT-C) are also very easy to apply[48]. With the CAGE questionnaire, two positive answers indicate alcohol dependence with a sensitivity of more than 70% and specificity of more than 90%. The AUDIT-C screening thresholds for the detection of alcohol abuse are ⩾ 4 points for men (sensitivity 86%, specificity 89%) and ⩾ 3 points for women (sensitivity 73%, specificity 91%).

As for laboratory tests, continued abuse can be read from higher GGT values, increased AST/ALT ratio or an increased volume of red blood cells (MCV). In advanced liver cirrhosis, however, the values of hepatic enzymes fall short of sufficient sensitivity or specificity levels. More information about the actual abuse of alcohol can be derived from the percentage of carboxy-deficient transferrin estimation (%CDT) in serum or ethyl glucuronide in urine or hair[49]. A CDT value greater than 2.8% has a 79% sensitivity and 92% specificity for active alcohol abuse detection in patients with advanced cirrhosis[50].

PREVENTION OF ALD

Prevention of or treatment for alcohol abuse are crucial steps in the prevention of ALD[51]. Alcohol dependence is a chronic relapsing medical disorder which is treatable when efficacious medicines are added to enhance the effects of psychosocial treatment. Medication with, e.g., naltrexone and acamprosate showed mixed results in previous clinical trials[52]. Rősner *et al*[53] recently performed a meta-analysis to determine the efficacy and tolerability of acamprosate in comparison with placebo and other pharmacological agents. Almost 7000 patients in 24 double-blind randomised controlled trials were evaluated. Compared to placebo, acamprosate was shown to significantly reduce the risk of any drinking (RR 0.86) and to significantly increase the cumulative abstinence duration. The only side effect that was more frequently reported under acamprosate than with placebo was diarrhea. The authors of this Cochrane review conclude that acamprosate appears to be an effective and safe treatment strategy for supporting continuous abstinence after detoxification in alcohol dependent patients. Indeed, without a pharmacological adjunct to psychosocial therapy, the clinical outcome is poor, with up to 70% of patients resuming drinking within one year[54].

The prevention of liver injury in active alcohol abusers is not clinically applicable. For example, in an experiment, the addition to the diet of polyunsaturated fatty acids prevented alcohol-induced fatty liver and mitochon-

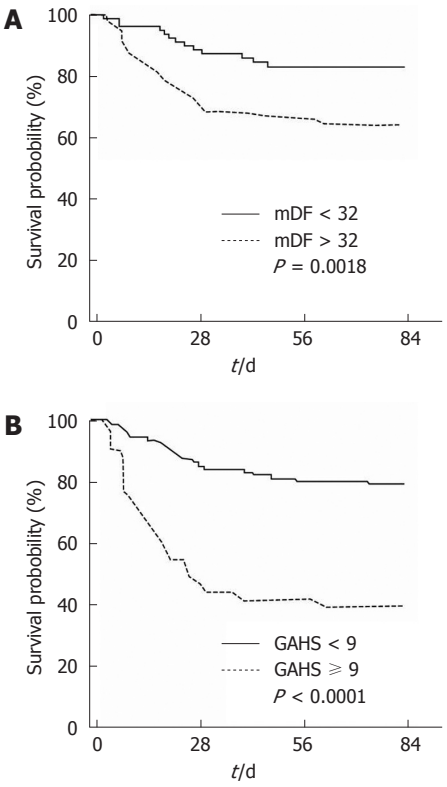

Figure 5 Kaplan-Meier survival analysis relative to the modified Maddrey discriminant function (mDF) (A) and the Glasgow alcoholic hepatitis score (GAHS) (B). The Glasgow score was developed on 241 patients and validated on 195 separate patients[44].

drial dysfunction in an animal model of ALD by protecting various mitochondrial enzymes, most likely through reducing oxidative/nitrosative stress[55]. The clinical use of similar medicaments would probably be always hampered by alcohol abusers´ failure to comply.

TREATMENT

Absolute abstinence is essential to consider any treatment for alcoholic liver disease. Even major changes, including cirrhotic restructuring, may show partial regression during total abstinence[56]. Portal hypertension declines and even regression of esophageal varices have been reported in abstainers. This, however, appears to have resulted from the remission of inflammatory changes and steatosis rather than from regressing fibrosis or cirrhosis. Sustained abstinence markedly improves the patient's prognosis in any phase of the liver disease[57], prevents the progression of the disease and fibrosis and, probably, also the development of hepatocellular carcinoma[58].

Pharmacotherapy of liver disease has but a supportive and rather dubious relevance. Treatment with silymarin, essential phospholipids or vitamin preparations was very popular in the past. Since an oxidative stress has been implicated in the pathophysiology of hepatic insult, the use of natural compounds with anti-oxidant properties represents an extremely popular therapeutic option for the treatment of liver disease. One such phytochemical, resveratrol, is remarkable as it is known as a major constituent of an alcoholic beverage, red wine. Resveratrol was shown to prevent liver injury by means of scavenging free radicals and inflammatory cytokines in experimental studies[59]. Its clinical utilization, though, is still far away. There are no conclusive data to prove the efficacy of any antioxidant medicaments for longer survival time or improved clinical conditions in the treatment of ALD. These are mostly cases of rather costly placebo. In contrast, dietary readjustment in the sense of sufficient energy intake and adequate supply of proteins is of value because malnutrition is a very poor prognostic factor in liver diseases[60]. What has been described as "liver diet" with increased supply of saccharides at the restriction of proteins and fats has no substantiation. Appropriate caloric intake with sufficient supply of proteins and polyunsaturated fats is important[34,61].

Severe alcoholic hepatitis has been treated with corticoids in many trials, with the best results in patients with hepatic encephalopathy, Maddrey score > 32 or Glasgow score > 9[62]. The Glasgow score is very simple to evaluate and its prognostic value is also greater than that of any other classification (Figure 5). The corticoid dose in that case is 40 mg prednisolone per day. The side effects of glucocorticosteroids must be also taken into consideration, as some patients on glucocorticosteroids experience adverse effects, mainly in the form hyperglycemia, Cushing's syndrome and increased risk of infection[63]. Despite the fact that the available trials are rather heterogeneous and some authors do not recommend the use of steroids in alcoholic hepatitis, recently published data emphasize the effect of corticosteroids on short-term survival of patients with severe alcoholic hepatitis[64], particularly in those with Maddrey score > 32.

Some trials and reviews of pentoxifylline (PTX) have shown a better risk/benefit profile than that of steroids and suggested that PTX could be a better first-line treatment in patients with severe AH. The efficacy of PTX in severe AH was first demonstrated by Akrividais *et al*[65] in 2000 on a group of 101 patients with severe AH. 24.5% of the patients who received PTX died during their index hospitalization, compared to a 46.1% mortality in the placebo group ($P = 0.037$). Remarkably, hepatorenal syndrome was the cause of death in 50% of patients on PTX compared to 91.7% of the HRS-related deaths in the placebo group ($P = 0.009$). According to the authors, the benefit appears to be related to a significant decrease in the risk of developing hepatorenal syndrome. In fact, renal dysfunction is frequent in patients with severe alcoholic hepatitis and, it seems, could be prevented with PTX[66].

Even in direct comparison with corticosteroids in a randomized trial, pentoxifylline was found to be superior to prednisolone for the management of severe alcoholic hepatitis regarding reduced mortality, improved risk-benefit profile and renoprotective effect[67]. Nevertheless, this observation should be confirmed on a larger cohort of patients[68]. A recent study by Lebrec *et al*[69] stopped short of confirming the effect of PTX on better survival but,

unlike a previous study, only Child-Pugh class C patients were included. However, the study did confirm a reduced risk of complications, such as bacterial infection, renal insufficiency, hepatic encephalopathy or gastrointestinal hemorrhage in patients treated with PTX compared to placebo.

Some centers recommend the use of PTX as the routine first line treatment of severe alcoholic hepatitis at a dose of 400 mg orally 3 times daily for a period of at least 4 wk[70]. They point to its safety, low cost and scope for long-term treatment. Significantly enough, the sweeping use of PTX as a first-line option is not generally recommended[71] and steroids should be used in patients with severe alcoholic hepatitis. Pentoxifylline could be used in patients with ineffectiveness or contraindications to steroids. The combination of pentoxifylline and steroids waits for clinical evaluation.

Biological treatment with anti- TNF-α antibodies fell short of expectations[72,73] so it can no longer be recommended for the management of alcoholic hepatitis[74].

Many studies with diverse conclusions have been published on the subject of nutrition and alcoholic hepatitis. In general, patients with alcoholic liver disease are frequently malnourished, a condition which worsens the prognosis[75]. However, the situation is not all that easy, as the spectrum of nutritional status in these patients may range from severe malnutrition to morbid obesity. The nutritional intervention on an outpatient basis depends on the degree of malnutrition, obesity and cooperation. In general, supplementation of multivitamins, folic acid and thiamine could be of value in chronic alcohol abuse, but data in the relevant literature are limited. Night-time nutritional supplements (approximately 700 kcal/d) may prevent muscle wasting and improve lean muscle mass in patients with liver cirrhosis[76] and should be considered, also relative to alcoholic hepatitis in patients with evidence of liver cirrhosis.

More data are available regarding the treatment of severe alcoholic hepatitis by enteral nutrition. The benefit of tube-feeding over the regular diet was demonstrated previously[77]. Patients on tube-fed nutrition had improved PSE scores, bilirubin and antipyrine clearance.

Many reviews and recommendations refer to a study by Cabre *et al*[78], which clearly demonstrated the efficacy of tube-fed nutrition. In their multi-center study, 71 patients with severe alcoholic hepatitis were randomized to receive 40 mg/d prednisolone or enteral tube feeding for 28 d and were followed up for 1 year. Mortality during the treatment was similar in both groups but during the follow-up significantly higher with steroids (37% *vs* 8%; $P = 0.04$), mainly because of infections with steroid treatment. The authors concluded that, unlike steroids, enteral nutrition had similar short-term mortality rates, improved 1 year mortality rates and reduced infectious complications. While some studies refrain from confirming any favorable effect of enteral feeding on survival, the implementation of tube-feeding in the treatment of acute alcoholic hepatitis is generally accepted[79]. There are only inconsistent data concerning the use of parenteral nutrition.

Despite the progress in the treatment of severe acute alcoholic hepatitis, the prognosis is still poor.

Alcoholic cirrhosis as such is treated in the same way as cirrhosis of other etiology; in particular, with adequate nutrition, bone disease prevention and prevention or treatment of liver cirrhosis complications (e.g., bleeding from esophageal varices, ascites, spontaneous bacterial peritonitis, hepatic encephalopathy)[80].

Quite a few medicinal products were tested for the treatment of alcoholic cirrhosis: antiphlogistics/propylthiuracil[81], colchicine[82], antioxidants/silymarin[83,84] and also phosphatidylcholine[85]. However, none of these were found to have a favorable effect on survival time and none are recommended for this particular indication any longer. Medicaments with a direct antifibrotic effect are still under evaluation[86].

Patients with advanced cirrhosis can be considered for liver transplantation, provided they are total abstainers[87]. In such cases, a five year post-transplantation survival can reach anything up to 85%[88].

CONCLUSION

Long-term intake of more than 30 g of absolute alcohol per day increases the risk of alcoholic liver disease; liver disease is nearly certain in long-term consumption in excess of 80 g of absolute alcohol per day. Alcoholic liver disease may take the chronic form (steatosis, steatohepatitis, fibrosis, cirrhosis) or that of acute hepatitis. Steatosis is fully reversible, which does not apply to the other conditions; cirrhosis is associated with a markedly shortened life expectancy. The results of laboratory testing in alcoholic liver disease usually include: increased GGT, AST/ALT ratio greater than 2 and increased MCV. Sonography will reveal enlarged liver and signs of steatosis. Absolute abstinence is an essential therapeutic precaution; no hepatoprotective treatment has been shown to improve the course of the disease. Likewise, there is no medicine that would demonstrably "protect" from the effects of alcohol.

The clinical course of severe alcoholic hepatitis could be improved with corticoids, enteral nutrition and pentoxifylline, although more clinical data are necessary to standardize or combine this treatment.

Patients with advanced cirrhosis should be considered for liver transplantation, provided they are verifiable abstainers.

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Online Tools

Alcohol-Related Disease Impact (ARDI) application (http://www.cdc.gov/ARDI)

To assist professionals in state and local public health departments in estimating the impact of alcohol-attributable deaths (AAD) and years of potential life lost (YPLL)—a measure of premature death—the Centers for Disease Control and Prevention (CDC) funded the development of the Alcohol-Related Disease Impact (ARDI) application (http://www.cdc.gov/ARDI). Originally released in 1989, ARDI was specifically designed to allow states to calculate AAD, YPLL, direct health care costs, indirect morbidity and mortality costs, and non-health-sector costs associated with alcohol misuse.

In 2002, with support from a grant from the Robert Wood Johnson Foundation, the Alcohol Program in CDC's National Center for Chronic Disease Prevention and Health Promotion began updating ARDI and migrating it to the Internet to be more accessible to state-based epidemiologists and other users. In September 2004, the new version of ARDI was released in conjunction with a *Morbidity and Mortality Weekly Report* article entitled Alcohol-Attributable Deaths and Years of Potential Life Lost — United States, 2001 (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5337a2.htm). That report highlighted that approximately 75,000 AAD occurred in 2001 resulting in approximately 2.3 million YPLLs. In February 2008, additional years of data were added, which resulted in 5-year average annual estimates of health effects based on the years 2001–2005. This was done to produce more stable estimates than can be obtained from a single year of data. In December 2013, the data in ARDI were updated once again to provide average annual estimates of AAD (88,000) and YPLL (2.5 million) for 2006-2010.

ARDI includes reports for all 50 states with options to view each report by gender and age groups. Since the original release in 2004, ARDI has been enhanced to include reports specifically focused on individuals younger than 21 years of age. In addition, the custom data feature has been enhanced to make it easier for users to conduct sub-state (e.g., county-level) analyses of alcohol-attributable deaths and YPLL.

Alcohol Policy Information System (APIS) (http://www.alcoholpolicy.niaaa.nih.gov/)

Managed by the National Institute on Alcohol Abuse and Alcoholism (NIAAA), the Alcohol Policy Information System (http://www.alcoholpolicy.niaaa.nih.gov/) (APIS) provides detailed information on 35 state and federal alcohol policies in the policies. These policies fall under the following categories:

- Alcohol beverages pricing
- Alcohol control systems
- Blood alcohol concentration (BAC) limits
- Health care services and financing

- Pregnancy and alcohol
- Retail sales
- Taxation
- Transportation
- Underage drinking

APIS is intended to support alcohol policy research and public health practice in preventing excessive alcohol use. APIS also provides a variety of informational resources of interest to alcohol policy researchers, public health practitioners, community coalitions, and others involved with alcohol policy issues. The system is based on in-depth legal research conducted by attorneys with special expertise in alcohol policy. APIS is updated annually, and the list of policies being tracked by the system is updated periodically on the basis of researchers' and public health practitioners' needs.

Consumer Costs and Job Impacts from State Alcohol Tax Increases
(http://www.camy.org/action/taxes/taxtool/)

The Community Preventive Services Task Force recommends increasing alcohol taxes based on strong evidence that this can reduce excessive alcohol consumption and related harms. However, questions have been raised about the potential impact of alcohol tax increases on the cost of alcohol to individual drinkers and on state employment. To help answer these questions, the Alcohol Program in the Centers for Disease Control and Prevention (CDC) funded a research collaborative involving the Johns Hopkins Bloomberg School of Public Health, the University of Florida, the University of Illinois at Chicago, and Boston Medical Center. This new web tool was developed based on these research findings to model how much extra adult drinkers would pay for alcohol following various hypothetical state alcohol tax increases based on how much they drink, income, and employment status, as well as the effect these hypothetical tax increases would be expected to have on state employment.

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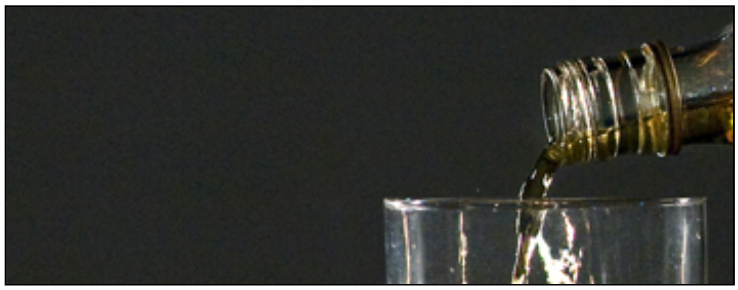
Excessive Drinking Costs U.S. $223.5 Billion



Excessive alcohol consumption cost the United States $223.5 billion in 2006, or about $1.90 per drink. By implementing effective community-based prevention strategies, we can reduce excessive alcohol consumption and its costs.

Excessive alcohol consumption is known to kill about 88,000 people in the United States each year, but a study released by the CDC and The Lewin Group shows that it has a huge impact on our wallets as well.

The cost of excessive alcohol consumption in the United States reached **$223.5 billion** in 2006 or about **$1.90 per drink**. Almost three-quarters of these costs were due to **binge drinking**. Binge drinking is defined as consuming four or more alcoholic beverages per occasion for women or five or more drinks per occasion for men, and is the most common form of excessive alcohol consumption in the United States.

The researchers found that the cost of excessive drinking was quite far-reaching, reflecting the effect this dangerous behavior has on many aspects of the drinker's life and on the lives of those around them. The costs largely resulted from losses in **workplace productivity** (72% of the total cost), **health care** expenses for problems caused by excessive drinking (11% of total), law enforcement and other **criminal justice** expenses related to excessive alcohol consumption (9% of total), and **motor vehicle crash** costs from impaired driving (6% of the total).

The study analyzed national data from multiple sources to estimate the costs due to excessive drinking in 2006, the most recent year for which data were available. The study did not consider a number of other costs such as those because of pain and suffering among either the excessive drinker or others that were affected by their drinking, and thus may be an underestimate. Nevertheless, the researchers estimated that excessive drinking cost **$746 for every man, woman, and child** in the United States in 2006.

What You Need to Know About Binge Drinking

- Binge drinking is reported by about 18% of U.S. adults.
- Binge drinking is most common among men, 18- to 34-year-olds, white people, and people with household incomes of $75,000 or more.
- Most binge drinkers are not alcohol dependent.

How Can We Prevent Excessive Alcohol Consumption and Reduce Its Economic Costs?

Communities can use evidence-based strategies, such as those recommended by the Community Preventive Services Task Force (http://www.thecommunityguide.org/alcohol) to prevent excessive drinking. These include:

- Increasing alcohol excise taxes.
- Reducing alcohol outlet density.
- Reducing the days and hours of alcohol sales.
- Holding alcohol retailers liable for injuries or damage done by their intoxicated or underage customers.

By implementing these evidence-based strategies, we can reduce excessive alcohol consumption and the many health and social costs related to it.

More Information

- Learn more about effective strategies communities can use to prevent excessive drinking and its costs (http://www.thecommunityguide.org/alcohol)
- Web Feature: The high cost of excessive drinking to states (http://www.cdc.gov/features/costsofdrinking/)
- Infographic: The real cost of excessive alcohol use (http://www.cdc.gov/alcohol/onlinemedia/infographics.htm)
- Find tools and resources related to the surveillance and prevention of excessive alcohol consumption and its costs. (http://www.cdc.gov/alcohol)
- Article: Economic costs of excessive alcohol consumption in the U.S., 2006 (http://www.ncbi.nlm.nih.gov/pubmed/22011424)
- Article: State costs of excessive alcohol consumption, 2006 (http://www.ncbi.nlm.nih.gov/pubmed/24050424)
- Excessive Alcohol Use Vital Signs Report (http://www.cdc.gov/vitalsigns/issues.html)
- Binge Drinking Fact Sheet (http://www.cdc.gov/alcohol/fact-sheets/binge-drinking.htm)
- Binge Drinking Video (http://www.cdc.gov/CDCTV/BingeDrinking/)
- Binge Drinking MMWR (http://www.cdc.gov/mmwr/preview/mmwrhtml/su6001a22.htm?s_cid=su6001a22_w)
- CDC Alcohol and Public Health Program (http://www.cdc.gov/alcohol)
- The High Price of Excessive Alcohol Consumption [PODCAST - 01:27 minutes] (http://www2c.cdc.gov/podcasts/player.asp?f=8621340)

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Licorice infusion: Chemical profile and effects on the activation and the cell cycle progression of human lymphocytes

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A licorice infusion (LI) and its major constituents were investigated for their capacity to stimulate the activation and the cell cycle progression of human lymphocytes, measured by the CD69 expression and DNA content, respectively. The chemical profile of LI was determined by high-performance liquid chromatography-diode array detection (HPLC-DAD). Results: Two major components of LI were identified as liquiritin (1) and glycyrrhizin (2); total flavones and flavonols were shown as its minor constituents. The LI (100-800 μg/ml) stimulated the expression of CD69 on lymphocytes in a concentration-independent manner. Values of the activation index (AI) of total lymphocytes treated with LI (100-800 μg/ml) did not differ significantly among them ($P < 0.05$), but were 50% lower than the AI value exhibited by cells treated with phytohemagglutinin (PHA). The LI showed a similar effect on T cells, but on a lower scale. Compounds 1 and 2 (12-100 μg/ml) did not stimulate the CD69 expression on lymphocytes. The LI, 1 and 2 showed no meaningful effect on cell cycle progression of lymphocytes. The experimental data indicates that LI stimulates the activation of lymphocytes as a result of a proliferation-independent process. This finding suggests that LI could be considered as a potential specific immune stimulator.

Key words: CD69 expression, flavonoids, HPLC, licorice, lymphocytes, phenolics

INTRODUCTION

Licorice, the dry roots of *Glycyrrhiza glabra* L. (Fabaceae), is considered one of the oldest and most widely used herbal drugs around the world, being present in most pharmacopoeias of Eastern and Western countries.[1] It has been traditionally used for respiratory, gastrointestinal, cardiovascular, genitourinary, eye, and skin disorders, and for its antiviral effects.[2] Glycyrrhizin and flavonoids such as liquiritin, isoliquiritin, and their aglycones have been reported as the major constituents of licorice and they are perceived as the active principles responsible for its pharmacological efficacy.[3]

The danger to global public health because of viral pandemic diseases such as those induced by influenza and HIV viruses requires the urgent evaluation of herbal drugs in widespread traditional use. Given that traditional sources mention licorice to treat symptoms attributable to viral infections, it is gaining attention as a potential immunomodulating agent.[4] The immunological action of herbs may involve the activation and induction of the cell cycle progression of immune cells, which play important roles in the generation of immune responses.[5] Licorice is consumed customarily in the form of teas and infusions,[6] but the immunomodulating properties of these aqueous preparations and the relation of such effect with its major constituents have been little explored.

The aim of the present study was to investigate the capacity of a licorice infusion (LI) and its major constituents to stimulate the activation and the cell cycle progression of human lymphocytes, using flow cytometry. The chemical profile of LI was determined by HPLC-DAD and spectrophotometric methods.

MATERIALS AND METHODS

Chemicals

Hide powder, standards (glycyrrhizin and quercetin), propidium iodide, ribonuclease A, phytohemagglutinin (PHA), and Tween-20 were obtained from Sigma Aldrich (Steinheim, Germany). Standard liquiritin was purchased

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from Wuhan Sunrise Technology Development Co., Ltd. (Hong Kong, China). Folin-Ciocalteu phenol reagent, aluminum chloride hexahydrate, gallic acid, and sodium carbonate were from Merck (Darmstadt, Germany). HPLC grade solvents were from Merck. Ultrapure water from the Milli-Q RG system (Millipore, Molsheim-France) was used. The monoclonal antibodies (phycoerythrin (PE), fluorescein isothiocyanate (FITC), and Allophycocyanin (APC) labeled) were obtained from Immunotech (France) and Dako (Denmark). X-Vivo medium was purchased from Bio-Wittaker (USA).

Sample collection and infusion preparation

Roots of *G. glabra* were collected in February 2008 from the Botanical Garden of the Faculty of Horticulture, Mendel University in Brno, Czech Republic (situated 164 m above sea level). The genetic resource was identified with the code 0001. The plant material was dried at 40°C in an oven and was subsequently ground to fine powders (mesh size 20). The infusion was prepared by adding 150 ml of distilled water (95-100°C) to a precisely weighed amount (1.50 g) of licorice powder.[7] The infusion was brewed for 20 minutes and was then filtered over Whatman No. 1 paper. The resulting aqueous extract was lyophilized and the extraction yield was calculated based on the dry weight of the licorice. The licorice lyophilized infusion (LI) obtained was assessed for its biological activities and chemical profile.

Determination of total content of phenolics

The total phenolic (TP) content was determined using the Folin-Ciocalteu procedure.[8] Briefly, the appropriate extract dilution was oxidized with the Folin-Ciocalteu reagent and the reaction was neutralized with sodium carbonate. The absorbance of the resulting blue color was measured at 760 nm after 30 minutes using a Shimadzu UV-1601 UV/Vis spectrophotometer. Quantification was plotted on a standard curve of gallic acid. The results were expressed as mg gallic acid equivalents (GAE)/100 mg of LI. Data are reported as means ± standard deviation (SD) to an accuracy of three replicates.

Determination of total content of tannins

After removal of tannins by adsorption on an insoluble matrix (hide powder), the total tannin (TT) content was determined by Folin-Ciocalteu procedure explained briefly in the previous paragraph. Calculated values were subtracted from the total phenolic contents and total tannin contents are expressed as mg gallic acid equivalents (GAE)/100 mg of LI. Data are reported as means ± standard deviation (SD) to an accuracy of three replicates.[9]

Determination of total content of flavones and flavonols

The total flavones and flavonols (TF) content was determined according to the aluminum chloride method.[8] Quercetin was used as a reference for the calibration curve. The absorbance of the reaction mixture was measured at 415 nm. Results were expressed as mg quercetin equivalents (QE)/100 mg of LI. Data are reported as means ± SD to an accuracy of three replicates.

HPLC-diode array detector analysis

The HPLC analysis was performed with a Jasco PU-2089 pump equipped with a Jasco MD-2015 diode array detector (DAD), and chromatographic separations were performed on a LiChrospher RP-18 column (4.0 × 250 mm i.d., 5 μm). Isocratic elution was used with a mobile phase containing acetonitrile: Methanol: Water: Glacial acetic acid (35:35:29:1, by volume) at a flow rate of 1 ml/min.[10] Separations were carried out at 25°C with an injection loop of 20 μl. The DAD detector was operated in the range of 200-650 nm, and the analysis was performed at 254 nm. Components of LI were identified by comparing their retention times and UV spectra with those of authentic standards (liquiritin and glycyrrhizin) under identical analysis conditions. Solutions at different concentrations of each standard were injected into the HPLC to check the linearity between concentration and peak areas, and a response factor was calculated. Quantifications of liquiritin and glycyrrhizin were done using these calibration factors.

Activation of immune cells

The activation of total lymphocytes and T cells was analyzed by flow cytometry, measured by CD69 expression.[11] Using sterile 96-well flat-bottomed plates, 100 μl of peripheral blood suspension were incubated along with 100 μl of X-Vivo medium containing test samples with increasing concentrations and without test samples (control). The mitogen PHA was used as a positive control at 10 μg/ml. Samples were filtered through 0.2 μm filters before use. Final concentrations of LI and compounds in the assay media were in the range of 100-800 μg/ml and 12-100 μg/ml, respectively. Plates were then incubated at 37°C with 5% CO_2 for 24 hours. After incubation, 40 μl of each incubated suspension was labeled with a cocktail of fluorescently labeled antibodies CD69 PE, CD69 FITC, CD3 APC. Flow cytometric analysis was performed on a Cytomics FC500 flow cytometer (Beckman Coulter, USA) and data were analyzed by CXP analysis software (Coulter Electronic, USA). Cell activation was measured by increases in mean fluorescence intensities (MFI) because of the expression of CD69 on the cell surface. Fluorescence signals were obtained as logarithmically amplified signals. Values of activation are presented as activation index (AI), which were calculated by dividing the MFI of treated cells with test samples by that of untreated cells (control). A positive immune cell response was defined as an AI ≥ 2. Data were presented as the mean ± SD for three experiments.

Cell cycle progression of lymphocytes

To assess the effect of samples on the cell cycle progression of lymphocytes, the DNA content of individual cells stained with propidium iodide was analyzed by flow cytometry.[5] Briefly, 100 µl of diluted peripheral blood (1:10) was incubated with test samples for 72 hours. Lymphocytes were isolated from red cells by lysis in hypotonic solution followed by centrifugation. Cells were fixed with 4% paraformaldehyde for at least five minutes and the supernatant was removed by centrifugation at 1100 rpm for 10 min. A volume of 500 µl of 0.5% Tween, 50 µl of ribonuclease A, and 50 µl of propidium iodide solution were added and the mixture was incubated for 60 minutes at 37°C with 5% CO_2. Fluorescence (DNA content) was measured on a Cytomics FC500 flow cytometer (Beckman Coulter, USA). A minimum of 10 000 cells analyzed in each sample served to determine the percentages of cells in each phase of the cell cycle; Multicycle AV software was used for the analysis (Phoenix Flow Systems Inc., USA).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the GraphPad Prism 5 software. Differences were considered significant when $P < 0.05$.

RESULTS

The present study reports the total content of phenolic, flavonoids, and tannins in a licorice infusion [Table 1]. The extraction yield of licorice lyophilized infusion (LI) was about 30%. The data from the HPLC-DAD analysis of LI is presented in Table 1, whereas, the HPLC chromatogram is presented in Figure 1. The two major components of LI were identified as liquiritin 1 and glycyrrhizin 2 [Figure 2] by comparing with the retention times and UV spectra of authentic standards. Peaks 3 and 4 of the chromatogram [Figure 1] showing UV λmax at 248 nm were preliminarily identified as derivatives of glycyrrhetic acid.

The effects of LI, 1, and 2 on the activation of total lymphocytes, measured by the CD69 expression, was analyzed by flow cytometry. As indicated by a shift to the right in histograms [Figure 3b, 4b], the CD69 expression on cells was increased after 24 hours. As the cells were activated, the amount of the fluorescently labeled antibodies bound to them increased. The LI moderately stimulated the expression of CD69 on lymphocytes in a concentration-independent manner in the range of 100-800 µg/ml. Values of the activation index (AI) of total lymphocytes treated with LI in the range of 100-800 µg/ml did not differ significantly among them ($P < 0.05$), but were lower than the AI value exhibited by cells treated with PHA [Figure 3d]. The LI showed a similar effect on T cells, but on a lower scale [Figure 4d]. Compounds 1 and 2 in the range of 12-100 µg/ml did not stimulate the CD69 expression on lymphocytes [Figure 3c, 4c]. It was observed that the LI, 1, and 2 showed no meaningful effect on cell cycle progression of lymphocytes. Most of the lymphocytes treated with LI, 1, and 2 were in G_1 phase, and there was only a very small number of cells in S or G_2 phases. The PHA stimulated lymphocytes to enter into the S and G_2 phases after 72 hours of culture [Figure 5].

DISCUSSION

Chemical analysis

About 300 kinds of phenolic compounds have been isolated from various species of *Glycyrrhiza* and many of them are described as exhibiting biological actions that supplement the efficacy of licorice.[3] Polyphenols are bioactive compounds believed to be involved in the defence process against oxidative damage in biological systems, owing at least in part to their antioxidant properties.[12] In a prior study, the content of TP (7.42 µg/mg) and TF (0.88 µg/mg) in a licorice extract were reported.[13] In another study, the TF content of *G. pallidiflora* was measured by two different methods. The TF contents were 2.09 mg/100 mg and 4.27 mg/100 mg of the extract, measured by the $AlCl_3$ and $Al(NO_3)_3$ methods, respectively.[14] Most reports on the flavonoid content in licorice are usually based on flavanones and chalcone such as liquiritin, isoliquiritin, and their corresponding aglycones.[3] Unlike flavanones from licorice, other types of minor flavonoids such as flavones and flavonols have been shown to exhibit strong antioxidant effects.[15] To date, there is very limited information on the TT content in the roots of

Table 1: Content of liquiritin, glycyrrhizin, and total polyphenolics in licorice infusion

Compounds	Content in dried roots	Content in lyophilized extract (LI)	Regression equation	Correlation coefficient (r^2)
Liquiritin	1.55 ± 0.05	5.18 ± 0.17	$Y = 246.02X + 1.9512$	0.9999
Glycyrrhizin	2.23 ± 0.04	7.43 ± 0.16	$Y = 161.05X + 0.0747$	0.9995
TP content[a]	1.75 ± 0.02	5.83 ± 0.06	$Y = 0.1011X + 0.0206$	0.9992
TF content[b]	0.21 ± 0.01	0.70 ± 0.02	$Y = 0.0535X + 0.0002$	0.9999
TT content[c]	0.24 ± 0.01	0.78 ± 0.01	$Y = 0.1011X + 0.0206$	0.9992

[a]Total phenolics (TP) and[c]total tannins (TT) contents are expressed as mg gallic acid equivalents (GAE)/100 mg LI.[b]Total flavones and flavonols (TF) content is expressed as mg quercetin equivalents (QE)/100 mg LI. Contents of liquiritin and glycyrrhizin are expressed as percentage (w/w) in terms of dry weight. Each value represents mean (n = 3) ± SD

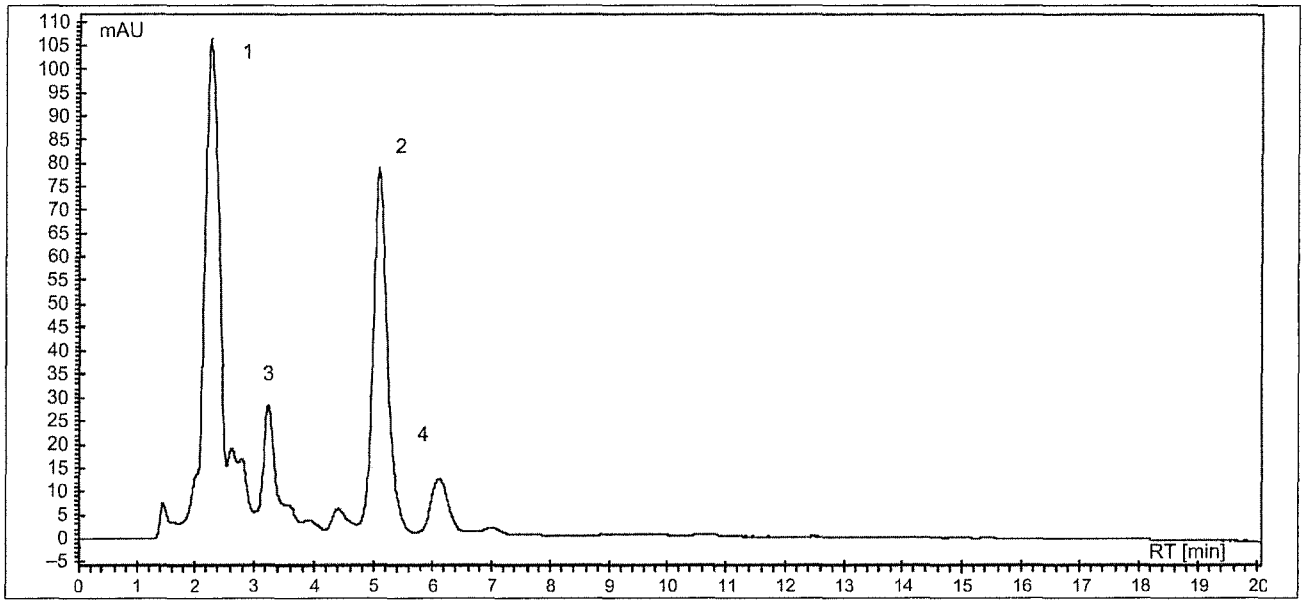

Figure 1: HPLC chemical profile of licorice infusion (LI). Detection at 254 nm. Peaks: (1) liquiritin; (2) glycyrrhizin; (3) and (4) glycyrrhetic acid derivatives (at column width)

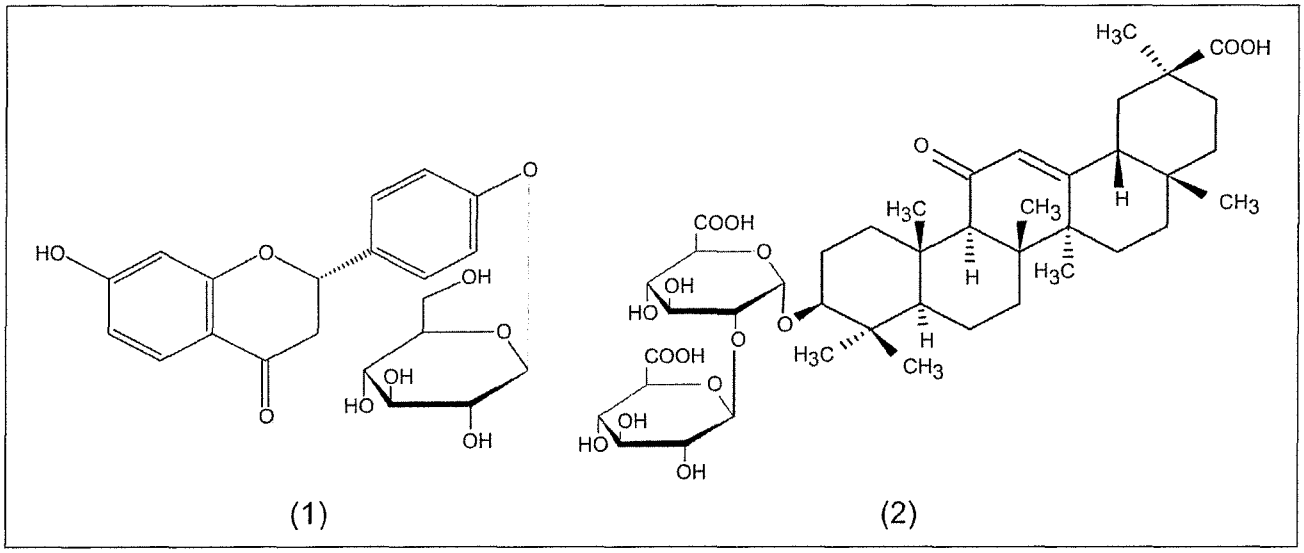

Figure 2: Structures of liquiritin (1) and glycyrrhizin (2) (at column width)

Glycyrrhiza species. Two flavonoids, named licochalcone B and glycyrrhisoflavone, were isolated from an acetonic- aqueous extract of licorice and they exhibited tannin-like activity.[15] Glycyrrhizin has been reported to constitute 10-25% of licorice extract[16] and the liquiritigenin glycosides were reported to constitute 1.6% of licorice aqueous extract.[17] As can be noticed, the comparison between the values reported by different laboratories can be complicated because of substantial differences in sample preparation, geographic sources, harvesting, and expression of results.

Activation of immune cells

The CD69 glycoprotein is a very early cell activation molecule expressed on the surface of T, B, and Natural Killer (NK) cells following activation.[18] It can appear within 1-2 hours of activation and exhibits maximal expression levels between 18 and 24 hours after stimulation.[19] Although a physiological ligand for CD69 has not yet been identified, its wide distribution and the observation that crosslinking of the molecule generates intracellular signals suggest a significant role for CD69 in immune response.[20] Three major types of lymphocytes include T, B, and NK cells. Adaptive immune responses are based on the activities of T lymphocytes (also called T cells) and B lymphocytes (B cells). The key to the adaptive immune system is the presence of the extremely variable

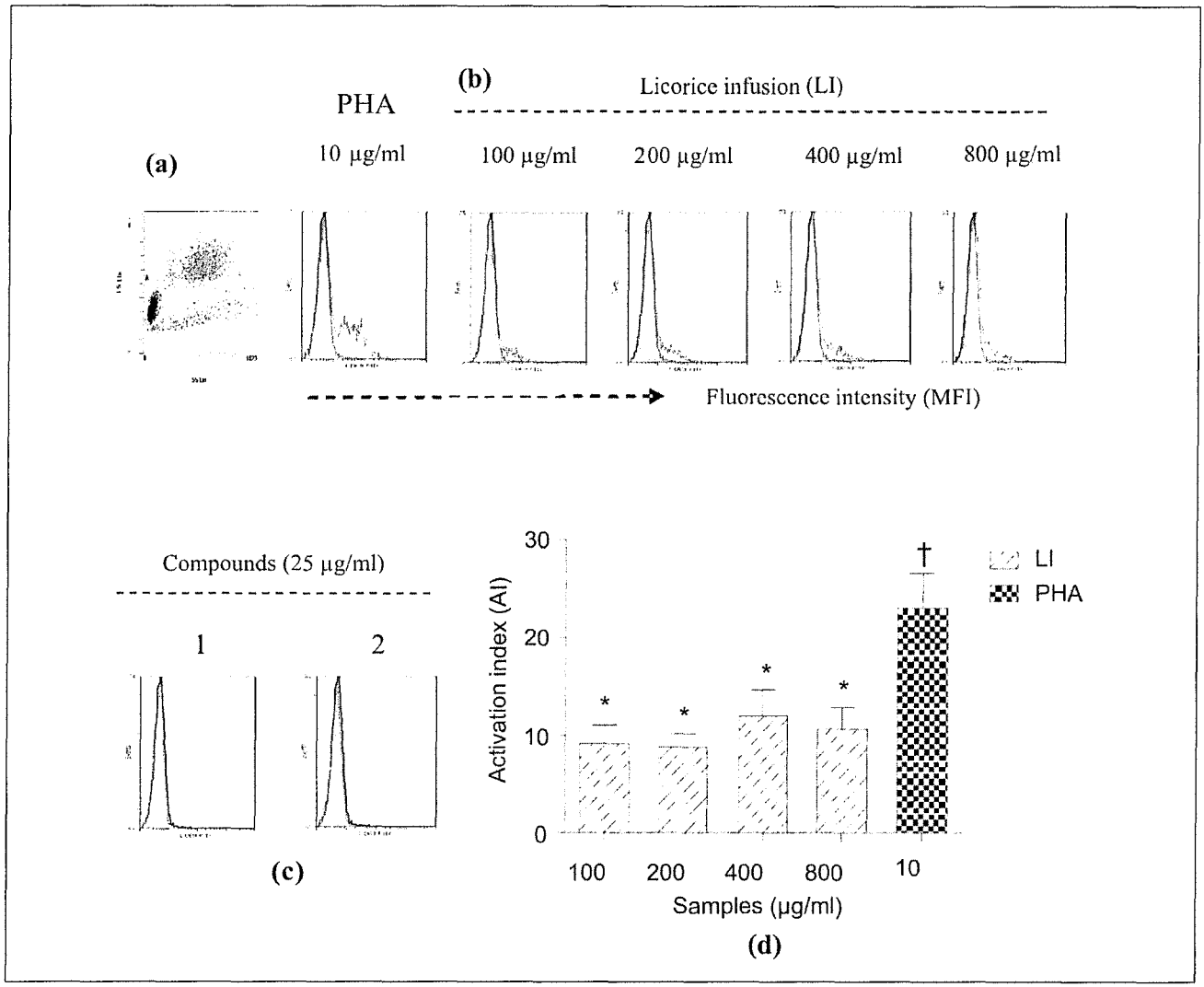

Figure 3: Effect of licorice infusion (LI), liquiritin (1) and glycyrrhizin (2) on activation of total lymphocytes, as measured by CD69 expression. Lymphocytes were initially gated by their characteristic forward (FSC) and side scatter (SSC) profiles, which represent size and granularity, respectively. The activated lymphocytes in circular gate A (a) were then analyzed for fluorescent intensity. The filled histograms represent the group control (untreated) and the open histograms the stimulated (treated) group (b). Compounds 1 and 2 (at range of 12-100 µg/ml) were inactive and histograms (c) are shown as representative figures. (at page width); Bar graphic (d) shows the effect of LI on the total lymphocytes as activation index (AI)

antigen-specific receptors of the T lymphocytes (called TCRs) and B lymphocytes (called B-cell receptors [BCRs], immunoglobulins, or antibodies). NK cells are part of the lymphoid lineage but are distinct from T and B lymphocytes because they do not express the specialized receptors associated with the adaptive immune response. Instead they have two other types of receptors that determine their ability to identify and kill targeted host cells: Killer activation receptors and killer inhibition receptors. NK cells are important elements in innate defenses against virally infected and cancerous host cells.[21]

As can be deducted from AI values [Figure 3d, 4d], the activation of total lymphocytes and T cells by LI represents about 50 and 34% of those promoted by PHA, respectively. In addition, by comparing the AI of total lymphocytes with those of T cells, it was observed that about 50% of the activation of total lymphocytes could proceed from the T cells activation. Although the effect of LI on the CD69 expression was moderate, this fact could be hypothetically interpreted as beneficial, given that increased level of CD69 expression on T cells has been associated with some autoimmune diseases.[22,24] Licorice has multiple constituents and not one active ingredient; therefore, it is possible that they are able to act in a regulatory way, both activating and modulating the immune response. It is also questionable whether the LI immunostimulating effect is long-term because the CD69 expression on the surface

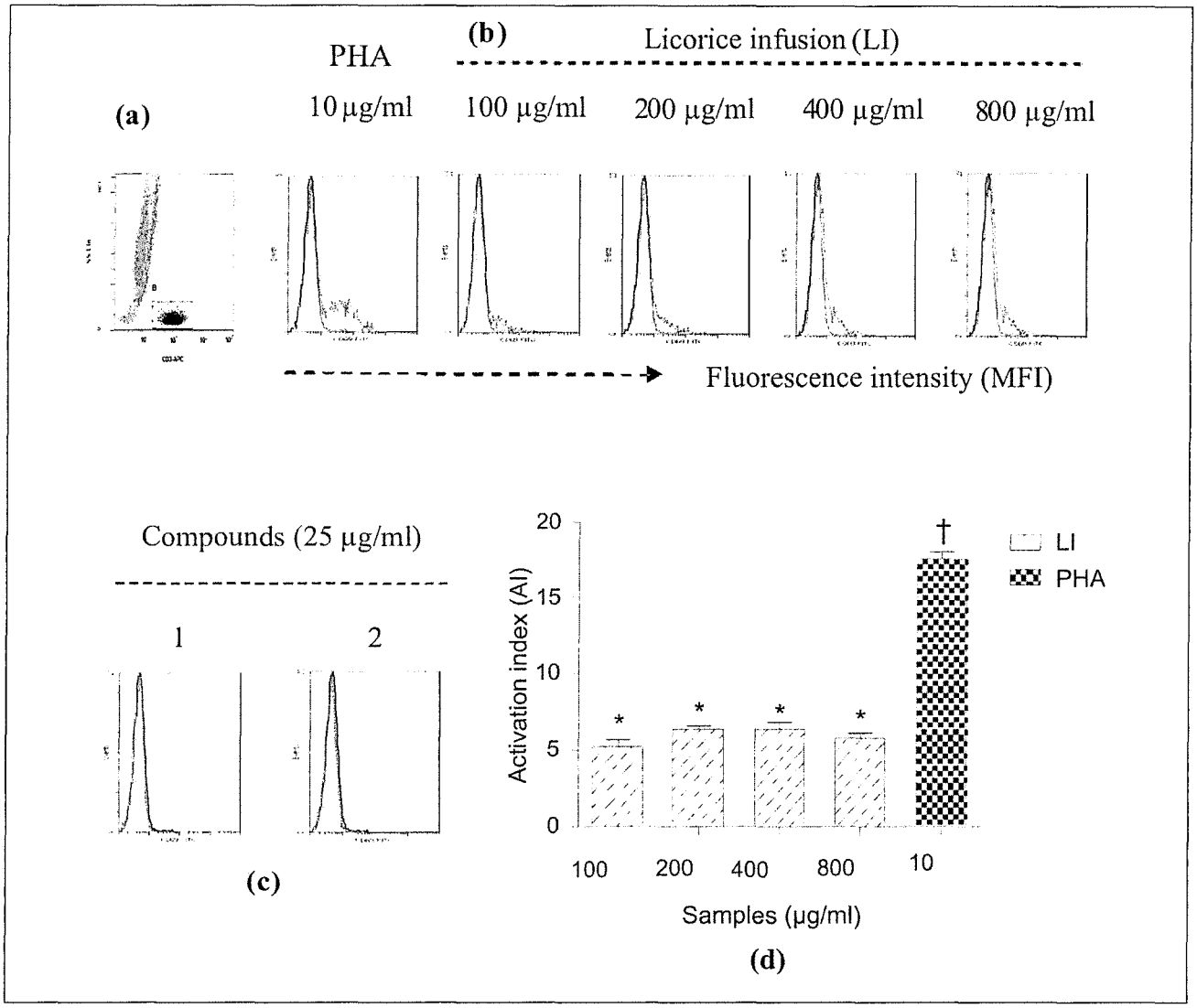

Figure 4: Effect of licorice infusion (LI), liquiritin (1) and glycyrrhizin (2) on activation of T lymphocytes, measured by CD69 expression. For visualization of T lymphocyte subset, the surface immunostaining with anti-human CD3 was used. The activated cells in rectangular gate B (a) were then analyzed for fluorescent intensity. The filled histograms represent the group control (untreated) and the open histograms the stimulated (treated) group (b). Compounds 1 and 2 (at range of 12-100 µg/ml) were inactive and histograms (c) are shown as representative figures. (at page width); Bar graphic (d) shows the effect of LI on the T lymphocytes as activation index (AI)

of immune cells not only occurs very rapidly, but also declines rapidly (disappearing after two days). Therefore, this study measured the CD25 expression, a later-expressed activation marker, but the expression was very low (data are not shown). Apparently, regular consumption of licorice infusion would be important for its long-term effect.

Recently, tinctures of *Echinacea purpurea*, *Astragalus membranaceus*, and *G. glabra* were shown to stimulate T cells, determined by CD69 expression. These three herbs had an additive effect on CD69 expression when used in combination and no chemical compound was related with the effects observed.[11] In contrast, previous *in vitro* studies revealed antiviral activity of glycyrrhizin against HIV-1, SARS related coronavirus, respiratory syncytial virus, arboviruses, vaccinia virus, and vesicular stomatitis virus. Mechanisms for antiviral activity included the reduced transport to the membrane and sialylation of viral surface antigen, reduction of membrane fluidity leading to inhibition of fusion of the viral membrane with the cell, induction of interferon gamma in *t*-cells, inhibition of phosphorylating enzymes in vesicular stomatitis virus infection, and reduction of viral latency.[4]

Licorice is popularly consumed in the form of teas and infusions;[6] however, studies on the immunomodulating effects of licorice by measuring the CD69 expression have been focused mainly on its tincture.[11] Infusion and tincture

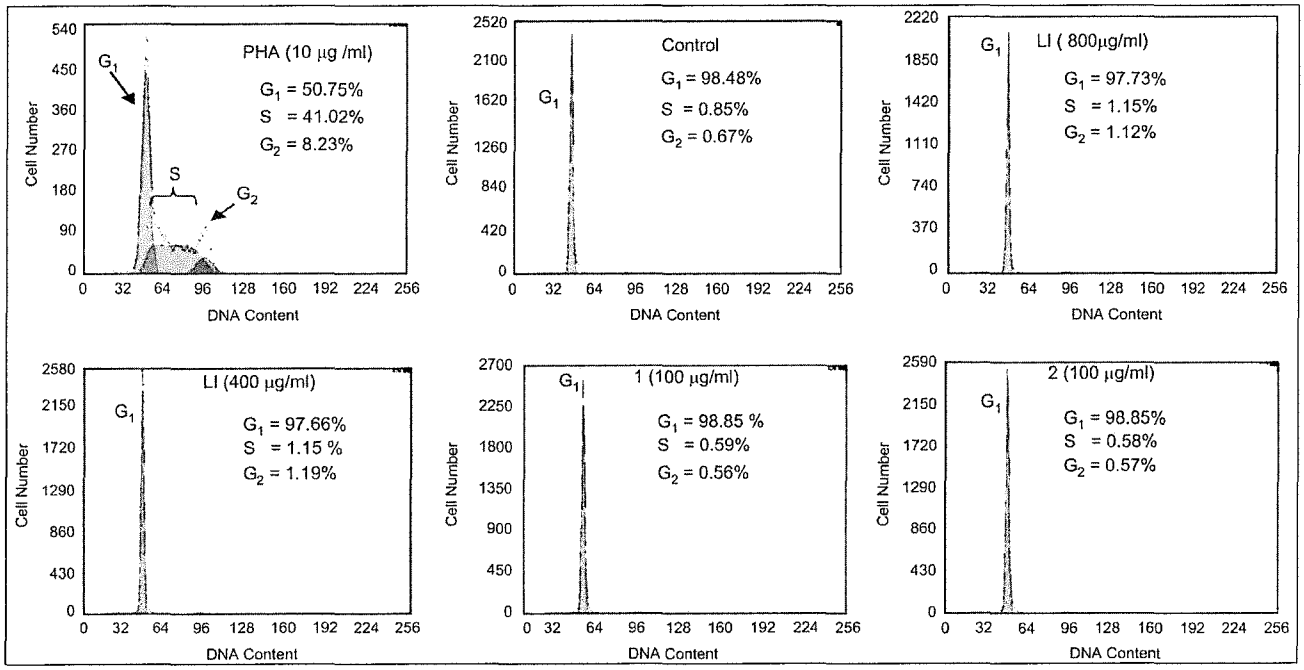

Figure 5: Effects of licorice infusion (LI), liquiritin (1) and glycyrrhizin (2) on the cell cycle progression of human lymphocytes. The LI, 1, and 2 showed no meaningful effect on cell cycle progression of lymphocytes. One representative experiment is shown and the percentages of cells in each phase of the cell cycle (G_1, S, and G_2 phases) are shown. (at column width)

of licorice have different chemical profiles[23,25] and therefore are expected to show different bioactivities. In the present study, the effect of a LI on the activation of lymphocytes was investigated for the first time. In contrast to a number of prior investigations,[26] the current study indicates that glycyrrhizin does not seem to be involved in the immunostimulating effect of LI, as measured by the CD69 expression.

Cell cycle progression of lymphocytes

The immunomodulating action of herbs may involve not only cell activation, but also cell proliferation. It is described that the interaction of lymphocytes with antigens or PHA initiates a cascade of biochemical events and gene expression, which induces the resting immune cells to enter the cell cycle, and then begin proliferating and differentiating.[27] By measuring the incorporation of propidium iodide in lymphocyte DNA using flow citometry, it was observed that LI, 1, and 2 had no effect on the cell cycle progression of lymphocytes. It has been suggested in different systems that CD69 expression may precede cell proliferation, maturation or differentiation.[28] Nevertheless, the CD69 expression may not completely overlap with cells that undergo DNA synthesis following antigen receptor engagement. Although the immunologic consequences of lymphocyte clonal expansion are well established and critical to the development of immunity, the relevance of CD69 expression to this process is less understood.[29] The emerging data from the present study suggests that the LI-activated lymphocytes express CD69 as a result of a proliferation-independent process. In the current investigation, the stimulating effect of LI and its two major constituents on the cell cycle progression of lymphocytes was investigated for the first time.

CONCLUSION

The present *in vitro* study demonstrates that licorice infusion (LI) stimulates the activation of lymphocytes, mainly T cells. The two major components of LI were identified as liquiritin (1) and glycyrrhizin (2) and they seem not to be involved in the activation of lymphocytes. Flavones and flavonols were identified as minor constituents of LI. This report establishes the need for future studies to determine the *in vivo* effect of LI on lymphocytes and to identify its active constituentes. This herbal preparation might be a potential alternative for mounting an effective immune response and as a preventive barrier against both viral and bacterial infections, and during immunosenescence.

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Clinical Analysis for Magnesium Isoglycyrrhizinate in the Treatment of Chronic Hepatitis

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Objective To observe the efficacy of magnesium isoglycyrrhizinate in the treatment of chronic hepatitis. Methods 72 patients with chronic hepatitis B were randomly divided into treatment group and control group.The 36 hepatitis sufferers were treated with 100mg magnesium isoglycyrrhizinate added into 250ml 10%glucose injection liquid,and the other 36 sufferers control group were treated with compound Glyeyrrhizin 150mg added into 250ml 10%glucose injection liquid,I.V.drip,once a day for 1 month.When the treatment was over,the practical effects were analyzed. Results The levels of ALT,AST and TBIL dropped more rapidly than those in control group and there was significant difference in symptom improvement between treatment group and control group(P0.05). Conclusion Clinic symptom and liver function index can be improved after treatment with magnesium isoglycyrrhizinate injection in chronic hepatitis sufferers,and there are few side reactions happened.

【Key Words】： **Chronic hepatitis Magnesium isoglycyrrhizinate Liver function**

【CateGory Index】： R512.62

【Citations】

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1 Liver Failure and Artficial Liver Group,Chinese Society of Infectious Diseases,Chinese Medical Association;Severe Liver Diseases and Artifcial Liver Group,Chinese Society of Hepatology,Chinese Medical Association.;Diagnostic and treatment guidelines for liver failure[J];Journal of Clinical Hepatology;2006-06

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Evaluation of oxidative stress at different time intervals after single dose of alcohol with or without NTX in rats (CTPL/ROS/01/2015)

Objective

The primary objective of the present study is to evaluate extent of oxidative stress at different time points after single dose of alcohol with or without NTX on selected parameters.

Table 1: Animal study groups

Groups	Dose of alcohol	NTX %
1. Alcohol	1 g/kg, p.o., bolus	-
2. NTX + Alcohol	1 g/kg, p.o., bolus	1.4

Brief Schematic Experimental Study Protocol:

Laboratory acclimatized animal (rat, n=16 in each group)

↓

Single Alcohol (1 gm/kg)/NTX+ alcohol (1 gm/kg) were administered orally

↓

Blood samples were collected at 0 min (pre dose), 30 min, 60 min, 120 min and 240 min post dose. Serum samples were separated and stored at -20 °C in aliquots.

↓

Following parameters were tested from serum samples:

1. Reactive oxygen species (ROS) using a) ROS ELISA kit and b) spectrophotometric assay of ROS metabolites (d-ROM test)
2. Serum MDA by ELISA method
3. Serum protein carbonyl content by ELISA method
4. Serum total GSH and GSSG
5. Total WBC count

Results:

Table 2: Changes in serum ROS level (U/ml) at different time points after single oral dose of alcohol (1 gm/kg bw) with or without NTX

Time points (hours)	**Alcohol** (1 gm/kg bw)	**SE**	**Alcohol + NTX** (1 gm/kg bw)	**SE**
0	77.38	12.76	80.24	9.34
0.5	96.43	10.26	98.26	17.33
1	102.17	14.22	93.33	21.61
2	50.24	10.23	51.12	16.92
4	54.19	11.36	40.48	9.78
AUC* value (Unit/ml/hr)	**273.74**		**256.35**	

Values are Mean ± SE (n=6); *AUC = Area under the curve

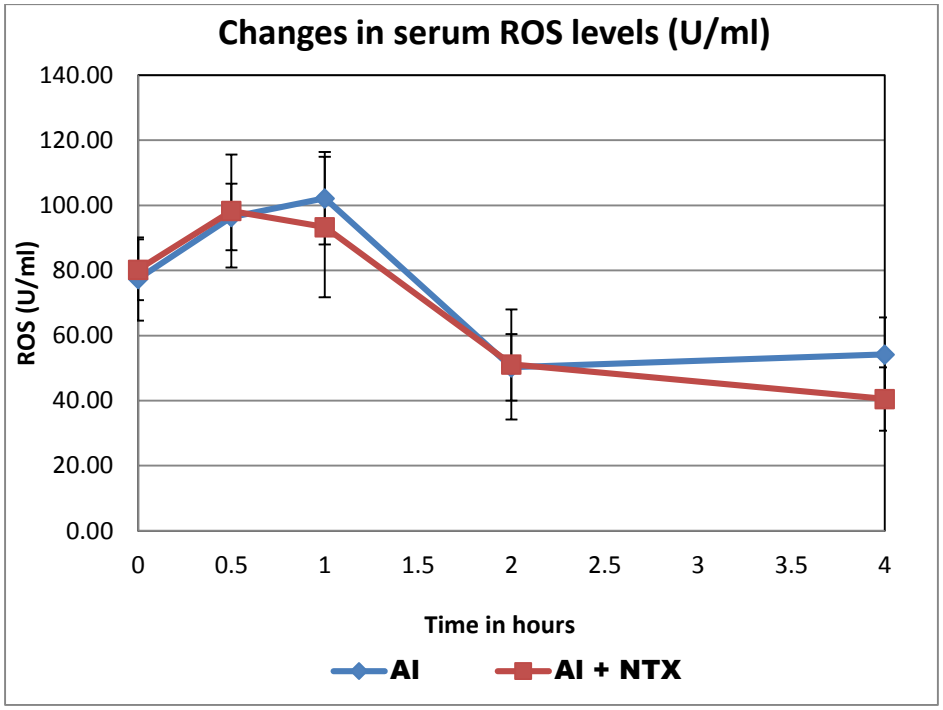
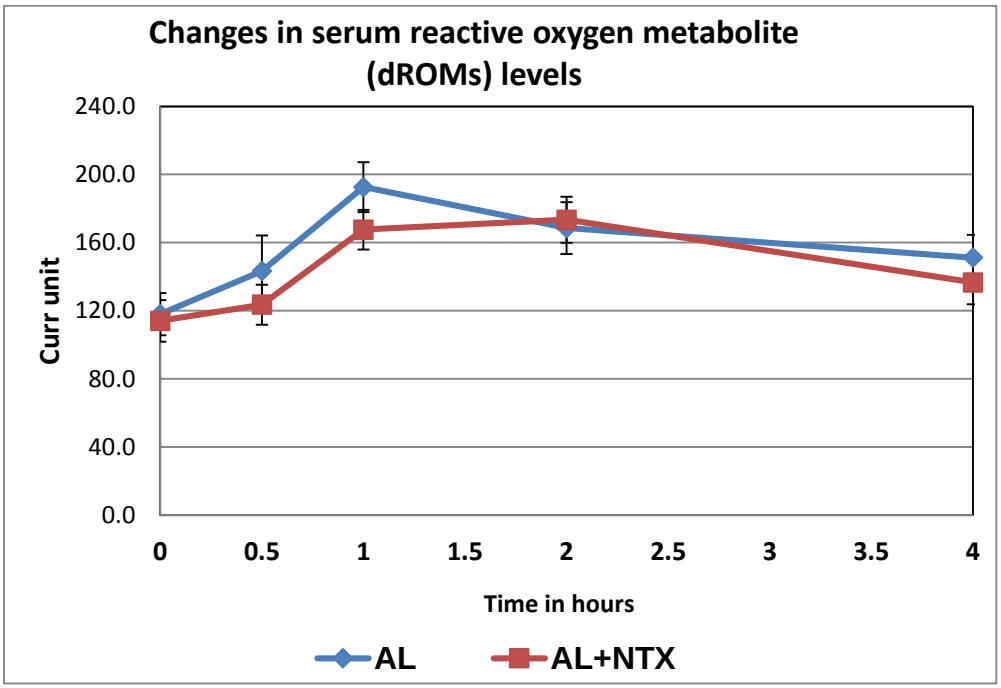


Table 3: Changes in serum reactive oxygen metabolites (ROMs, CURR unit) level at different time points after single oral dose of alcohol (1 gm/kg bw) with or without NTX

Time points (hours)	**Alcohol** (1 gm/kg bw)	**SE**	**Alcohol + NTX** (1 gm/kg bw)	**SE**
0	118.0	12.4	114.0	12.2
0.5	143.3	20.9	123.5	11.8
1	192.6	14.7	167.6	11.7
2	168.5	15.3	173.4	13.5
4.00	151.2	13.4	136.6	12.8
AUC* value (Curr Unit/ml/hr)	**649.55**		**612.65**	

Values are Mean ± SE (n=6); *AUC = Area under the curve



Values are in CURR unit

Table 4: Changes in serum malondialdehyde (MDA, ng/ml) levels at different time points after single oral dose of alcohol (1 gm/kg bw) with or without NTX

Time points (hours)	**Alcohol** (1 gm/kg bw)	**SE**	**Alcohol + NTX** (1 gm/kg bw)	**SE**
0	8.75	0.89	8.24	1.04
0.5	8.47	0.66	7.65	0.9
1	9.52	0.42	7.49	0.53
2	7.26	0.73	7.29	0.51
4.00	6.88	0.95	6.80	0.89
AUC* value (ng/ml/hr)	**31.33**		**29.24**	

Values are Mean ± SE (n=6); *AUC = Area under the curve

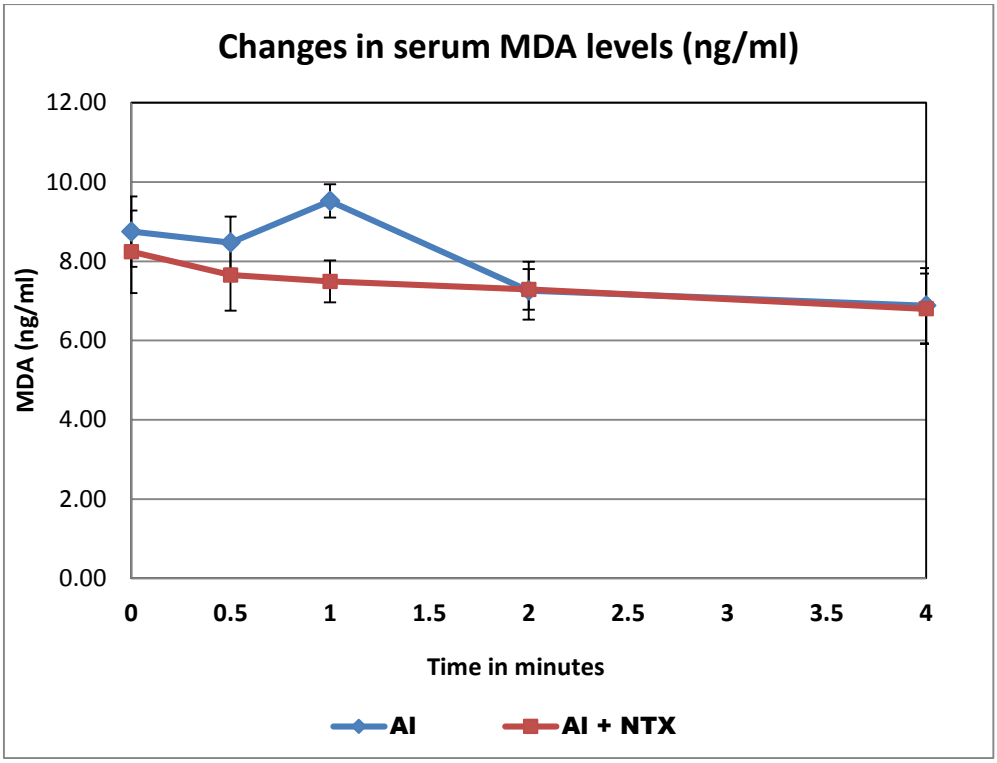


Table 5: Changes in serum total glutathione (tGSH, nmol/ml) levels at different time points after single oral dose of alcohol (1 gm/kg bw) with or without NTX

Time points (hours)	**Alcohol** (1 gm/kg bw)	**SE**	**Alcohol + NTX** (1 gm/kg bw)	**SE**
0	0.243	0.029	0.248	0.020
0.5	0.227	0.028	0.231	0.021
1	0.212	0.010	0.235	0.023
2	0.207	0.019	0.248	0.019
4.00	0.197	0.010	0.217	0.010
AUC* value (nM/ml/hr)	**0.84**		**0.94**	

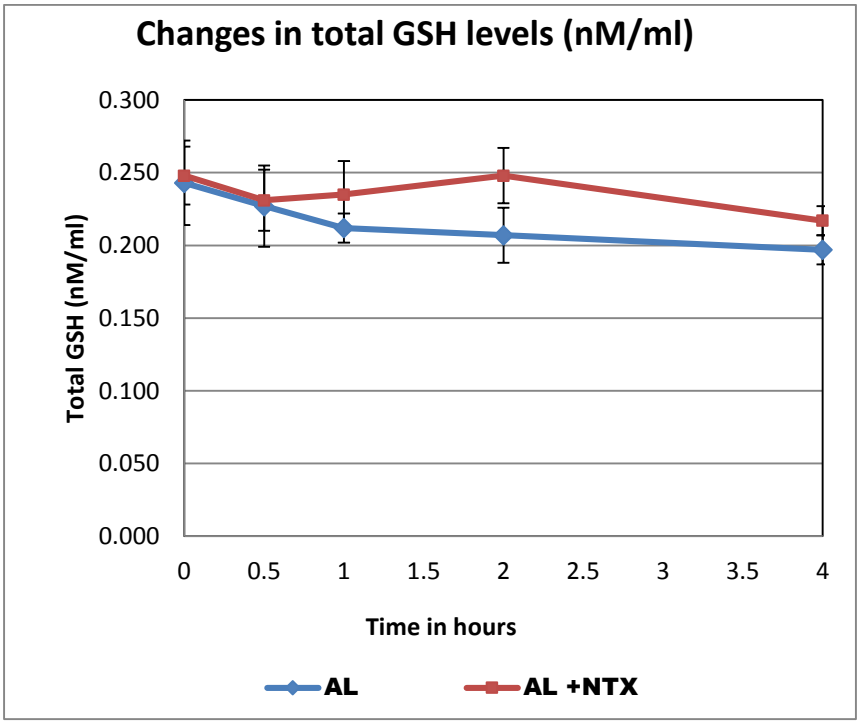


Table 6: Changes in serum oxidized glutathione (GSSG, nmol/ml) levels at different time points after single oral dose of alcohol (1 gm/kg bw) with or without NTX

Time points (hours)	**Alcohol** (1 gm/kg bw)	**SE**	**Alcohol + NTX** (1 gm/kg bw)	**SE**
0	0.045	0.006	0.039	0.006
0.5	0.057	0.005	0.051	0.004
1	0.057	0.009	0.065	0.012
2	0.055	0.006	0.056	0.007
4.00	0.053	0.006	0.051	0.007
AUC* value (nM/ml/hr)	**0.218**		**0.219**	

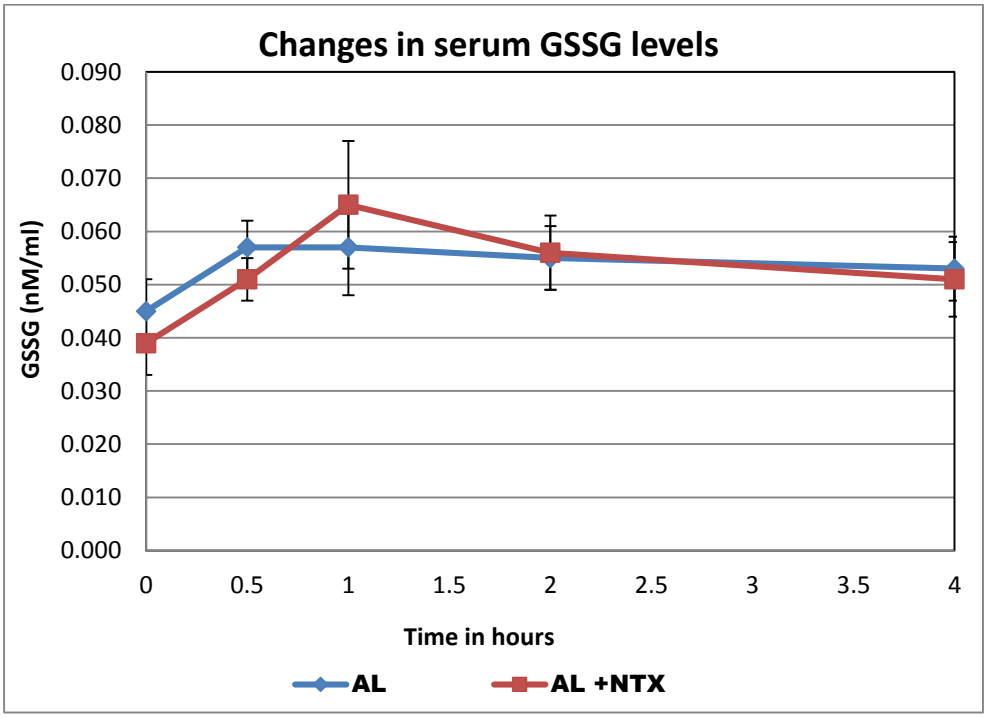


Table 7: Changes in serum protein carbonyl (ng/ml) levels at different time points after single oral dose of alcohol (1 gm/kg bw) with or without NTX

Time points (hours)	**Alcohol** (1 gm/kg bw)	**SE**	**Alcohol + NTX** (1 gm/kg bw)	**SE**
0	0.259	0.077	0.217	0.054
0.5	0.265	0.038	0.230	0.058
1	0.212	0.062	0.223	0.026
2	0.210	0.052	0.252	0.049
4.00	0.235	0.07	0.269	0.06
AUC* value (ng/ml/hr)	**0.906**		**0.984**	

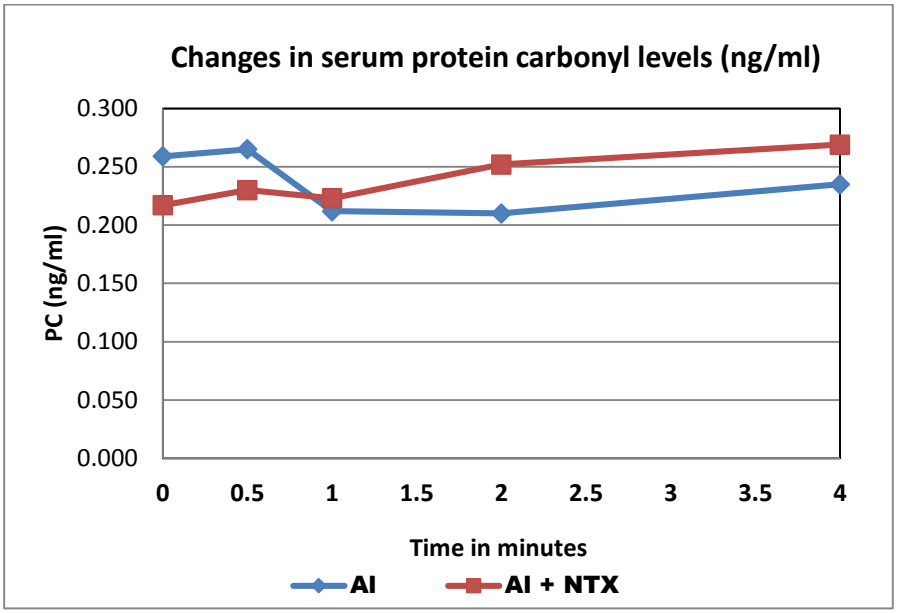
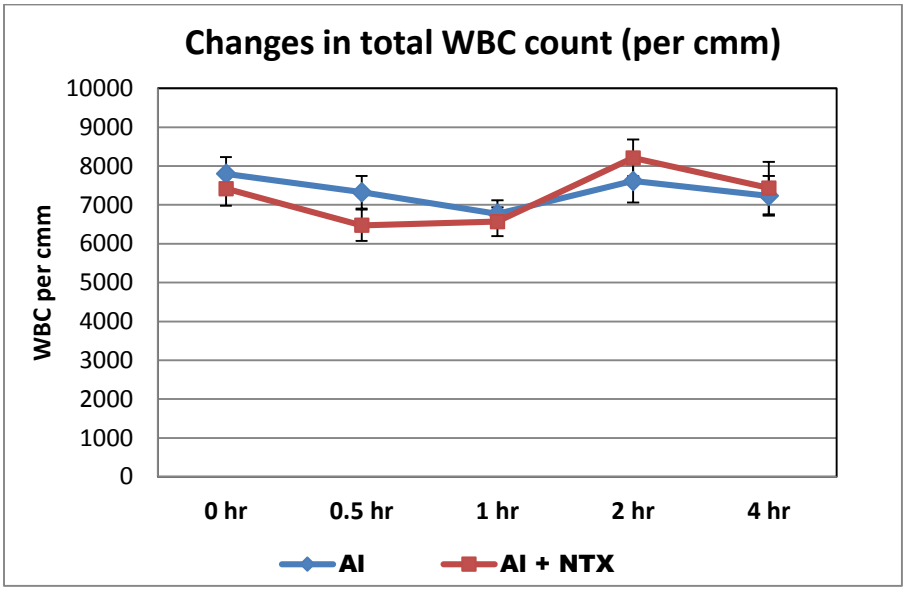


Table 8: Changes in total WBC count (per cmm) at different time points after single oral dose of alcohol (1 gm/kg bw) with or without NTX

Time points (hours)	Alcohol (1 gm/kg bw)	SE	Alcohol + NTX (1 gm/kg bw)	SE
0	7800	427	7417	437
0.5	7325	420	6475	402
1	6770	347	6567	373
2	7617	558	8208	473
4.00	7233	508	7433	677



Conclusions:

1. Orally administered alcohol at a dose of 1 gm/kg/bolus showed transient rise in ROS and ROS metabolites levels. Alcohol containing NTX (1.4%) showed slightly lower ROS generation both in terms of direct ROS and ROS metabolites. The comparison between AUC values of alcohol with or without NTX showed that NTX could dampen the serum ROS level by around 6%.

2. Single oral dose of alcohol (1 gm/kg/bolus) showed a rise in serum MDA level around 1 hour after administration. Animals received alcohol+ NTX did not show such peak. In terms of AUC values, NTX showed 6.7% less MDA value, compared to only alcohol group.

3. Similarly, addition of NTX prevents slight fall of serum total GSH level after alcohol administration and maintains total GSH level. In terms of AUC values, NTX showed nearly 12% more total GSH values compared to only alcohol.

4. Protein carbonyl, total WBC showed very similar trends in both the groups.

Evaluation of reactive oxygen species (ROS) from blood at different time intervals after oral consumption of two different alcohol formulations: A comparative, double blind, crossover, pilot clinical trial

Introduction

Exposure to acute or chronic alcohol triggers production of reactive oxygen species (ROS) with concomitant drop in the level or activity of antioxidants. The resulting state, which is characterized by a disturbance in the balance between ROS production, on one hand and ROS removal and repair of damaged complex molecules, on the other results in a comprehensive oxidative stress condition.

The major pathways of alcohol metabolism responsible for the generation of ROS are alcohol dehydrogenase in the cytosol, microsomal ethanol oxidizing system in the endoplasmic reticulum and aldehyde oxidase in the mitochondria (Lieber CS, 1997, Mira L et al., 1995). It has been comprehensively demonstrated that ROS accumulation contributes to alcohol-induced syndrome including liver injury (Reinke LE, 1985). The interaction of ROS with bio-molecules leads to alcohol induced toxicity includes enhanced lipid peroxidation, protein carbonyl formation, decreases in hepatic antioxidant defense especially GSH (Knecht et al., 1990) (Tsukamoto and Lu, 2001).

Objective of the study

The primary objective of the study will be to evaluate levels of ROS and other related oxidative stress markers from blood after oral consumption of two different alcohol formulations.

Inclusion criteria

- Male healthy participants (volunteers, who are regular/frequent/social alcohol drinkers)
- Aged between 25-50 years
- Body weight range of 55-85 kg

Exclusion criteria

- Volunteers with acid peptic disorder, diabetes mellitus, gallstones, neurological and psychiatric disorders, history of drug abuse.
- Volunteers consuming certain concomitant medications like antibiotics, antidepressants, antihistamines, anticonvulsants, cardiovascular medications, sedatives and hypnotic.

Study procedure:

- All subjects were asked to maintain their normal life style and were advised not to have excess food, cold drinks, coffee and avoid heavy physical exercise during the study period.

- The alcohol was provided in separate bottle and the amount was adjusted so that the alcohol intake would be around 1.5 gm/kg, bw (around 225-350 ml vodka). The subjects were allowed to drink with some diluents (limca, sprite, coke, water) with some snacks within a period of 1.0 hours.
- Blood will be collected at following intervals:
    - Before drinking initiated (mentioned as: -1 hr)
    - Just after the completion of drinking (mentioned as 0 min)
    - 30 min after the completion of drinking
    - 60 min after the completion of drinking
    - 120 min after the completion of drinking and
    - 240 min after the completion of drinking
- The study has been conducted in day time (between 10.00 a.m. to 6.00 p.m.)
- The subjects were allowed to have a light lunch after collection of 2 hours blood.

Statistical analysis

Percentage changes for measures were calculated from the difference between means of the each time points of alcohol and NTX groups (viz., 1 hr alcohol vs 1 hr NTX). Paired 'student's t-test' was carried out between the values of each time points of two groups (alcohol and NTX) and $p < 0.05$ was considered statistically significant.

Results:

Table 1: Demographic pattern of participants:

Parameters		
No. of completed subjects in each arm	12	
Age (Year)	33.83 ± 9.12	
Body weight (Kg)	65.42 ± 17.89	
Height (cm)	1.66 ± 0.06	
BMI	23.36 ± 4.56	
Al-consumed (both groups) (ml)	240.67 ± 49.13	
Gm/kg body wt	1.49 ± 0.12	
	Alcohol	NTX
Sys-BP (mm Hg)	119.17 ± 14.28	120 ± 10.44
Dia-BP (mm Hg)	77.17 ± 4.61	78.25 ± 3.74
Pulse rate (beats/min)	73.08 ± 8.08	72.42 ± 6.57
Drinking time (min)	47.08 ± 11.77	49.17 ± 12.40

Table 2. Changes in serum ROS levels after alcohol consumption:

Time points (hr)	ALCOHOL (ROS-U/ml)		NTX (ROS-U/ml)		% change*	p value (paired t-test)	Significance
	Mean	SD	Mean	SD			
-1	138.92	38.01	142.83	37.23	2.82	0.315	NS
0	174.33	44.66	164.33	40.75	-5.74	0.417	NS
0.5	201.08	80.56	190.33	91.47	-5.35	0.488	NS
1	**203.25**	**68.00**	**162.42**	**34.80**	**-20.09**	**0.035**	**S**
2	209.08	60.47	188.42	49.25	-9.88	0.164	NS
4	**194.83**	**67.96**	**166.25**	**48.48**	**-14.67**	**0.024**	**S**

n=12 in each arm, *% change has been calculated between two groups in each time point.

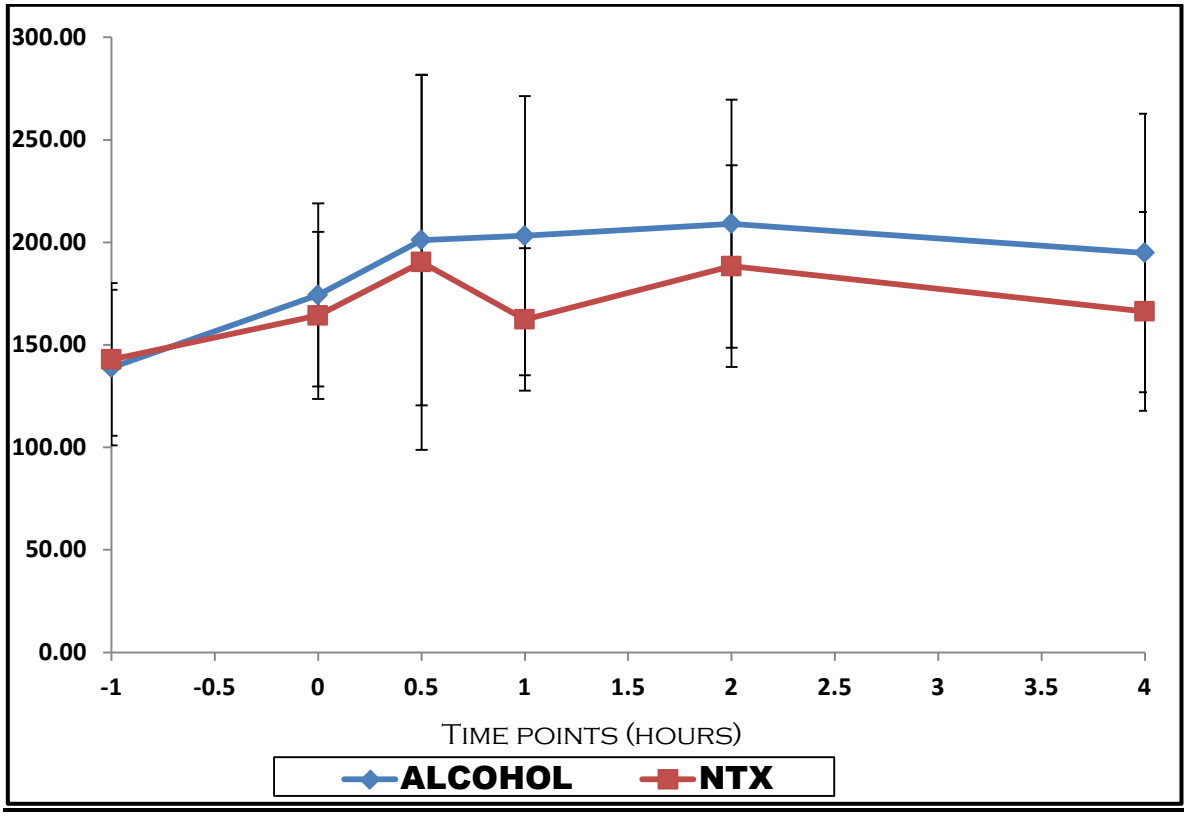


Fig.1: Changes in serum ROS levels at different time intervals after alcohol consumption.

Table 3. Changes in serum derivatives of reactive oxygen metabolites (dROM) levels after alcohol consumption:

Time points (hr)	ALCOHOL (dROM-CURR unit)		NTX (dROM-CURR unit)		% change*	p value (paired t-test)	Significance
	Mean	SD	Mean	SD			
-1	81.45	15.96	80.125	41.11	-1.63	0.662	NS
0	**107.23**	**24.48**	**94.425**	**55.51**	**-11.94**	**0.028**	**S**
0.5	104.70	34.93	96.075	56.22	-8.24	0.280	NS
1	113.85	27.68	106.425	54.67	-6.52	0.414	NS
2	**108.43**	**22.26**	**95.825**	**53.42**	**-11.62**	**0.021**	**S**
4	100.08	22.77	88.725	47.50	-11.34	0.064	NS

n=12 in each arm, *% change has been calculated between two groups in each time point.

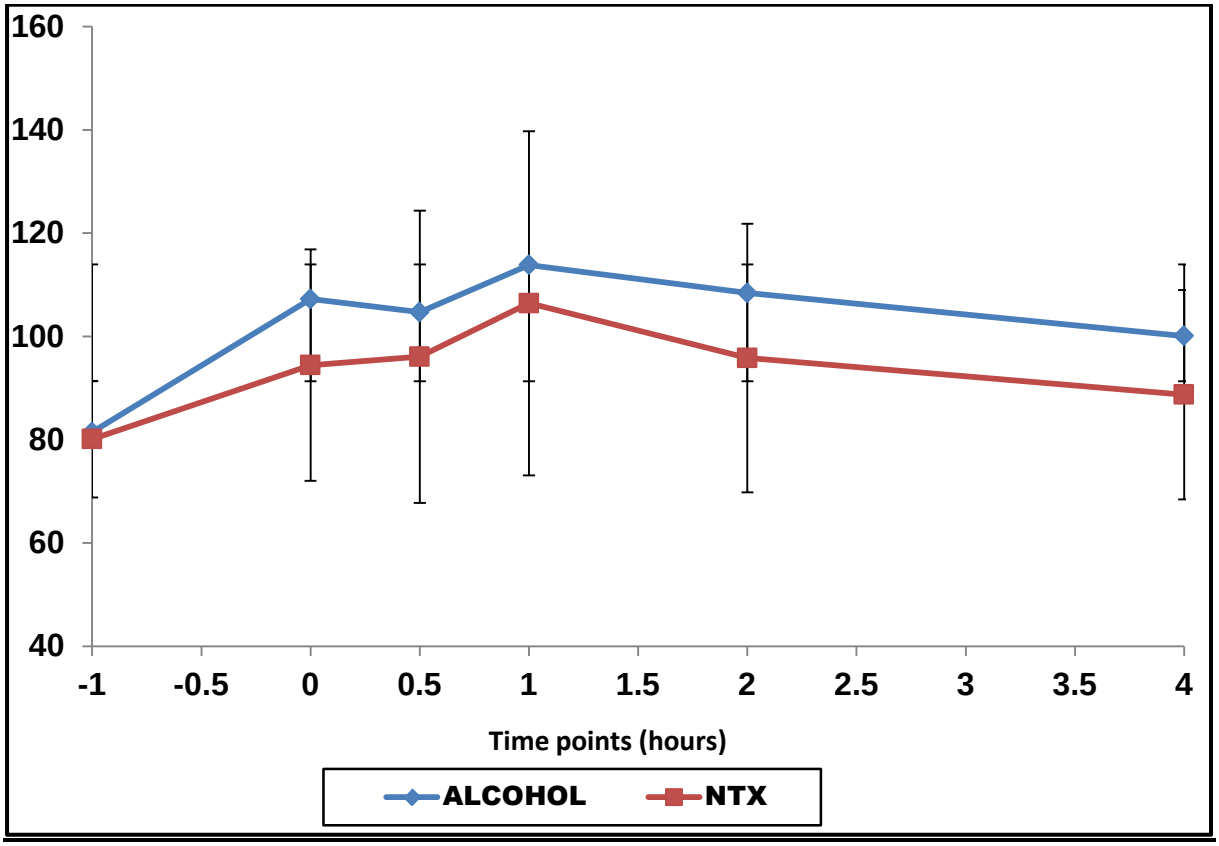


Fig.2: Changes in serum dROM levels at different time intervals after alcohol consumption.

Table 4. Changes in serum total glutathione (GSH) levels after alcohol consumption:

Time points (hr)	ALCOHOL (GSH nM/ml)		NTX (GSH nM/ml)		% change*	p value (paired t-test)	Significance
	Mean	SD	Mean	SD			
-1	0.160	0.019	0.159	0.020	-0.58	0.738	NS
0	0.133	0.023	0.142	0.023	6.50	0.120	NS
0.5	**0.147**	**0.031**	**0.163**	**0.034**	**10.83**	**0.026**	**S**
1	**0.143**	**0.033**	**0.161**	**0.029**	**11.91**	**0.020**	**S**
2	0.145	0.025	0.143	0.032	-1.29	0.805	NS
4	0.153	0.019	0.162	0.032	5.81	0.151	NS

n=12 in each arm, *% change has been calculated between two groups in each time point.

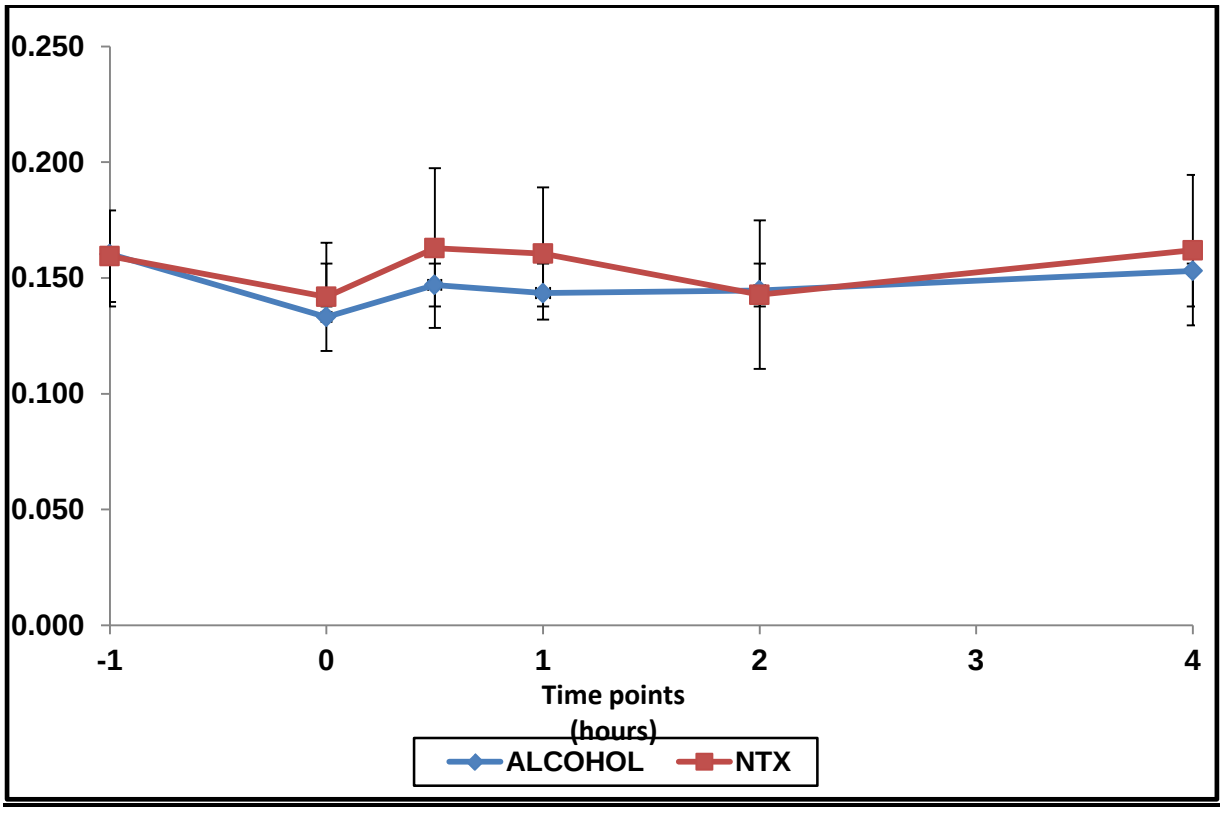


Fig.3: Changes in serum GSH levels at different time intervals after alcohol consumption.

Table 5. Changes in serum Malondialdehyde (MDA) levels after alcohol consumption:

Time points (hr)	ALCOHOL (MDA uM/ml)		NTX (MDA uM/ml)		% change*	p value (paired t-test)	Significance
	Mean	SD	Mean	SD			
-1	6.806	0.53	6.736	0.69	-1.02	0.482	NS
0	7.863	1.03	7.489	1.15	-4.76	0.288	NS
0.5	**8.098**	**1.19**	**7.105**	**1.29**	**-12.27**	**0.011**	**S**
1	7.409	0.71	6.960	0.92	-6.06	0.163	NS
2	8.034	1.51	7.735	1.86	-3.72	0.595	NS
4	**8.040**	**0.63**	**7.153**	**0.69**	**-11.03**	**0.001**	**S**

n=12 in each arm, *% change has been calculated between two groups in each time point.

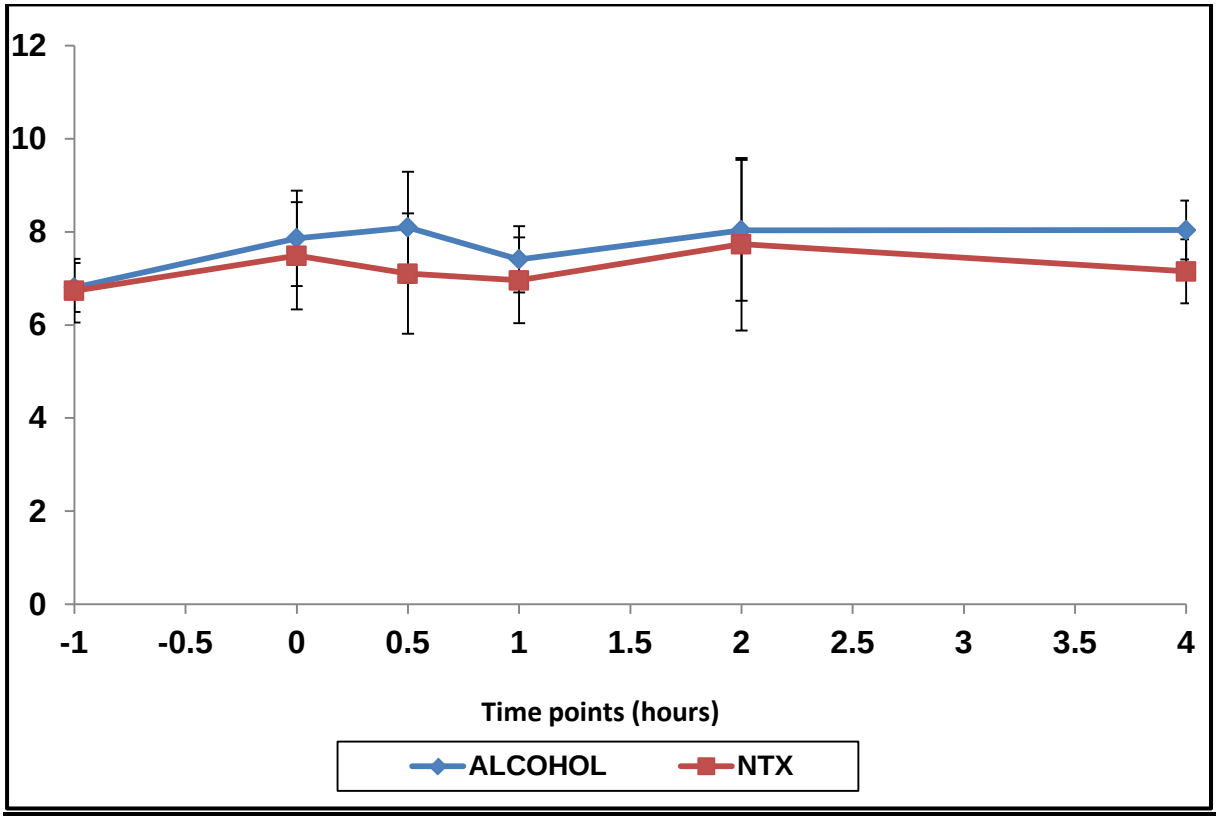


Fig.4: Changes in serum MDA levels at different time intervals after alcohol consumption.

Table 6. Changes in serum protein carbonyl (PC) levels after alcohol consumption

Time points (hr)	ALCOHOL (PC nM/L)		NTX (PC nM/L)		% change*	p value (paired t-test)	Significance
	Mean	SD	Mean	SD			
-1	1.119	0.347	1.153	0.317	3.04	0.498	NS
0	1.809	0.831	1.831	0.764	1.22	0.880	NS
0.5	2.459	0.833	2.330	0.952	-5.24	0.550	NS
1	**2.341**	**0.507**	**1.901**	**0.421**	**-18.81**	**0.051**	**S**
2	1.797	0.981	1.858	0.541	3.41	0.784	NS
4	1.445	0.413	1.409	0.429	-2.55	0.586	NS

n=12 in each arm, *% change has been calculated between two groups in each time point.

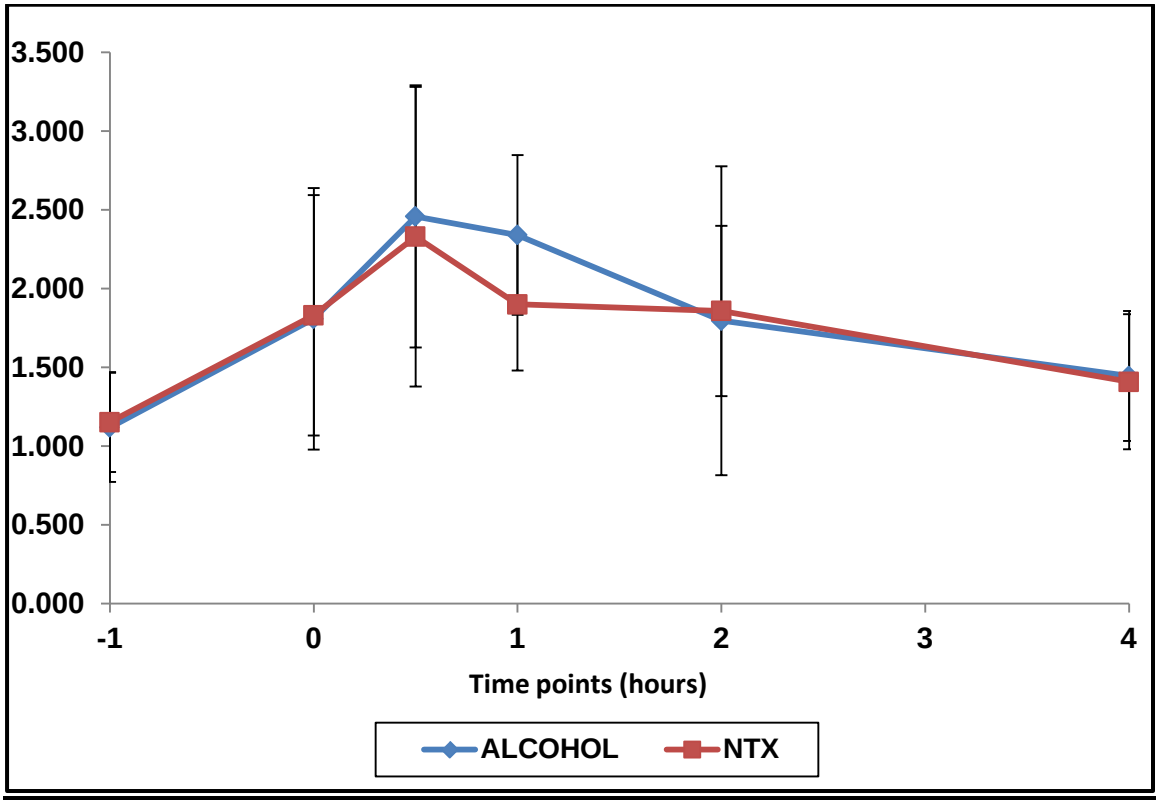


Fig.5: Changes in serum PC levels at different time intervals after alcohol consumption.

Conclusions:

1. Consumption of alcohol at a dose of around 1.5 gm/kg within a period of 40-60 minutes showed transient rise in ROS and ROS metabolites (dROM) levels. Alcohol containing NTX showed slightly lower ROS generation both in terms of direct ROS and ROS metabolites. A significant ($p<0.05$) drop in ROS levels were observed at 1 and 4 hour (20.09% and 14.67% respectively). Levels of ROS metabolites (as judged by dROM test) also significantly ($p<0.05$) decreased at +0 and 2 hour (11.94% and 11.34% respectively) indicating that NTX could dampen the serum ROS level.
2. Initial loss of serum total GSH level was slightly better managed by NTX group (+6.5%) though that was not statistically significant. However, NTX could able to maintain a significantly ($p<0.05$) higher level of serum total GSH during 30 minutes and 1 hour period (10.83% and 11.91% respectively). Whereas only alcohol group showed slow recovery of serum GSH level.

3. Throughout the study period, the increase in serum MDA level was found to be less pronounced in NTX group, particularly 30 minute and 4 hour time points (-12.27% and -11.03% respectively; $p<0.05$).

4. Serum protein carbonyl level showed initial transient rise in both the groups. However, serum level of PC in NTX group was significantly reduced at 1 hour (-18.81%, $p=0.051$) compared to only alcohol group at same time point. However, serum PC levels of both the groups slowly coming down to near baseline value at the end of 4 hours.

5. The present pilot study indicates the probable contribution of NTX in reducing ROS level in alcohol drinkers which could be effective in reducing alcohol induced diseases in long run.

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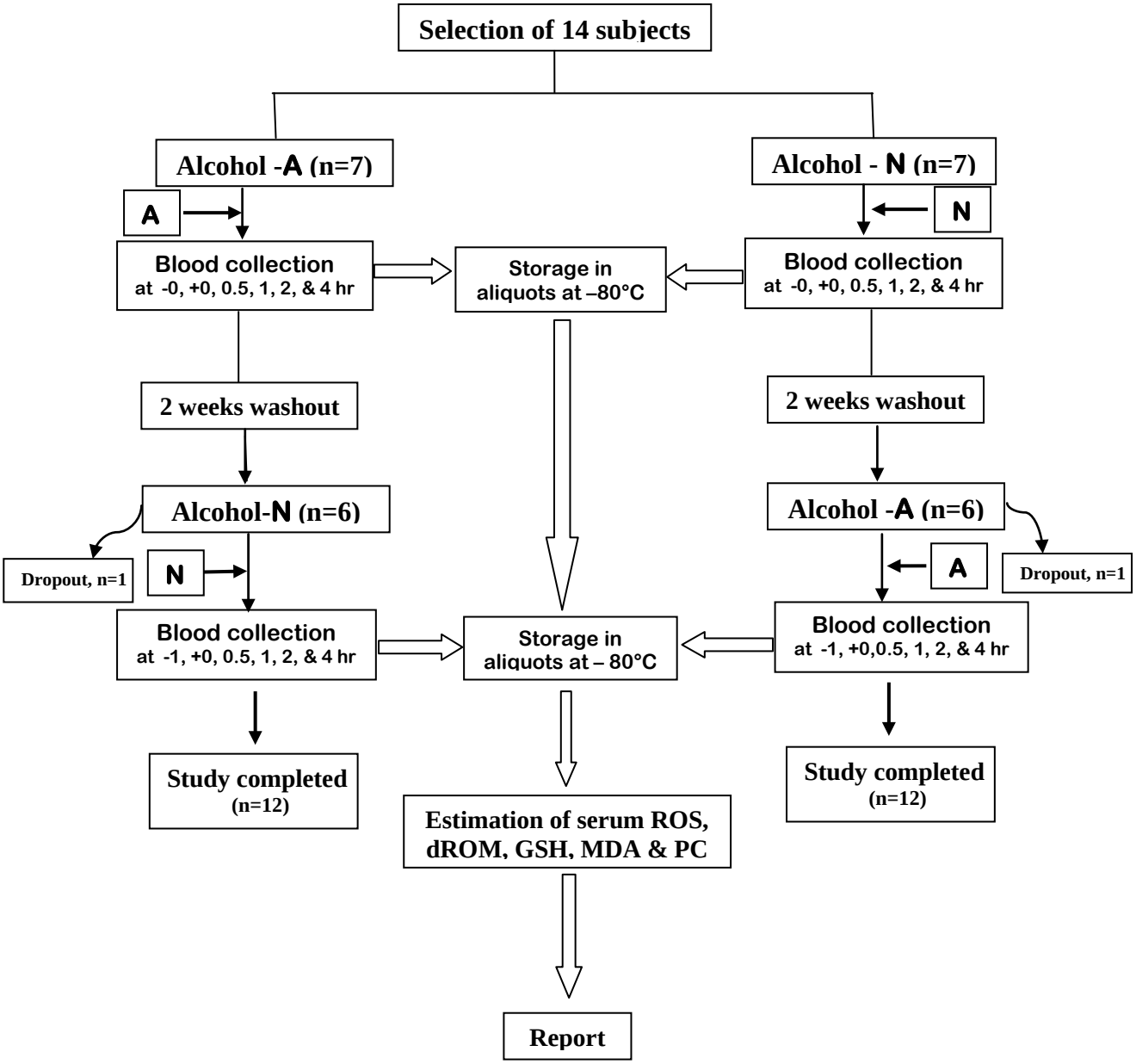
Fig. 6: Schematic of study design



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(12) **United States Patent**
Chigurupati et al.

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(45) **Date of Patent: Oct. 6, 2015**

(54) **REDUCED TOXICITY IN ALCOHOLIC BEVERAGES**

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USPC 514/33

See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to reduced toxicity of functional alcoholic beverage composition comprising distilled alcohol, deionized water, 18β-Glycyrrhizin or 18α-Glycyrrhizin and a sugar alcohol or sugars, having pH in the range of 4.0-9.0. More particularly, alcoholic beverage composition comprises distilled alcohol, deionized water, 18β-Glycyrrhizin or 18α-Glycyrrhizin and a sugar alcohol/sugars as hepato-protectants. The present invention provides an alcoholic beverage for reducing hepatotoxicity caused by its consumption and a process to manufacture the said alcoholic beverage.

23 Claims, No Drawings

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REDUCED TOXICITY IN ALCOHOLIC BEVERAGES

The following specification particularly describes the invention and the manner in which it is to be performed.

FIELD OF INVENTION

The present disclosure provides an alcoholic beverage having reduced hepato-toxicity. The invention also relates to a process for the preparation of the said beverage.

BACKGROUND OF THE INVENTION

Ethanol consumption could lead to 60 medical conditions. Acute as well as chronic toxic effect of ethanol may ensue in irreversible organ damage (Das S. K. et. al., Indian journal of Biochemistry & Biophysics, 2010, Vol. 47, 32). The widely accepted forms of alcoholic liver diseases (ALD) are simple fatty liver (steatosis), which is reversible with abstinence, fatty liver accompanied by inflammation (steato-hepatitis) leads to scar tissue formation (fibrosis), the destruction of the normal liver structure (liver cirrhosis), which may or may not improve with abstinence and subsequently lead to liver cancer (hepatocellular carcinoma). In 2010, WHO suggests 10% of the adult population in the United States suffering from alcohol use disorders and liver cirrhosis is the 12^{th} leading cause of death in United States (Alcohol and Health, Focus on: Alcohol and the Liver, 2010, Vol. 33, No. 1 and 2, 87). It is known that 5% of the ethyl alcohol i.e. ethanol (hereinafter alcohol), ingested by a human being is excreted unchanged while the remaining 95% is degraded to acetaldehyde. Alcohol is rapidly absorbed from the GI tract. In fasting state the peak blood alcohol concentration reaches within 30 minutes. Distribution is rapid with tissue levels approximating blood concentrations. Liver accounts for nearly 90% of alcohol metabolism the remainder is excreted through the lungs & urine. The typical adult can metabolize 7-10 g of alcohol/hour (U.S. Pat. No. 7,666,909B2).

The primary pathway of alcohol metabolism, when consumed in low to moderate amount, is mainly catalyzed in the cytoplasm of hepatocytes by alcohol dehydrogenase (ADH) to form acetaldehyde. The accumulation of NADH (excess reducing equivalents) in the liver plays a role in liver damage seen more prominently with chronic alcohol use. Acetaldehyde produced through microsomal ethanol oxidation system (MEOS) initially represents a minor pathway of ethanol oxidation probably accounting for less than 10%, of the liver capacity to oxidize ethanol.

At higher alcohol level (>100 mg/dl), MEOS is dependent on CYP450 (2E1, 1A2 & 3A4) plays significant role in alcohol metabolism using NADPH as a cofactor & O_2. Catalase is especially capable of oxidizing ethanol in fasting state in the presence of hydrogen peroxide generating system. Acetaldehyde is oxidized in the liver via mitochondrial nicotinamide adenine dinucleotide (NAD^+) dependent aldehyde dehydrogenase (ALDH) to acetate. Activity of ALDH is nearly 3 times lower that ADH, hence accumulation of Acetaldehyde takes place. Acetate is further metabolized to acetyl CoA and can enter min TCA cycle or synthesis fatty acids. Each of these pathway results in the formation of free radicals (like reactive oxygen species {ROS}) with concomitant changes in the cells redox state (i.e. in the ratio of NADH to NAD^+ results in production of more NADH (Nicotinamide Adenine Dinucleotide (NAD^+) reduced by two electrons). The cell has a limited capacity to oxidize NADH back to NAD^+ in mitochondrial respiratory chain at the maximum capacity of this system, which determines the kinetics of the reaction. The redox state in relation to alcohol metabolism causes inhibition of NAD^+-mediated enzyme reactions typical to the normal metabolism of the hepatocyte. The citric acid cycle is affected the most as it gets inhibited. This leads to positive NADH/NAD ratio, which is considered the most important reason for the development of alcohol-induced fatty liver. The maximum capacity of the mitochondrial respiratory chain depends on the overall level of metabolism of the body. The consequence of altered redox state includes Hypoxia (oxygen deficit cell). The other plausible pathway of alcohol induced hepatotoxicity includes excess production of pro-inflammatory cytokines by gut-endotoxin stimulated Kupffer cells. ROS is mainly generated in association with the mitochondrial electron transport system; it is also produced by CYP2E1 and by activated Kupffer cells in the liver. Both acute and chronic alcohol consumption can increase ROS production, which leads to oxidative stress through a variety of pathways mentioned above [(Zakhari, S. Alcohol Research & Health, 2006, 29, 4, 245), (Wheeler M. D. et al, Free Radical Biology & Medicine, 2001, Vol. 31, No. 12, 1544), (Koop, D. R., Alcohol Research & Health, 2006, 29, 4, 274), (U.S. Pat. No. 7,666,909B2)].

The mechanisms involved by which alcohol causes cell injury are complex and combination of several inter-related pathways. ROS react primarily with the cell membrane (tight junction becomes more permeable) and in turn leaks lipopolysaccharides (LPS), as a consequence impaired gut structural integrity. The increases in transaminase enzymes [aspartate amino-transferase (AST) and alanine aminotransferase (ALT)] indicate cellular leakage and loss of functional integrity of cell membrane (Yue et. J, 2006). Loss of cellular integrity affects hepato-biliary function leading to elevated alkaline phosphatase (ALKP) activities with concurrent increase in serum bilirubin level and decrease in the total plasma protein content. Both increases and decreases in the levels of ROS can lead to apoptosis of hepatocytes (Wheeler M. D. Alcohol Res. Health, 2003; 27, 300). For the cell to function normally, GSH is critical to protect itself against ROS generated during activity of the mitochondrial respiratory chain. Alcohol consumption rapidly depletes GSH levels; alcohol interferes with Cytochrome c to leak from the mitochondria into the cytosol, which can activate enzymes known as caspases that can trigger apoptosis.

ROS induces LPO [ROS reacting with Malondialdehyde (MDA), 4-hydroxy nonenal (HNE)] and recognized as important starting place of hepatocytes damage. Endotoxin-activated Kupffer cells affects mitochondria leading to release of ROS (hydrogen peroxide radical, hydroxyl radical, particularly superoxide radical) and several cytokines (viz., Tumour necrotic factor {TNF-α}) leading to hepatocytes necrosis and apoptosis. It has been established by clinical studies that patients with alcoholic liver disease have increased levels of the inflammatory cytokines IL-1, IL-6, and TNF-α as well as the chemokine IL-8 and other cytokines.

Alcohol might enhance the sensitivity of hepatocytes, consequently which could lead to an increased production of ROS in the mitochondria. ROS could activate a regulatory protein called nuclear factor kappa B (NFκB), which plays critical role in regulation of immune response and controls the activities of numerous genes, including those that expresses TNF-α & its receptor as well as genes encoding proteins that promote apoptosis. Thus, a vicious cycle would be established in the hepatocytes: TNF-α promotes ROS production, which in turn activates NFκB, leading to enhanced production of additional TNF-α and its receptor as well as to production of factors that promote apoptosis. This cycle eventu-

ally alters the structure of the hepatocytes, impairs their function, and can lead to hepatocyte apoptosis. TNF-α also facilitates hepatocyte regeneration by promoting the proliferation [(Wheeler M. D. Alcohol Res Health, 2003; 27, 300), (Molina P., Happel, K. I., Zhang P., Kolls J. K., Nelson S., Focus on: alcohol and the immune system. Alcohol Res. Health, 2010, 33 (1 & 2), 97)1)].

TGF-β (transforming growth factor beta) might be involved in the development of alcohol-induced liver damage, which could cause the hepatocytes to produce molecules like trans-glutaminase, cytokeratins that are normally responsible for giving the cells their shapes. In excess, these molecules are cross-linked to form microscopic structures called Mallory bodies, which are markers of alcoholic hepatitis. TGF-β can also contribute to liver damage by activating stellate cells. In a normal state, these cells primarily serve to store fat and vitamin A in the liver. When activated, stellate cells produce collagen, the major component of scar tissue it leads to the development of liver fibrosis. Alcohol might trigger the activation of TGF-β and thereby contribute to the initiation of apoptosis if this molecule enters the blood in higher concentrations (Wheeler M. D., Alcohol Res. Health, 2003; 27, 300).

Acetaldehyde or ROS with DNA or protein or protein building blocks and ROS with MDA or MAA (mixed MDA-acetaldehyde-protein adduct) or HNE etc. in the cell could form stable or unstable adduct, which could be carcinogenic, immunogenic, induce inflammatory process, damage to the mitochondria etc. [(Zakhari, S. Alcohol Research & Health, 2006, 29 (4)245); (D. Wu, Alcohol Research & Health, 2106, 27, 4, 277); (Wheeler M. D., Alcohol Res. Health, 2003; 27, 300); (Molina P., Happel K. I., Zhang P., Kolls J. K., Nelson S., Focus on: alcohol and the immune system; (Alcohol Res. Health, 2010, 33, Vol. 1 & 2, 97); (Neuman M. G., Cytokine-central factor in alcoholic liver disease, Alcohol Res. Health, 2003, 27, 307)].

Varieties of endogenous enzymatic and non-enzymatic mechanisms have evolved to protect cells against ROS. This includes the superoxide dismutases (SOD), which remove O_2^-; Catalase (CAT) and the glutathione peroxidase (GP_X) system, which remove H_2O_2 and non-enzymatic low-molecular-weight antioxidants such as reduced glutathione (GSH), Vitamin E, Vitamin C, Vitamin A, Ubiquinone, Uric acid, and bilirubin. But these are capable to protect the cells to limited extent. Additional protection could be achieved by orally administrating the glutathione precursor like S-adenosyl-L-methionine (SAMe), N-acetyl cysteine (NAC) or anti-oxidant like Vitamin E. Vitamin C, plant bioactives (gallic acid, quercetinete) etc. (D. Wu, Alcohol Research & Health, 2006, 27, 4, 277).

PRIOR ART OF THE INVENTION

Literature discloses alcoholic beverages with various types of additives. The following literature exists in the field of this invention and has been considered in entirety.

US Patent Publication No. 20100086666 discloses alcoholic beverages in which a protein like casein hydrolysate to enhance smoother taste and gives some nutritional benefit to the consumer.

Das S. K. et. al. (Indian Journal of Biochemistry & Biophysics, 2010, vol 47, 32) describes concomitant treatment of resveratrol or vitamin E with alcohol in mice ameliorates; alcohol induced oxidative stress, angiogenesis process and aid in controlling immune-modulatory activity.

US Patent Publication No. 20100086666 discloses alcoholic beverages, which comprises phenol like epigallocatechingallate (EGCG), epigallocatechine (EGC), epicatechin (EC), epicatechingallate (ECG), proanthocyanin, tannin and quercetin etc. known to reduce oxidative stress by scavenging free radicals generated by alcohol.

U.S. Pat. No. 7,666,909B2 reveals alcoholic beverages comprising D-Glyceric acid and its salts enhancing the metabolism of alcohol reducing the adverse event caused due to alcohol consumption.

GA or Matrine (Mat) alkaloid isolated from *S. flavescens* alone, or GA+Mat, when administered to rat models of hepatic fibrosis induced by abdomen injection of dimethyl nitrosamine (DMN) in acetaminophen overdosed mice, reduces the mortality by attenuating acetaminophen-induced hepatotoxicity. This is probably due to reduced number and area of γ-GT positive foci. In addition, GA+Mat had a protective effect on immunosuppression, a strong non-specific anti-inflammatory effect, and an effect of reducing the incidence of sodium and water retention (W. Xu-yingae, Chemico-Biological Interactions, 181 (2009) 15-19).

WO No. 2008/055348A1 discloses that alcoholic beverages comprising turmeric reduces hangover.

Das S. K. et al. (Indian Journal of Experimental Biology, 2006, Vol 44, 791) reveals concomitant treatment of lecithin with Vitamin B complex or Vitamin E with alcohol in Wistar rats was performed. It was established that lecithin with Vitamin B complex with alcohol was promising therapeutic approach than Vitamin E with alcohol in allaying oxidative stress.

El-Fazaa S. et al. (Alcoholism & Alcoholism, 2006, Vol. 41, No 3, 236) exemplifies alcoholic beverages comprising resveratrol inhibits the alcohol induced lipid peroxidation and have protective effect against injury.

WO1989004165A1 or EP0336960A4 divulges alcoholic beverages with combination of any one or more sugars from the group consisting of D-Galactose, D-Lactose, D-Xylose. L-Fructose, D-Mannitol, D-Sorbitol, D-Glucose etc.

JP06014746 discloses alcoholic beverages comprising a glycoside of quercetin, divalent metallic ion and licorice extract (Glycyrrhizin). This beverage enhances alcohol metabolism and has hepatopathy-suppressive activity, due to ethanol and acetaldehyde. Thus, it reduces hangover.

CP Patent Publication No. 1736270 discloses liver-protecting drink constituting Chitosan oligosaccharide, glycyrrhizin, aqueous extract of kudzuvine flower and aqueous extract of hovenine.

US Patent Publication No. 20090196951 reveals alcoholic beverages comprising resveratrol a strong anti-oxidant, also activates the Sirtuin 1 (SIRT1) and Peroxisome proliferator-activated (PPAR)-gamma coactivator-1[PGC-1'] gene, which are key regulator of energy and metabolic homeostasis.

JP2008266203 and EP0502554 discloses an increase in amount of an enzyme activity of the Reactive oxygen species (ROS) scavenging enzyme group such as superoxide dismutase, catalase or peroxidase with one or more kinds of substances selected from the group consisting of Erythritol, Mannitol, Sorbitol and Xylitol.

CN1448497 discloses an alcoholic drink comprising of ethanol and Glycyrrhizin, but a synergistic mixture of alcohol with hepato-protectants that include certain sugar alcohols or sugars as integral part of the present composition, apart from Glycyrrhizin has not been described.

CN101744865 discloses a method of producing a liver protecting tablet comprising Xylitol and Glycyrrhizin. CN101744865 focuses on a method for preparing Xylitol liver tablets and nowhere demonstrates biological activity of such tablets. Moreover, the present patent is focused to an alcoholic beverage having reduced toxicity and a method of preparing the same. The present application demonstrates a

synergistic mixture of alcohol with hepato-protectants that include certain sugar alcohols or sugars as integral part of the composition and such synergistic mixture offers a good degree of hepato-protection.

Various other prior art documents are known (US 20080226787, U.S. Pat. No. 3,282,706, U.S. Pat. No. 1,720,329, U.S. Pat. No. 4,537,763, U.S. Pat. No. 8,524,785) where glycyrrhizin and sugar alcohols like Mannitol, Erythritol, Xylitol etc. have been used for imparting various functions in the beverages as non-nutritive sweetening agent having low calorific value or as flavoring agent, but the aspect of hepato-protection has not been disclosed.

Documents are available in prior art, which show that Glycyrrhizin, sugar alcohols and sugars are independently known to exhibit hepato-protective activity, but their combination to exhibit synergistic hepato-protection has not been reported so far. Applicant in this application reports for the first time synergistic activity imparted by a combination of 18β or α-Glycyrrhizin and sugar alcohols, more particularly 18β/α-Glycyrrhizin and D-Mannitol exhibiting exemplified synergistic hepato-protection to provide a beverage with reduced toxicity.

SUMMARY OF THE INVENTION

The present disclosure relates to an alcoholic beverage, particularly to alcoholic distilled spirits like vodka, flavored vodka, whisky, etc. having reduced hepato-toxicity comprising distilled alcohol, deionized water, glycyrrhizin and a sugar alcohol or sugar having a pH in the range of 4.0-9.0.

More particularly the invention provides an alcoholic beverage having reduced hepatotoxicity comprising distilled alcohol, deionized water, 18β-Glycyrrhizin or 18α-Glycyrrhizin and a sugar alcohol or sugar. The invention also relates to a process for the preparation of the said beverage. The exemplified reduced hepato-toxicity provided by the beverage has been achieved by synergistic hepato-protection exhibited by the combination of 18β or 18α-glycyrrhizin and a sugar alcohol/sugar present in the said alcoholic beverage.

OBJECTS OF THE INVENTION

An object of the present invention is to provide an alcoholic beverage having reduced toxicity.

Another object of the present invention is to provide an alcoholic beverage having synergistic activity and providing enhanced hepato-protection.

Yet another object of the present invention is to provide a beverage comprising hepato-protective agent(s) to achieve the reduced hepato-toxicity.

Yet another object of the present invention is to provide an alcoholic beverage comprising 18β-Glycyrrhizin or 18α-Glycyrrhizin to achieve the reduced hepato-toxicity.

Yet another object of the present invention is to provide an alcoholic beverage comprising hepato-protective agent(s) like sugar alcohols and sugar.

Yet another object of the present invention is to provide an alcoholic beverage comprising the sugar alcohols selected from D-Mannitol, D-Erythritol, D-Xylitol and like.

Yet another object of the present invention is to provide an alcoholic beverage comprising sugars selected from D-Xylose, D-Mannose, D-Sucrose and D-Lactose.

Still another object of the present invention is to provide an alcoholic beverage comprising pH adjusting agent(s), flavoring agent(s).

Further object of the present invention is provide an alcoholic beverage comprising optionally of the flavoring agents selected from vanilla, strawberry and like.

Still another object of the present invention is to provide an alcoholic beverage having acceptable taste, flavor, odor, clarity and buzz factor.

Another important object of the present invention is to provide a process for the preparation of alcoholic beverage composition comprising (a) alcohol or alcohol:water mixture (b) 18β-Glycyrrhizin/18α-Glycyrrhizin (c) sugar alcohol or sugar (d) pH adjusting agents and optionally a flavoring agent.

Still another object of the present invention provides an alcoholic beverage composition having enhanced hepato-protection.

The alcoholic beverage is for use in a method of amelioration of diseases involving acute and chronic alcoholic toxicity like alcoholic liver diseases (ALD) like steatosis.

BRIEF DESCRIPTION OF THE TABLES

Table 1: % Protection of D-Mannitol
Table 2: % Protection of D-Xylitol & D-Erythritol
Table 3: % Comparative Protection of 18β and 18α-Glycyrrhizin
Table 4: % Protection and % Synergism of 18β-Glycyrrhizin-Mannitol combinations
Table 5: Comparative % Protection and % Synergism of 18β or 18α-Glycyrrhizin-Mannitol combinations
Table 6: Comparative % Protection and % Synergism of 18β-Glycyrrhizin-Mannitol, Xylitol & Erythritol)
Table 7: Comparative data of % Protection and % Synergism of (180 Glycyrrhizin/Mannitol, Xylitol & Erythritol)
Table 8: % Protection of Sucrose, Mannose, Xylose & Lactose
Table 9: % Protection and % Synergism of (18β-GA: Sucrose, Mannose, Xylose & Lactose)

DETAILED DESCRIPTION OF THE INVENTION

Accordingly, the present invention provides a beverage, more specifically an alcoholic beverage having reduced hepato-toxicity comprising distilled alcohol, deionized water, 18β or 18α-Glycyrrhizin and a sugar alcohol or sugar and having pH in the range of 4.0-9.0. More particularly the hepato-toxicity is caused by the intake of alcohol. The reduced hepatotoxicity of the beverage of the present invention is achieved by the enhanced hepato-protective activity provided by the synergistic combination of 18β or 18α-Glycyrrhizin and a sugar alcohol or Glycyrrhizin and a sugar incorporated in the said alcoholic beverage. The synergistic effect of the components has been established by dose dependent study for hepato-protection of 18β or 18α-Glycyrrhizin, sugar alcohol and a combination of Glycyrrhizin and sugar alcohol/sugar by performing experiment on animal models.

Ingredient Description:

Glycyrrhizin (or Glycyrrhizic acid or Glycyrrhizinic acid: abbreviated as GA) is the chief sweet-tasting constituent of *Glycyrrhiza glabra* (liquorice) root. It has also been given intravenously in Japan as a treatment for hepatitis C and as an emulsifier and gel-forming agent in foodstuff and cosmetics. Glycyrrhizin (GA) is a triterpenoid saponin glycoside. It is available as in racemic or pure form of 2 isomers: 18β-Glycyrrhizin and 18α-Glycyrrhizin. Hepato-protective mechanism of GA is due to its aglycone, glycyrrhetic acid, which inhibits both free radical generation as well as lipid peroxidation. 18α-GA has anti-hepato fibrosis effect—it is fre-

quently used as a hepato-protective agent. The sweetness of GA has a slower onset than sugar, and lingers in the mouth for some time. GA is partly absorbed as an intact drug. (W. Xuyinga et. al.) Chemico-Biological Interactions 181 (2009) 15-19), (T, Zing et. al. Chinese Journal of Modern Applied Pharmacy 2006, 02, 15-19). GA and its metabolites exhibit steroid-like anti-inflammatory activity, due, in part, to inhibition of Phospholipase A2 activity, an enzyme critical to numerous inflammatory processes. They inhibit hepatic metabolism of aldosterone and suppress hepatic 5-α-reductase. Because Cortisol and aldosterone bind with the same affinity to the mineralocorticoid receptor, an increase in renal Cortisol will result in a hyper-mineralocorticoid effect (Akamatsu, H. Planta Med., 1991, 57: 119-121), (Armanini, D., Clin. Endocrinol. 1983, 19: 609).

GA completely suppressed viral antigen expression possibly by causing a decrease in the negative charge on the cell surface and/or by decreasing the membrane fluidity thereby preventing Hepatitis A virus entry in cells by receptor mediated endocytosis (W. Xu-Yinga et. al., Chemico-Biological Interactions 181 (2009) 15-19).

GA induces phase II enzymes involved in the detoxification and excretion of carcinogenic or toxic substances and other antioxidant enzymes responsible for maintaining a balanced state between free radicals/oxidants and the antioxidants within the cellular environment. Oxidative injury in AR mice (Aldose reducrase deficient mice) is reduced by GA, by increasing GSH content and decreased MDA formation in a dose dependent manner. Concomitant decreases were observed in glutathione peroxidase (GPx), catalase (CAT), total antioxidant capacity (TAOC) and SOD activities in AR mice. IFN-α, or type II interferon, is a cytokine that is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumour control. GA led to a significant, increase of IFN-α level in medicine treated mice. IL-4 is a cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells. Upon activation by IL-4, Th2 cells subsequently produce additional IL-4 (Xiao-Lan Li *Int. J. Mol. Sci.* 2011, 12, 905). GA could increase infection resistance as [monocyte chemo-attractant (chemotactic) protein-1] is a CC chemokine MCP-1 inhibitor (United States Patent Application 20060116337).

The mice were treated intra-peritoneally with CCl_4 (0.5 ml/kg). They received GA (50, 300, 200, 400 mg/kg) 24 h and 0.5 h before and 4 h after administering CCl_4, This protection is likely due to the induction of heme oxygenase-1 and the down-regulation of pro-inflammatory mediators (Biol Pharm Bull. 2007, 30, 10, 11898). 18α-GA could dose-dependently inhibits CCl_4 induced liver fibrosis, by promoting the proliferation of hepatocytes, but inhibited that of Hepatic stellate cells (HSCs) GA blocks the translocation of NF-kB into the nucleus; this could suppress the activation and induce the apoptosis of HSCs (Q Ying, Med Sci. Monit., 2012, 18, 1: BR24).

GA was shown to attenuate histological hepatic changes and significantly reduced serum levels of AST, ALT, and lactic dehydrogenase (LDH), at all the indicated times. GA also significantly inhibited hepatocyte apoptosis by down-regulating the expression of caspase-3 and inhibiting the release of Cytochrome c from mitochondria into the cytoplasm. The anti-inflammatory activity of GA may rely on the inhibition of release of tumour necrosis factor-α, myeloperoxidase activity, and translocation of nuclear factor-kappa B into the nuclei. GA also up-regulated the expression of proliferating cell nuclear antigen, implying that it might be able to promote regeneration of livers harmed by LPS. In summary, GA may represent a potent drug protecting the liver against endotoxin-induced injury, especially after massive hepatectomy (Brazilian journal of Medical and Biological Research, 2007, 40, 1637). Pretreatment with GA (50 mg/kg) and the MMP inhibitor (5 mg/kg) suppressed increases in serum levels of ALT and AST in mice treated with LPS/Gal N due to a down-regulation of MMP-9 (J Pharm Pharmacol. 2008, 60, 1, 91).

The metabolic syndrome (MetS) is a cluster of metabolic abnormalities comprising visceral obesity, dyslipidaemia and insulin resistance (IR). Oral administration of 50 mg/kg of GA for one week could counteract the development of visceral obesity and improve dyslipidaemia via selective induction of tissue lipoprotein lipase (LPL), expression and a positive shift in serum lipid parameters respectively, and retard the development of IR associated with tissue steatosis (Lipids Health Dis. 2009, 29, 8, 31).

Diammoniumglycyrrhizinate (DG) protected mice against Concanavalin A (ConA)-induced elevation of serum ALT levels and apoptosis of hepatocytes; DG may possibly protect the liver from injury via two pathways: direct protection of hepatocytes from apoptosis through an IL-6 dependent way and indirect inhibition of T-cell-mediated inflammation through an IL-1 independent way (Int Immunopharmacol. 2007 October: 7(10): 1292).

Magnesium isoglycyrrhizinate 100 or 150 mg once daily, drugs are effective and safe treatment for chronic liver diseases (Zhoiighua Gan Zang Bing Za Zhi. 2009, 11, 847).

A sugar alcohol is a kind of alcohol prepared from sugars. These organic compounds are a class of polyols, also called polyhydric alcohol, polyalcohol, or glycitol. They are white, water-soluble solids that occur naturally and are used widely in the food industry as thickeners and sweeteners. Sugar alcohols such as Mannitol, Erythritol, Sorbitol, Xylitol etc., which are chemically stable can be used as a radical scavenger (hydroxyl radical). Similarly, it has been found that compounds like Erythritol, Mannitol, Sorbitol, Xylitol etc. up-regulated different types of superoxide dismutase (SOD) like Cu/Zn-, Mn- and EC-SOD isozymes. In particular, the SOD activity of the erythritol-added group increased by 2-5 times. Further it is reported that diabetics have a low SOD activity due to the Maillard reaction, because the Maillard reaction remarkably causes a decrease in the SOD activity (US Patent Application 20100037353 A1). Mannitol containing hyperosmolar solution has been shown to protect ethanol-induced gastric mucosal damage (Gharzouli K, Exp. Toxic. Pathol., 2001; 53: 175). Both rats and humans absorb and metabolize partially the Mannitol ingested in gastro intestinal tract (GIT). However, intestinal microflora convert Mannitol in to more absorbable form. In rat, absorbed mannitol is converted in to hepatic glycogen probably via fructose (J. Nutr. 1985, 115: 890). The mechanism of protecting living cells by Mannitol is not fully understood.

The beverage comprises of certain other ingredients like pH adjusting agent(s), and flavoring agent(s) etc.

Some important embodiments of the beverage of the present invention are as follows:

An important embodiment of the present invention relates to a beverage having reduced toxicity.

Yet another embodiment of the present invention relates to an alcoholic beverage having reduced hepato-toxicity.

Yet another embodiment of the present invention relates to an alcoholic beverage comprising hepato-protective agent(s) to achieve the reduced hepato-toxicity.

In an important embodiment of the present invention, the beverage comprises of 18β-Glycyrrhizin in combination with sugar alcohols selected from the group consisting D-Mannitol, D-Xylitol, D-Erythritol and mixtures thereof and reducing

or non-reducing sugars selected from D-Xylose, D-Mannose, D-Sucrose and D-Lactose and mixtures thereof.

In yet another important embodiment of the present invention, the beverage comprises of 18α-Glycyrrhizin in combination with sugar alcohols selected from the group consisting D-Mannitol, D-Xylitol, D-Erythritol and mixtures thereof.

In an important embodiment, the beverage composition comprises 18β-Glycyrrhizin in the range of 0.05 to 0.4%, preferably 0.1 to 0.3% and D-Mannitol, D-Xylitol, D-Erythritol, D-Xylose, D-Mannose, D-Sucrose, D-Lactose and mixture thereof is in the range of 0.5 to 3.0%, preferably 1.0 to 2.5%.

In an important embodiment, the beverage composition comprises 18β-Glycyrrhizin in range of 0.05 to 0.3%, preferably 0.1 to 0.3% and D-Mannitol, D-Xylitol, D-Erythitol and mixtures thereof is in the range of 0.5 to 3.0%, preferably 1.0 to 2.5%.

In an important embodiment, the most preferable combination of hepato-protective agents is a combination of 18β-Glycyrrhizin or 18α-Glycyrrhizin and D-Mannitol.

In an important embodiment, the beverage composition comprises 18β-Glycyrrhizin in the range of 0.05 to 0.3% and the D-Mannitol is in the range of 0.5 to 3.0% and preferably 18β-Glycyrrhizin in the range of 0.1 to 0.3% and the D-Mannitol is in the range of 1.0 to 2.5%.

In an important embodiment, the beverage composition comprises 18α-Glycyrrhizin in the range of 0.1 to 0.3% and the D-Mannitol in the range of 1.0 to 2.5%.

In yet another embodiment, the process for the preparation of alcoholic beverage composition comprising steps of (a) obtaining alcohol or water or a mixture thereof, (b) mixing 18β-Glycyrrhizin or 18α-Glycyrrhizin with the alcohol or water or a mixture of alcohol and water of step (a), (c) adding sugar alcohol or sugar to the mixture of step (b), (d) adjusting the pH of the resulting solution of step (c) between 4.0-9.0, (e) optionally adding the flavoring agent and (t) obtaining the required alcoholic beverage composition.

Still another embodiment of the present invention is to provide an alcoholic beverage composition comprising the pH adjusting agent(s).

In yet another embodiment, the pH adjusting agent is an organic or inorganic base/buffer, preferably selected from potassium sorbate or sodium phosphate (monobasic or dibasic or tribasic).

Further embodiment of the present invention provides a beverage optionally comprising of flavoring agents selected from, vanilla and strawberry.

Still another embodiment of the present invention is to provide a beverage having acceptable taste, flavor, odor, clarity and buzz factor.

In a further embodiment of the present invention variation in dosages of sugar alcohols, glycyrrhizin and a combination of sugar alcohols and 18β or 18α-Glycyrrhizin has also been evaluated for its hepato-protective activity.

The scope of the present invention also includes the study in respect of acute and chronic hepatotoxicity caused by the variation in the alcohol dosage and its time of duration in administration.

Still another embodiment of the beverage composition relates to providing reduced hepato-toxicity.

Yet another embodiment of the beverage composition is the use in a method of amelioration of diseases involving acute and chronic toxicity such as alcoholic liver diseases (ALD) like steatosis, steatohepatitis, fibrosis, liver cirrhosis and hepatocellular carcinoma etc. which are caused by alcohol induced toxicity.

Another important embodiment of the present invention is that the beverage composition can be packed as ready-to-drink produce in food grade bottles, cans, tetra packs, pouches, etc. The packaging can be done by conventional methods.

For the establishment of synergism existing in the formulation of the present invention, markers/marker enzymes viz. SOD, Catalase, GPx, TNF-α were primarily taken into consideration for evaluating the % synergism. However, enzymes ALT, AST, ALKP and MDA were also analyzed to support the same.

Reasons for Estimating ALT, AST, ALKP:

Chronic misuse of alcohol changes marker enzymes of liver functions such as serum aspartate aminotransferase and alanine aminotransferase (AST, ALT), alkaline phosphatase (ALKP) and so these enzymes were studied.

ALT and AST are found in hepatocytes but AST is also found in skeletal and myocardial cells. In alcohol related liver damage, the AST is elevated more than the ALT, at least in part as a reflection of alcohol related skeletal damage. This is the reverse of the normal pattern in acute hepatocellular disease (for example acute viral hepatitis) where the ALT exceeds the AST.

ALKP is an enzyme in the cells lining the biliary ducts of the liver. ALKP levels in plasma will rise almost concomitantly with liver disease related with altered bile production and/or secretion and chronic liver diseases.

Reasons for Estimating Oxidative Stress Markers (MDA, Antioxidant Enzymes [SOD, CAT, Glutathione Peroxidase Etc.] Reduced Glutathione [GSH]):

Alcohol metabolism in the liver results in the formation reactive oxygen species (ROS). Alcohol also stimulates the activity of cytochrome P450, which contribute to ROS production. Further, alcohol can alter the levels of certain metals in the body, thereby facilitating ROS production. Finally, alcohol reduces the levels of agents that can eliminate ROS (i.e., endogenous antioxidants). The resulting state of the cell, known as oxidative stress, can lead to cell injury. ROS production and oxidative stress in liver cells play a central role in the development of alcoholic liver disease.

MDA (Malondialdehyde) is the end product of cell membrane lipid peroxidation. ROS degrade (oxidative degradation) polyunsaturated fatty acids of cell membrane resulting cell damage. The extent of lipid peroxidation can be well correlated with tissue MDA content.

SOD (Superoxide dismutase) catalyzes the breakdown of the superoxide radical into oxygen and hydrogen peroxide. Liver cells are enriched with SOD as it is the major organ related with metabolism numerous substances.

CAT (Catalase) catalyzes the conversion of hydrogen peroxide (H_2O_2) to water and oxygen. This enzyme is localized to peroxisomes in most eukaryotic cells.

GPx (Glutathione peroxidase) is the most abundantly available in the cytoplasm of most of the cells. It neutralizes hydrogen peroxide (H_2O_2) in presence of GSH.

$$2GSH + H_2O_2 \xrightarrow{GPx} GS\text{-}SG + 2H_2O_2$$

(GSH-reduced glutathione, GSSG-oxidized glutathione)

GSH is the most abundant antioxidant in aerobic cells. GSH is critical for protecting the cells from oxidative stress, acting as a free radical scavenger and inhibitor of lipid peroxidation. (GSH also participates in the degradation of H_2O_2 by glutathione peroxidases (GPx). The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) is an indicator of cellular health (status of cellular redox potential). In normal

healthy conditions GSH constituting nearly 90% of cellular glutathione (i.e., GSH/GSSG is around 9). However, the GSH/GSSG ratio is reduced in ROS related disorders.

Reasons for Estimating Tumor Necrotic Factor Alpha (TNF-α):

Alcohol consumption increases the translocation of endotoxins from intestine to portal circulation and interacts with Kuppfer cells (immunocytes) leading to secretion of several pro-inflammatory cytokines including tumor necrotic factor alpha (TNF-α).

Based on the Above Description, we Identified Some Key Marker and Justify the Importance of the Parameter Chosen: SOD, Catalase & GPx: In system SOD catalyzes the dismutation of superoxide to H_2O_2. GPx and Catalase then independently convert this H_2O_2 to water. SOD together with GPx and catalase form the main enzyme defense against harmful effect of ROS.

GSH is the main endogenous antioxidant that protects cells from xenobiotics including alcohol. Alcohol is known to deplete GSH levels on the process to neutralize oxidants. Apart from this, endogenous glutathione-glutathione peroxidase system acts as an important antioxidants and cyto-protective machinery in the hepatocytes exposed to ethanol. Thus, depletion of cellular GSH level plays an important role in ethanol-mediated hepato-cellular dysfunction.

The following tables (1 to 9) illustrate the % of hepatoprotection of individual ingredients, combination of ingredients and the % synergism exhibited using respective combinations. All animal experiments were conducted for a period of one month by per oral administration of 2.5 g/kg dose of alcohol.

TABLE 1

% Protection of D-Mannitol						
Sample Code	Man %	GSH % Prot.	SOD etc. % Prot.	TNF-α % Prot.	ALT etc % Prot.	MDA % Prot.
A	0.5	10.35	12.71	7.19	12.26	19.17
3	1	20.06	19.32	16.74	20.37	31.63
B	1.5	25.76	26.21	29.89	25.94	48.56

TABLE 1-continued

% Protection of D-Mannitol						
Sample Code	Man %	GSH % Prot.	SOD etc. % Prot.	TNF-α % Prot.	ALT etc % Prot.	MDA % Prot.
C	2.5	31.53	35.83	31.46	29.71	50.8
11	3	32.37	36.08	30.76	29.48	50.31

TABLE 2

% Protection of D-Xylitot & D-Erythritol					
	GSH % Prot.	SOD etc % Prot.	TNF-α % Prot.	ALT etc % Prot.	MDA % Prot.
Xyl %					
1%	19.76	18.91	15.77	17.62	26.9
2.5%	35.57	36.88	30.05	26.72	45.38
Ery %					
1%	18.71	17.94	16.57	17.84	24.71
2.5%	37.29	36.29	35.96	32.13	48.61

TABLE 3

% Comparative Protection of 18β and 1.8α-Glycyrrhizin						
Sample Code	GA %	GSH % Prot.	SOD etc % Prot.	TNF-α % Prot.	ALT etc % Prot.	MDA Prot. %
	18β-GA					
D	0.1	3.29	11.45	7.64	8.38	15.97
U	0.2	12.1	16.72	12.31	13.25	27.12
W	0.3	19.1	27.95	21.18	20.99	46.35
X	0.4	31.34	31.05	29.28	26.42	56.74
	18α-GA					
4	0.1	8.93	14.33	10.58	11.98	15.1
5	0.3	16.96	25.84	23.45	18.3	41.69

TABLE 4

% Protection and % Synergism of 18β-Glycyrrhizin-Mannitol combinations												
Sample Code	GA %	Man %	GSH % Prot.	GSH % Syn.	SOD etc % Prot.	SOD etc % Syn	TNF-α % Prot.	TNF-α % Syn	ALT etc. % Prot.	ALT etc. % Syn	MDA % Prot.	MDA % Syn
H	0.1	2.5	48.24	38.51	60.15	26.65	50.56	29.31	40.35	10.52	85.62	28.23
L	1	2.5	83.29	10.45	78.75	21.31	87.64	29.99	52.35	−11.15	93.29	−20.87
O	0.3	2.5	61.95	22.43	71.57	13.44	69.63	32.28	49.4	−1.09	76.54	−21.21
M	0.4	2.5	76.38	21.55	79.83	20.59	81.62	34.38	53.15	−4.17	80.41	−25.23
C	0.1	0.5	17.64	28.76	25.34	3.72	19.16	29.2	21	7.32	39.63	12.78
4	0.1	1	29.58	26.68	39.33	28.1	32.68	34.04	29.13	5.25	55.41	16.41
12	0.1	3	45.53	27.68	58.15	22.74	47.2	22.92	37.23	0.37	70.87	6.93

TABLE 5

Comparative % Protection and % Synergism of 18β or 18α-Glycyrrhizin - Mannitol combinations												
Sample Code		Man %	GSH % Prot.	GSH Syn %	SOD etc % Prot.	SOD etc % Syn	TNF-α % Prot.	TNF-α % Syn	ALT etc % Prot.	ALT etc % Syn	MDA % Prot.	MDA Syn %
	18β-GA %											
4	0.1	1	29.58	26.68	39.33	28.1	32.68	34.04	29.13	5.25	55.41	16.41
H	0.1	2.5	48.24	38.51	60.15	26.65	50.56	29.31	40.35	10.52	85.62	28.23
O	0.3	2.5	61.95	22.43	71.57	13.44	69.63	32.28	49.4	−1.09	76.54	−21.21

TABLE 5-continued

Comparative % Protection and % Synergism of 18β or 18α-Glycyrrhizin - Mannitol combinations

Sample Code		Man %	GSH % Prot.	GSH Syn %	SOD etc % Prot.	SOD etc % Syn	TNF-α % Prot.	TNF-α % Syn	ALT etc % Prot.	ALT etc % Syn	MDA % Prot.	MDA Syn %
	1.8α-GA %											
6	0.1	1	32.74	12.94	42.42	26.01	34.05	24.63	30.97	−0.29	54.16	15.9
8	0.1	2.5	52.68	30.2	60.16	19.8	53.21	26.57	41.35	3.51	76.6	16.24
10	0.3	2.5	57.44	18.46	69.06	12.57	68.1	24.02	46.49	−1.35	75.8	−18.05

TABLE 6

Comparative % Protection and % Synergism of 18β-Glycyrrhizin-Mannitol, Xylitol & Erythritol)

		SOD etc. % Prot.	SOD etc. % Syn	GSH % Prot.	GSH % Syn	TNF-α % Prot.	TNF-α % Syn
0.10%	1%						
GA %	Man %	39.33	28.1	29.58	26.68	32.68	34.04
GA %	Ery %	35.64	21.5	28.85	31.14	30.37	25.44
GA %	Xyl %	38.26	26.35	28.19	22.3	29.72	26.95
Man: Ery		—	1.3	—	0.85	—	1.33
Man: Xyl		—	1.06	—	1.19	—	1.26
0.10%	2.50%						
GA %	Man %	60.15	26.65	48.24	38.51	50.56	29.31
GA %	Ery %	56.47	18.21	43.35	6.83	49.26	12.98
GA %	Xyl %	56.94	17.61	44.8	15.29	46.29	22.82
Man: Ery		—	1.46	—	5.63	—	2.25
Man: Xyl		—	1.51	—	2.51	—	1.28
0.30%	2.50%						
GA %	Man %	71.57	13.44	61.95	22.43	69.63	32.28
GA %	Ery %	71.86	11.94	66.14	17.29	64.36	12.64
GA %	Xyl %	71.18	10.04	60.61	10.87	55.65	8.63
Man: Ery		—	1.12	—	1.29	—	2.55
Man: Xyl		—	1.33	—	2.06	—	3.74

TABLE 7

Comparative data of % Protection and % Synergism of (18β Glycyrrhizin/Mannitol, Xylitol and Erythritol)

		ALT etc % Prot.	ALT etc % Syn	MDA % Prot.	MDA % Syn
0.10%	1%				
GA %	Man %	29.13	5.25	55.41	16.41
GA %	Ery %	24.48	−5.83	46.38	14.01
GA %	Xyl %	27.19	6.63	50.02	16.68

TABLE 7-continued

Comparative data of % Protection and % Synergism of (18β Glycyrrhizin/Mannitol, Xylitol and Erythritol)

		ALT etc % Prot.	ALT etc % Syn	MDA % Prot.	MDA % Syn
0.10%	2.50%				
GA %	Man %	40.35	10.52	85.62	28.23
GA %	Ery %	40.06	−0.62	75.29	16.58
GA %	Xyl %	38.2	10.18	76.51	24.71
0.30%	2.50%				
GA %	Man %	49.4	−1.09	76.54	−21.21
GA %	Ery %	52.68	−0.89	80.3	−15.44
GA %	Xyl %	46.9	−1.86	80.52	−12.22

TABLE 8

% Protection of Sucrose, Mannose, Xylose & Lactose

	GSH % Prot.	SOD etc % Prot.	TNF-α % Prot.	ALT etc % Prot.	MDA % Prot.
Suc %					
1	6	5.16	6.13	6.70	8.27
2.5	11.63	10.49	14.18	13.89	18.92
Mans %					
1	6.12	3.93	7.85	6.14	10.65
2.5%	13.59	11.18	16.49	16.34	23.67
Xyls %					
1	6.23	7.83	6.44	8.06	6.28
2.5	11.84	19.1	13.98	14.73	15.38
Lac %					
1	4.36	6.78	8.19	8.21	7.70
2.5	14.8	17.38	15.26	17.41	21.47

TABLE 9

% Protection and % Synergism of (18β-GA: Sucrose, Mannose, Xylose & Lactose)

Sample Code	GA %		GSH % Prot.	GSH % Syn	SOD etc % Prot.	SOD etc % Syn	TNF-α % Prot.	TNF-α % Syn	ALT etc % Prot.	ALT etc % Syn	MDA % Prot.	MDA % Syn
		Suc %										
10	0.1	1	10.65	14.64	18.32	10.37	15.14	9.95	14.63	1.69	25.87	6.72
11	0.3	2.5	33.41	8.72	41.3	8.37	40.12	13.46	31.4	−7.47	56.53	−13.39
		Mans %										
14	0.1	1	11.02	17.11	18.05	17.29	17.07	10.2	15.71	8.66	28.82	8.26
15	0.3	2.5	37.58	14.96	42.02	9.16	43.19	14.65	33.88	−7.97	59.27	−15.35

TABLE 9-continued

% Protection and % Synergism of (18β-GA: Sucrose, Mannose, Xylose & Lactose)

Sample Code	GA %		GSH % Prot.	GSH % Syn	SOD etc % Prot.	SOD etc % Syn	TNF-α % Prot.	TNF-α % Syn	ALT etc % Prot.	ALT etc % Syn	MDA % Prot.	MDA % Syn
		Xyls %										
18	0.1	1	10.9	14.05	20.97	8.83	15.6	10.8	16.84	4.26	22.23	−0.09
19	0.3	2.5	34.27	10.76	53.23	13.21	38.1	8.36	32.28	−9.47	52.64	−14.66
		Lac %										
22	0.1	1	8.57	12.03	19.47	6.79	17.2	8.65	16.75	3.17	25.1	6.04
23	0.3	2.5	38.16	12.57	47.19	5.07	39.55	8.53	34.6	−9.98	57.88	−14.66

The data provided in the above tables clearly indicates that the 18β-GA/D-Mannitol combination exhibits superior order of synergism over the combination of 18β-GA/D-Erythritol and 18β-GA/Xylitol combinations.

The data provided in the above tables also indicates that overall the 18β-GA/D-Mannitol combinations exhibit almost similar order of synergism as that of 18α-GA/D-Mannitol combinations.

Also it can be concluded that the combination of 18β-GA/reducing or non-reducing mono or disaccharide has exhibited lesser degree of synergistic effect.

The present invention is illustrated with the following examples. However, it should be understood that the scope of the present invention is not limited by the examples in any manner. It will be appreciated by any person skilled in this art that the present investigation includes following examples and further can be modified and altered within the scope of the present invention.

Materials and Methods

Reagents

Distilled ethanol was obtained from Bengal Chemicals, West Bengal, India. Biochemical kits like AST, ALT, ALKP and total protein were obtained from Span Diagnostics Ltd. Surat, India. Time course study of oxidative and nitrosative stress and antioxidant enzymes in $K_2Cr_2O_7$-induced nephrotoxicity. BMC Nephrol., 6: 4). TNF-α was estimated by standard procedures as mentioned in Rat TNF-α ELISA kit (Bio Legend, Inc. San Diego, Calif., USA).

All the chemicals used in the present study were of analytical grade and obtained from the following companies: Sigma (St. Louis, Mo. USA), Merck (Mumbai, India), S. D. Fine Chemicals (Mumbai, India) and Qualign (Mumbai, India).

Alcohol Induced Sub-Acute Hepatotoxicity in Rats

Male Wistar albino rats weighing 150-200 g are procured from local registered traders (CPCSEA Regd No. 1443/po/6/4/CPCSEA), Kolkata. India and were acclimatized for 7 days at standard housing condition (26° C.±2° C., 60-70% RH with 12±1 hours light and dark cycle). Animals were fed with commercially available diet (Upton India Pvt. Ltd, India) and water ad-libitum during the experiment period.

EXAMPLES

Example 1

a) Model for Biological Testing

Male Wistar albino rats weighing 150-200 g are procured and randomly divided into groups consisting of six animals in each group. Sub-acute toxicity is induced by alcohol in rats by oral administration of 25% alcohol (2.5 gm/kg/day, p.o.) for 28 days and this group served as the negative control and the positive control group received distilled water only.

b) Preparation of Drug Solution

All drug solutions were prepared in 15-40% aqueous alcohol, adjusting the pH in the range of 4.0-9.0 for evaluation of hepato-protective activity. This solution is further diluted with distilled water to obtain 25%, aqueous alcoholic solution and administered orally by gavage to different rats group of step (a).

c) Evaluation of Hepato-Protective Activity

On day 28th day the animals are anaesthetized with ether and blood samples are collected by cardiac puncture and the serum is used for the assay of marker enzymes viz. serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP). The rats are sacrificed by exposure to an overdose of ether, immediately after the collection of blood; their livers are removed, washed in cold saline. Part of the liver is used for preparation of liver homogenate in phosphate buffer (pH 7.4). The supernatant is used for the estimation of malondialdehyde (MDA), super oxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and Glutathione peroxidase (GPx).

Example 2

D-Mannitol (0.5 g) is dissolved in aqueous alcohol (100 ml) to provide 0.5% solution. This solution is administered in several portions to one of the rats group of Example (1a). The administration is carried out over a period of 28 days; each day 10 ml sample is diluted with 6 nil distilled water to make 25% aqueous alcoholic solution (16 ml) and fed orally (10 ml/kg/day). Evaluation of hepato-protective activity is carried out as per Example (1c).

Mean % hepato-protection:	
ALT, AST and ALKP	12.26%
SOD, CAT and GPx	12.71%
GSH	10.35%
Hepatic MDA	19.17%
TNF-α	7.19%

Example 3

D-Mannitol (2.5 g) is dissolved in aqueous alcohol (100 ml) to provide 2.5% solution. This solution is administered in several portions to one of the rats group of Example (1a). The administration, sample dilution, oral feeding and evaluation of hepato-protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection:	
ALT, AST and ALKP	29.71%
SOD, CAT and GPx	35.83%
GSH	31.53%
Hepatic MDA	50.80%
TNF-α	31.46%

Example 4

18β-Glycyrrhizin (0.1 g) is dissolved in aqueous alcohol (100 ml) to provide 0.1% solution. This solution is administered in several portions to one of the of rats group of Example (1a). The administration, sample dilution, oral feeding and evaluation of hepato-protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection	
ALT, AST and ALKP	8.38%
SOD, CAT and GPx	11.45%
GSH	3.29%
Hepatic MDA	15.97%
TNF-α	7.64%

Example 5

D-Mannitol (2.5 g) and 18β-Glycyrrhizin (0.1 g) are dissolved in aqueous alcohol (100 ml) to provide 2.6% solution. This solution is administered in several portions to one of the rats group of Example (1a). The administration, sample dilution, oral feeding and evaluation of hepato-protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection:	
ALT, AST and ALKP	40.35%
SOD, CAT and GPx	60.15%
GSH	48.24%
Hepatic MDA	85.62%
TNF-α	50.56%

Example 6

D-Mannitol (2.5 g) and 18β-Glycyrrhizin (1.0 g) are dissolved in aqueous alcohol (100 ml) to provide 3.5% solution. This solution is administered in several portions to one of the rats groups of Example 1(a). The administration, sample dilution, oral feeding and evaluation of hepato-protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection:	
ALT, AST and ALKP	52.35%
SOD, CAT and GPx	78.75%
GSH	83.29%
Hepatic MDA	93.29%
TNF-α	87.64%

Example 7

D-Mannitol (0.5 g) and 18β-Glycyrrhizin (0.1 g) are dissolved in aqueous alcohol (100 ml) to provide 0.6% solution. This solution is administered in several portions to one of the rats group of Example (1a). The administration, sample dilution, oral feeding and evaluation of hepato-protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection:	
ALT, AST and ALKP	21.0%
SOD, CAT and GPx	25.34%
GSH	17.64%
Hepatic MDA	39.63%
TNF-α	19.16%

Example 8

D-Mannitol (3.0 g) and 18β-Glycyrrhizin (0.1 g) are dissolved in aqueous alcohol (100 ml) to provide 3.1% solution. This solution is administered in several portions to one of the rats group of Example (1a). The administration, sample dilution, oral feeding and evaluation of hepato-protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection:	
ALT, AST and ALKP	37.23%
SOD, CAT and GPx	58.15%
GSH	45.53%
Hepatic MDA	70.87%
TNF-α	47.20%

Example 9

D-Mannitol (2.5 g) and 18β-Glycyrrhizin (0.4 g are dissolved in aqueous alcohol (100 ml) to provide 2.9% solution. This solution is administered in several portions to one of the rats group of Example (1a). The administration, sample dilution, oral feeding and evaluation of hepato-protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection:	
ALT, AST and ALKP	53.15%
SOD, CAT and GPx	79.83%
GSH	76.38%
Hepatic MDA	80.41%
TNF-α	81.62%

Example 10

D-Mannitol/D-Xylitol/D-Erythritol (1.0 g) and 18β-Glycyrrhizin (0.1 g) are dissolved in aqueous alcohol (100 ml) to provide 1.1% solution. This solution is administered in several portions to one of the rats group of Example (1a). The administration, sample dilution, oral feeding and evaluation of hepato-protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection:			
	Sugar alcohols		
Enzymes/Markers	D-Mannitol	D-Xylitol	D-Erythritol
ALT, AST and ALKP	29.13%	27.19%	24.48%
SOD, CAT and GPx	39.33%	38.26%	35.64%
GSH	29.58%	28.19%	28.25%
Hepatic MDA	55.41%	50.02%	46.38%
TNF-α	32.68%	29.72%	30.37%

Example 11

D-Mannitol/D-Xylitol/D-Erythritol (2.5 g) and 18β-Glycyrrhizin (0.3 g) are dissolved in aqueous alcohol (100 ml) to provide 2.8% solution. This solution is administered in several portions to one of the rats group of Example (1a). The administration, sample dilution, oral feeding and evaluation of hepato protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection:			
	Sugar alcohols		
Enzymes/Markers	D-Mannitol	D-Xylitol	D-Erythritol
ALT, AST and ALKP	49.40%	46.90%	52.68%
SOD, CAT and GPx	71.57%	71.18%	71.86%
GSH	61.95%	60.61%	66.14%
Hepatic MDA	76.54%	80.52%	80.30%
TNF-α	69.63%	55.65%	64.36%

Example 12

DI-Mannose/D-Xylose/D-Lactose/D-Sucrose (2.5 g) and 18β-Glycyrrhizin (0.3 g) are dissolved in aqueous alcohol (100 ml) to provide 2.8% solution. This solution is administered in several portions to one of the rats group of Example (1a). The administration, sample dilution, oral feeding and evaluation of hepato-protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection:				
	Sugars			
Enzymes/Markers	D-Mannose	D-Xylose	D-Lactose	D-Sucrose
ALT, AST and ALKP	33.88%	32.28%	34.60%	31.40%
SOD, CAT and GPx	42.02%	53.23%	47.19%	41.30%
GSH	37.58%	34.27%	38.16%	33.41%
Hepatic MDA	59.27%	52.64%	57.88%	56.53%
TNF-α	43.19%	38.10%	39.55%	40.12%

Example 13

D-Mannose/D-Xylose/D-lactose/D-Sucrose (1.0 g) and 18β-Glycyrrhizin (0.1 g) are dissolved in aqueous alcohol (100 ml) to provide 1.1% solution. This solution is administered in several portions to one of the rats group of Example (1a). The administration, sample dilution, oral feeding and evaluation of hepato-protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection:				
	Sugars			
Enzymes/Markers	D-Mannose	D-Xylose	D-Lactose	D-Sucrose
ALT, AST and ALKP	15.71	16.84%	16.75%	14.63%
SOD, CAT and GPx	18.05	20.97%	19.47%	18.32%
GSH	11.02	10.90%	8.57%	10.65%
Hepatic MDA	28.82	22.23%	25.10%	25.87%
TNF-α	17.07	15.60%	17.20%	15.14%

Example 14

D-Mannitol (1.0 g) and 18α-Glycyrrhizin (0.1 g) are dissolved in aqueous alcohol (I 00 ml) to provide 1.1% solution. This solution is administered in several portions to one of the rats group of Example (1a). The administration, sample dilution, oral feeding and evaluation of hepato-protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection:	
ALT, AST and ALKP	30.97%
SOD, CAT and GPx	42.42%
GSH	32.74%
Hepatic MDA	54.16%
TNF-α	34.05%

Example 15

D-Mannitol (2.5 g) and 18α-Glycyrrhizin (0.3 g) are dissolved in aqueous alcohol (100 ml) to provide 2.8% solution. This solution is administered in several portions to one of the rats group of Example (1a). The administration, sample dilution, oral feeding and evaluation of hepato-protective activity is carried out as mentioned in Example 2 and as per Example (1c).

Mean % hepato-protection:	
ALT, AST and ALKP	46.49%
SOD, CAT and GPx	69.06%
GSH	57.44%
Hepatic MDA	78.80%
TNF-α	68.1%

Example 16

Method of Preparation:

0.1 to 0.4 grams of 18β/α-Glycyrrhizin is dissolved in 15-40% alcohol or alcohol:water mixture (in 100 ml). To this solution (0.5 to 3.0 grams) of sugar alcohol or sugar is added. The resulting solution is mixed thoroughly to obtain a clear solution. Thereafter the pH of the resulting solution is adjusted to between 4.0-9.0 and optionally desired flavoring agent (vanilla) is added to obtain the final alcoholic beverage composition.

The expansion for the abbreviations used in this application is enumerated as below:

GA: Glycyrrhizin (Glycyrrhizic acid or Glycyrrhizinic acid or 18β-Glycyrrhizin)

Man: Mannitol

Xyl: Xylitol

Ery: Erythitol
Mans: Mannose
Suc: Sucrose
Xyls: Xylose
Lac: Lactose
SOD etc: SOD, CAT & GPx
ALT etc: ALT, AST and ALKP
Mat: Matrine

ADVANTAGES OF THE PRESENT INVENTION

1. The alcoholic beverage of the present invention has better hepato-protection.
2. The alcoholic beverage of the present invention has an acceptable odor, taste, clarity and acceptable buzz factor.

What is claimed is:

1. An alcoholic beverage composition providing synergistic hepato-protection, the alcoholic beverage comprising:

a) a first hepato-protective agent;

b) alcohol or an alcohol-water combination; and

c) a second hepato-protective agent;

d) wherein said first hepato-protective agent comprises 18α-Glycyrrhizin in a mass concentration range of 0.05% to 0.3% or 18β-Glycyrrhizin in a mass concentration range of 0.05% to 0.4% or a combination thereof;

e) wherein said second hepato-protective agent comprises at least one sugar or at least one sugar alcohol in a mass concentration range of 0.5% to 3.0%.

2. The alcoholic beverage composition as claimed in claim **1**, wherein said second hepato-protective agent comprises at least one sugar alcohol selected from the group consisting of D-Mannitol, D-Xylitol, and D-Erythritol, or a combination thereof.

3. The alcoholic beverage composition as claimed in claim **1**, wherein said second hepato-protective agent comprises at least one sugar selected from the group consisting of D-Sucrose, D-Mannose, D-Xylose and D-Lactose, or a combination thereof.

4. The alcoholic beverage composition as claimed in claim **2**, wherein: said first hepato-protective agent comprises 18α-Glycyrrhizin in a mass concentration range of 0.05% to 0.3%; and said sugar alcohol is in a mass concentration range of 0.5% to 3.0%.

5. The alcoholic beverage composition as claimed in claim **2**, wherein: said first hepato-protective agent comprises 18α-Glycyrrhizin in a mass concentration range of 0.1% to 0.3%; and said sugar alcohol is in a mass concentration range of 1.0% to 2.5%.

6. The alcoholic beverage composition as claimed in claim **3** wherein: said first hepato-protective agent comprises 18α-Glycyrrhizin in a mass concentration range of 0.05 to 0.3%; and said sugar is in a mass concentration range of 0.5% to 3.0%.

7. The alcoholic beverage composition as claimed in claim **3**, wherein: said first hepato-protective agent comprises 18α-Glycyrrhizin in a mass concentration range of 0.1% to 0.3%; and said sugar is in a mass concentration range of 1.0% to 2.5%.

8. The alcoholic beverage composition as claimed in claim **1**, further comprising at least one pH adjusting agent.

9. The alcoholic beverage composition as claimed in claim **1**, herein said alcoholic beverage composition further comprises at least one flavoring agent.

10. A process for the preparation of alcoholic beverage composition of claim **1**, comprising steps of (a) obtaining alcohol or alcohol-water combination, (b) mixing 18β-Glycyrrhizin or 18α-Glycyrrhizin with the alcohol or alcohol-water combination of step (a), (c) adding sugar alcohol or sugar to the mixture of step (b), (d) adjusting pH of resulting solution of step (c) between 4.0-9.0, (e) optionally adding flavoring agent and (f) obtaining the alcoholic beverage composition.

11. A method of using the alcoholic beverage composition as claimed in claim **1**, comprising the step of consuming the alcoholic beverage composition by a human, for providing reduced hepato-toxicity in said human.

12. The alcoholic beverage composition as claimed in claim **1** for use in a method of amelioration of diseases involving acute and chronic toxicity caused by alcohol consumption.

13. The alcoholic beverage composition claimed in claim **9**, wherein said flavoring agent comprises vanilla flavor or strawberry flavor.

14. The alcoholic beverage composition as claimed in claim **2**, wherein: said first hepato-protective agent comprises 18β-Glycyrrhizin in a mass concentration range of 0.05% to 0.4%; and said sugar alcohol is in a mass concentration range of 0.5% to 3.0%.

15. The alcoholic beverage composition as claimed in claim **2**, wherein: said first hepato-protective agent comprises 18β-Glycyrrhizin in a mass concentration range of 0.1% to 0.3%; and said sugar alcohol is in a mass concentration range of 1.0% to 2.5%.

16. The alcoholic beverage composition as claimed in claim **3**, wherein: said first hepato-protective agent comprises 18β-Glycyrrhizin in a mass concentration range of 0.05 to 0.4%; and said sugar is in a mass concentration range of 0.5% to 3.0%.

17. The alcoholic beverage composition as claimed in claim **3**, wherein: said first hepato-protective agent comprises 18β-Glycyrrhizin in a mass concentration range of 0.1% to 0.3%; and said sugar is in a mass concentration range of 1.0% to 2.5%.

18. The alcoholic beverage composition as claimed in claim **1**, wherein said first hepato-protective agent comprises 18β-Glycyrrhizin or 18α-Glycyrrhizin and said second hepato-protective agent comprises D-Mannitol.

19. The alcoholic beverage composition as claimed in claim **18**, wherein: said first hepato-protective agent comprises 18β-Glycyrrhizin in a mass concentration range of 0.05% to 0.4%; and said D-Mannitol is in a mass concentration range of 0.5% to 3.0%.

20. The alcoholic beverage composition as claimed in claim **18**, wherein: said first hepato-protective agent comprises 18β-Glycyrrhizin in a mass concentration range of 0.1% to 0.3%; and said D-Mannitol is in a mass concentration range of 1.0% to 2.5%.

21. The alcoholic beverage composition as claimed in claim **18**, wherein: said first hepato-protective agent comprises 18α-Glycyrrhizin in a mass concentration range of 0.05% to 0.3%; and said D-Mannitol is in a mass concentration range of 0.5% to 3.0%.

22. The alcoholic beverage composition as claimed in claim **18**, wherein: said first hepato-protective agent comprises 18α-Glycyrrhizin in a mass concentration range of 0.1% to 0.3%; and said D-Mannitol is in a mass concentration range of 1.0% to 2.5%.

23. The alcoholic beverage composition as claimed in claim **8**, wherein said pH adjusting agent comprises an organic or inorganic base/buffer selected from the group consisting of potassium sorbate, sodium dihydrogen phosphate, sodium hydrogen phosphate, and trisodium phosphate.

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ORIGINAL ARTICLE

The use of mannitol in partial and live donor nephrectomy: an international survey

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Abstract
Purpose Animal studies have shown the potential benefits of mannitol as renoprotective during warm ischemia; it may have antioxidant and anti-inflammatory properties and is sometimes used during partial nephrectomy (PN) and live donor nephrectomy (LDN). Despite this, a prospective study on mannitol has never been performed. The aim of this study is to document patterns of mannitol use during PN and LDN.

Materials and methods A survey on the use of mannitol during PN and LDN was sent to 92 high surgical volume urological centers. Questions included use of mannitol, indications for use, physician responsible for administration, dosage, timing and other renoprotective measures.

Results Mannitol was used in 78 and 64 % of centers performing PN and LDN, respectively. The indication for use was as antioxidant (21 %), as diuretic (5 %) and as a combination of the two (74 %). For PN, the most common

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dosages were 12.5 g (30 %) and 25 g (49 %). For LDN, the most common doses were 12.5 g (36.3 %) and 25 g (63.7 %). Overall, 83 % of centers utilized mannitol, and two (percent or centers??) utilized furosemide for renoprotection.

Conclusions A large majority of high-volume centers performing PN and LDN use mannitol for renoprotection. Since there are no data proving its value nor standardized indication and usage, this survey may provide information for a randomized prospective study.

Keywords Mannitol · Live donor nephrectomy · Partial nephrectomy · Renoprotective agent · Diuretics

Introduction

Both in living donor (LDN) and partial nephrectomy (PN), preserving renal function is crucial. It is well known that warm ischemia-time affects renal function by organ-induced ischemia and subsequent Ischemia–Reperfusion Injury (IRI). IRI is known to affect renal function which may lead to organ failure. Its biological effect lays on sub-cellular injuries with generation of free radicals leading to acute tubular necrosis, reduced glomerular filtration rate (GFR) and, eventually, renal failure [1–4]. There has been evidence that implementation of diuresis may in fact reduce the warm ischemia-related renal damage [1, 3, 5–8]. Therefore, diuretics are commonly used in partial and live donor nephrectomy. It has been speculated that mannitol may have some advantages over other diuretics due to a possible antioxidant activity [9, 10]. Despite this, there is insufficient evidence to recommend and standardize the use of mannitol.

The aim of this study is to document the trend in mannitol use during partial and live donor nephrectomy, through an international survey sent to high-volume tertiary centers.

Materials and methods

A survey on the use of mannitol during partial and live donor nephrectomy was sent by e-mail to 92 high surgical volume urological centers around the world. Survey was performed considering indications, doses and modalities of infusion and use of mannitol reported in literature. The survey was distributed and returned via e-mail in February 2011. We initially queried about the type of Institution and related surgical activities. Questions specifically related to the use of mannitol included the following: if mannitol was utilized in surgical practice, indications (partial and or donor nephrectomy), rationale of using mannitol, physician in charge for mannitol administration (urologist, nephrologist or anesthetist), dose administered in case of partial or donor nephrectomy, timing of administration (before or after clamping), usage of other type of kidney protectors and number of partial of donor nephrectomies performed per year at the corresponding Institution.

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Results

We obtained surveys from 47 centers (51 %). Forty-seven of 47 (100 %) and 17 of 47 (36 %) performed partial and live donor nephrectomy, respectively. Mannitol was used in 78.7 and 64.7 % of centers performing partial and live donor nephrectomy, respectively. The indication for mannitol use was as an antioxidant (20.5 %), as a diuretic (5.2 %) and as a combination of the two (74.3 %). The physician in charge of mannitol administration was the urologist in 53.8 % of the cases, the anesthesiologist in 30.7 % of the cases and both in 12.8 %. In case of partial nephrectomy, dosages administered were 12.5 g in 29.7 % of the cases, 25 g in 48.6 % and different dosages in 21.7 % of the cases. The timing of mannitol administration was before clamping in 75.6 % of the cases, after clamping in 5.4 % and both before clamping and at reperfusion in 19 % of the cases.

In case of live donor nephrectomy, it was administered at dosages of 12.5 g in 36.3 % of the cases and at 25 g in 63.7 %. The timing for administration was before clamping in all cases.

Overall, 83 % ($N = 39$) of the centers utilized mannitol. Of the centers not using mannitol, two utilized Furosemide as kidney protector instead of mannitol.

Discussion

Ischemia, and subsequent reperfusion injury, is a consequence of LDN and PN with arterial clamping. In both circumstances, the induced injury in the renal parenchyma is caused by the alteration of the microcirculatory compliance and by the increase in the perfusion pressure. This effect is magnified in cases of prolonged ischemia-time. Therefore, it is widely accepted that the duration of the ischemia-time is proportionally correlated with the magnitude of ischemia/reperfusion injury [2]. Although the understanding of the cell-death mechanism subsequent to ischemia/reperfusion injury has been deeply investigated during the last few years [1–3, 11], little is known about the efficacy of strategies to reduce this event during LDN and PN. Hypothermic kidney storage (ice) is the principal secure method for kidney preservation. It has been shown that hypothermia reduces cellular metabolism and slows the process that impair cell viability [1].

Diuretics have been also evaluated for kidney protection during LDN and PN. Some authors have evaluated the role of mannitol and other diuretics as kidney protector agents, and there is still a controversial opinion on its real efficacy as kidney protector agent [4, 8, 12–15]. The rationale of giving diuretics during PN or LDN is that a non-oliguric state may be maintained by promoting diuresis and protect the kidney from ischemic/reperfusion injury; furthermore, diuretics have been shown to prevent tubular obstruction, to reduce medullary oxygen consumption and to increase renal blood flow [16]. A multinational survey [14] has recently confirmed that 70 % of intensivists use loop diuretics in a large spectrum of acute renal injury conditions like major surgery, sepsis, shock, contrast or drug-induced nephrotoxicity.

Few prospective randomized trials have addressed the role of diuretics in prevention of acute renal injury, and most of them have been conducted to study the effect of contrast administration [17] and cardiac surgery [18]. Unfortunately, the results have shown that diuretics offer no protection against contrast-induced nephropathy, and in cardiac surgery, higher postoperative creatinine levels were seen in patients treated with furosemide [18]. Similar results were published in four different randomized controlled trials examining the role of diuretics in patients with established renal failure treated in intensive care unit, with no improvements reported in recovering renal function or in decreasing mortality [19–21].

Moreover, three meta-analyses published similar results and noted a significant higher risk of side effects (like hearing-loss) [13, 15, 22] for patients receiving diuretics treatment in cardiac surgery, established renal failure or contrast nephropathy. Similarly, other studies compared diuretics with dopamine or placebo without demonstrating benefits from this drug-regimen therapy [23, 24].

Mannitol is a potent osmotic fluid, with 50 g of mannitol causing an intracellular to extra-cellular shift of 1 liter of water [25]. It is metabolically inert and mostly excreted by the kidney with a minimal reabsorption (7 %) in the renal tubule [26]. Furthermore, it is known that it has a protective role against oxidative injuries acting as a scavenger of the hydroxyl radical that may result in reducing oxidant-derived injury to the kidney, heart and lung [27, 28].

In a recent review [29], results from an international collaboration of the Critical Care Nephrology Working Group of the European Society of Intensive Care medicine (ESICM), the roles of different substances used in acute renal injury were critically evaluated and, accordingly,

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recommendations for clinical practice were given. In the review, loop diuretics and mannitol were not recommended to prevent or ameliorate acute renal injury (grade 1B).

In a recent paper on mannitol use during minimally invasive partial nephrectomy, renal function outcomes were compared between patients who received i.v. Mannitol versus those who did not. Conclusions of this retrospective series were that mannitol did not influence renal function recovery and that an appropriately designed study of mannitol is needed [30].

Despite the lack of scientific evidence, our survey shows that a high proportion of surgeons both from Europe and US regularly use mannitol to protect renal function during PN and LDN (100 and 36 %, respectively); moreover, the different dosages employed at different timing during surgeries underline also the lack of standardization on its use in this setting.

Based on these findings we recently designed a prospective randomized study at our center on the use of mannitol during partial nephrectomy. All patients undergoing partial nephrectomy (open, laparoscopic or robotic) at our center will be randomly assigned to 25 g of mannitol administered as a single dose before clamping versus no mannitol. Patients will be followed postoperatively with serum creatinine, GFR and nuclear GFR at 12 h, 7 and 30 days. The study already obtained the internal ethical committee approval (IRB) and will start recruiting patients on October 15th. A second prospective randomized trial for the use of mannitol during laparoscopic live donor nephrectomy is under development.

Conclusions

Two important conclusions can be highlighted from this survey. First, the majority of the centers performing high-volume partial and live donor nephrectomy prefer to use mannitol as a kidney protector. Second, it appears that there are neither unified criteria nor standardization for mannitol indication and usage. The hope is that our ongoing prospective randomized trial will help solving the dilemma.

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Appendix: Survey

Use of mannitol in partial nephrectomy and living donor nephrectomy

Please answer the questions by adding an X to the right answer

1. Institution (Name of the Institution, City, Country)
2. Type of Institution
   a. Academic
   b. Peripheral University Affiliate
   c. Public Hospital (or County Hospital)
   d. Private Hospital
   e. Other
3. Faculty number in the department

   Number
4. Number of beds of the Urology Department

   Number
5. Is mannitol (or other similar agent) used in partial or/and donor nephrectomy at your institution?
   a. Yes
   b. No

   If you answer YES, please proceed to question 7.
   If you answer NO please proceed to question 6.
6. Reason why mannitol is not available at your institution
   a. Not part of the internal protocol
   b. Do not believe in its benefits
   c. Other
7. At your institution, mannitol is used for
   a. Partial nephrectomy
   b. Living donor nephrectomy
   c. Both
8. What is the reason of using mannitol during these surgeries?
   a. Kidney protector/Antioxidan
   b. Stimulate diuresis

c. Both
d. Other

9. Who does indicate mannitol administration?

a. Urologist
b. Nephrologist
c. Anesthetist
d. Other

In case of partial nephrectomy

10. Dosage

a. 12.5 g
b. 25 g
c. Other

11. Timing

a. Before clamping
b. After clamping
c. Other

12. Other kidney protector agent

In case of living donor nephrectomy

13. Dosage

a. 12.5 g
b. 25 g
c. Other

14. Timing

a. Before clamping
b. After clamping
c. Other

15. Other kidney protector agent
16. How many partial nephrectomies are performed at your institution per year?

a. <10
b. Between 10 and 20
c. >20
d. >50
e. >100

17. How many live donor nephrectomies have you performed at your institution?

a. <10
b. Between 10 and 20
c. >20
d. >50
e. >100

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Final Report on the Safety Assessment of Glycyrrhetinic Acid, Potassium Glycyrrhetinate, Disodium Succinoyl Glycyrrhetinate, Glyceryl Glycyrrhetinate, Glycyrrhetinyl Stearate, Stearyl Glycyrrhetinate, Glycyrrhizic Acid, Ammonium Glycyrrhizate, Dipotassium Glycyrrhizate, Disodium Glycyrrhizate, Trisodium Glycyrrhizate, Methyl Glycyrrhizate, and Potassium Glycyrrhizinate[1]

Glycyrrhetinic Acid and its salts and esters and Glycyrrhizic Acid and its salts and esters are cosmetic ingredients that function as flavoring agents or skin-conditioning agents—miscellaneous or both. These chemicals may be isolated from licorice plants. Glycyrrhetinc Acid is described as at least 98% pure, with 0.6% 24-OH-Glycyrrhetinic Acid, not more than 20 μg/g of heavy metals and not more than 2 μg/g of arsenic. Ammonium Glycyrrhizate has been found to be at least 98% pure and Dipotassium Glycyrrhizate has been found to be at least 95% pure. Glycyrrhetinic Acid is used in cosmetics at concentrations of up to 2%; Stearyl Glycyrrhetinate, up to 1%; Glycyrrhizic Acid, up to 0.1%; Ammonium Glycyrrhizate, up to 5%; Dipotassium Glycyrrhizate, up to 1%; and Potassium Glycyrretinate, up to 1%. Although Glycyrrhizic Acid is poorly absorbed by the intestinal tract, it may be hydrolyzed to Glycyrrhetinic Acid by a β-glucuronidase produced by intestinal bacteria. Glycyrrhetinic Acid and Glycyrrhizic Acid bind to rat and human albumin, but do not absorb well into tissues. Glycyrrhetinic Acid and Glycyrrhizic Acid and metabolites are mostly excreted in the bile, with very little excreted in urine. Dipotassium Glycyrrhizate was undetectable in the receptor chamber when tested for transepidermal permeation through pig skin. Glycyrrhizic Acid increased the dermal penetration of diclofenac sodium in rat skin. Dipotassium Glycyrrhizate increased the intestinal absorption of calcitonin in rats. In humans, Glycyrrhetinic Acid potentiated the effects of hydrocortisone in the skin. Moderate chronic or high acute exposure to Glycyrrhizic Acid, Ammonium Glycyrrhizate, and their metabolites have been demonstrated to cause transient systemic alterations, including increased potassium excretion, sodium and water retention, body weight gain, alkalosis, suppression of the renin-angiotensis-aldosterone system, hypertension, and muscular paralysis; possibly through inhibition of 11β-hydroxysteroid dehydrogenase-2 (11β-OHSD2) in the kidney. Glycyrrhetinic Acid and its derivatives block gap junction intracellular communication in a dose-dependent manner in animal and human cells, including epithelial cells, fibroblasts, osteoblasts, hepatocytes, and astrocytes; at high concentrations, it is cytotoxic. Glycyrrhetinic Acid and Glycyrrhizic Acid protect liver tissue from carbon tetrachloride. Glycyrrhizic Acid has been used to treat chronic hepatitis, inhibiting the penetration of the hepatitis A virus into hepatocytes. Glycyrrhetinic Acid and Glycyrrhizic Acid have anti-inflammatory effects in rats and mice. The acute intraperitoneal LD_{50} for Glycyrrhetinic Acid in mice was 308 mg/kg and the oral LD_{50} was >610 mg/kg. The oral LD_{50} in rats was reported to be 610 mg/kg. Higher LD_{50} values were generally reported for salts. Little short-term, subchronic, or chronic toxicity was seen in rats given ammonium, dipotassium, or disodium salts of Glycyrrhizic Acid. Glycyrrhetinic Acid was not irritating to shaved rabbit skin, but was considered slightly irritating in an in vitro test. Glycyrrhetinic Acid inhibited the mutagenic activity of benzo[a]pyrene and inhibited tumor initiation and promotion by other agents in mice. Glycyrrhizic Acid inhibited tumor initiation by another agent, but did not prevent tumor promotion in mice. Glycyrrhizic Acid delayed mortality in mice injected with Erlich ascites tumor cells, but did not reduce the mortality rate. Ammonium Glycyrrhizate was not genotoxic in in vivo and in vitro cytogenetics assays, the dominant lethal assay, an Ames assay, and heritable translocation tests, except for possible increase in dominant lethal mutations in rats given 2000 mg/kg day^{-1} in their diet. Disodium Glycyrrhizate was not carcinogenic in mice in a drinking water study at exposure levels up to 12.2 mg/kg day^{-1} for 96 weeks. Glycyrrhizate salts produced no reproductive or developmental toxicity in rats, mice, golden hamsters, or Dutch-belted rabbits, except for a dose-dependent increase (at 238.8 and 679.9 mg/kg day^{-1}) in sternebral variants in a study using rats. Sedation, hypnosis, hypothermia, and respiratory depression were seen in mice given 1250 mg/kg Glycyrrhetinic Acid intraperitoneally. Rats fed a powdered diet containing up to 4% Ammonium Glycyrrhizate had no treatment related effects in motor function tests, but active avoidance was facilitated at 4%, unaffected at 3%, and depressed at 2%. In a study of 39 healthy volunteers, a no effect level of 2 mg/kg/day was determined for Glycyrrhizic Acid given orally for 8 weeks. Clinical tests in seven normal individuals given oral Ammonium Glycyrrhizate at 6 g/day for 3 days revealed reduced renal and thermal sweat excretion of Na^+ and K^+, but carbohydrate and protein metabolism were not affected. Glycyrrhetinic Acid at concentrations up to 6% was not a skin irritant or a sensitizer in clinical tests. Neither Glycyrrhizic Acid, Ammonium Glycyrrhizate,

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nor Dipotassium Glycyrrhizate at 5% were phototoxic agents or photosensitizers. Birth weight and maternal blood pressure were unrelated to the level of consumption of Glycyrrhizic Acid in 1049 Finnish women with infants, but babies whose mother consumed >500 mg/wk were more likely to be born before 38 weeks. The Cosmetic Ingredient Review (CIR) Expert Panel noted that the ingredients in this safety assessment are not plant extracts, powders, or juices, but rather are specific chemical species that may be isolated from the licorice plant. Because these chemicals may be isolated from plant sources, however, steps should be taken to assure that pesticide and toxic metal residues are below acceptable levels. The Panel advised the industry that total polychlorobiphenyl (PCB)/pesticide contamination should be limited to not more than 40 ppm, with not more than 10 ppm for any specific residue, and that toxic metal levels must not contain more than 3 mg/kg of arsenic (as As), not more than 0.002% heavy metals, and not more than 1 mg/kg of lead (as Pb). Although the Panel noted that Glycyrrhizic Acid is cytotoxic at high doses and ingestion can have physiological effects, there is little acute, short-term, subchronic, or chronic toxicity and it is expected that these ingredients would be poorly absorbed through the skin. These ingredients are not considered to be irritants, sensitizers, phototoxic agents, or photosensitizers at the current maximum concentration of use. Accordingly, the CIR Expert Panel concluded that these ingredients are safe in the current practices of use and concentration. The Panel recognizes that certain ingredients in this group are reportedly used in a given product category, but the concentration of use is not available. For other ingredients in this group, information regarding use concentration for specific product categories is provided, but the number of such products is not known. In still other cases, an ingredient is not in current use, but may be used in the future. Although there are gaps in knowledge about product use, the overall information available on the types of products in which these ingredients are used and at what concentration indicate a pattern of use. Within this overall pattern of use, the Expert Panel considers all ingredients in this group to be safe.

INTRODUCTION

Glycyrrhetinic Acid, its salts and esters, and Glycyrrhizic Acid, and its salts and esters are used as cosmetic ingredients. This review presents information relevant to the safety of these ingredients as considered by the Cosmetic Ingredient Review (CIR) Expert Panel.

These ingredients are not plant extracts, powders, or juices, but rather are specific chemical species that may be isolated from the licorice plant. Plant-derived extracts, powders, juices, etc. [e.g., Glycyrrhiza Glabra (Licorice) Leaf Extract] also used as cosmetic ingredients, which may contain these organic compounds, but likely will include other chemicals, will be reviewed in another report.

The ingredients in this safety assessment are:

- Glycyrrhetinic Acid,
- Potassium Glycyrrhetinate,
- Disodium Succinoyl Glycyrrhetinate,
- Glyceryl Glycyrrhetinate,
- Glycyrrhetinyl Stearate,
- Stearyl Glycyrrhetinate,
- Glycyrrhizic Acid,
- Ammonium Glycyrrhizate,
- Dipotassium Glycyrrhizate,
- Disodium Glycyrrhizate,
- Trisodium Glycyrrhizate,
- Methyl Glycyrrhizate, and
- Potassium Glycyrrhizinate.

This review presents information relevant to the safety of these cosmetic ingredients as considered by the CIR Expert Panel.

No published information was identified for Disodium Succinoyl Glycyrrhetinate, Glyceryl Glycyrrhetinate, Glycyrrhetinyl Stearate, Methyl Glycyrrhizate, Potassium Glycyrrhetinate, Potassium Glycyrrhizinate, or Trisodium Glycyrrhizate, but they are considered to be sufficiently structurally related to the rest of the ingredients that the data may be extrapolated to address their safety.

CHEMISTRY

Definition and Structure

Glycyrrhetinic Acid (CAS no. 471-53-4) is an organic compound derived from Glycyrrhizic Acid or shredded licorice roots. It conforms generally to the structure presented in Figure 1 (Gottschalck and McEwen 2004).

Synonyms for Glycyrrhetinic Acid include Glycyrrhetic Acid; Olean-12-En-29-Oic-Acid, 3-Hydroxy-11-Oxo-, (3β, 20β)-; and (3β, 20β)-3-Hydroxy-11-Oxo- Olean-12-En-29-Oic-Acid. All 13 ingredients reviewed in this report have in common the basic core structure of Glycyrrhetinic Acid (Gottschalck and McEwen 2004). There are two stereoisomers of Glycyrrhetinic Acid described in the literature, 18α- and 18β-Glycyrrhetinic Acid. It is unclear which stereoisomer is used in cosmetics or if it is a mixture.

Glycyrrhetinic Acid has the empirical formula of $C_{30}H_{46}O_4$ and conforms to the formula presented in Figure 1, with both R_1 and R_2 being OH. Table 1 lists its derivatives and the R groups corresponding to each one.

Potassium Glycyrrhetinate (CAS no. 85985-61-1) is the potassium salt of Glycyrrhetinic Acid. Potassium Glycyrrhetinate is also known as Olean-12-En-29-Oic Acid, 3-Hydroxy-1, 1-Oxo-, Monopotassium Salt (Gottschalck and McEwen 2004).

Disodium Succinoyl Glycyrrhetinate (no CAS no. listed) is the disodium salt of the ester of succinic alcohol and Glycyrrhetinic Acid that conforms to the structure shown in Figure 1, with R groups given in Table 1 (Gottschalck and McEwen 2004).

Glyceryl Glycyrrhetinate (CAS no. 108916-85-4) is the monoester of glycerine and Glycyrrhetinic Acid. Synonyms for Glyceryl Glycyrrhetinate include Glycyrrhetinic Acid, Glyceryl Ester; Glyceryl Glycyrrhetate; and Olean-12-En-29-Oic Acid, 3-Hydroxy-11-Oxo-, Monoester with 1,2,3 Propanetriol, (3β, 20β) (Gottschalck and McEwen 2004).

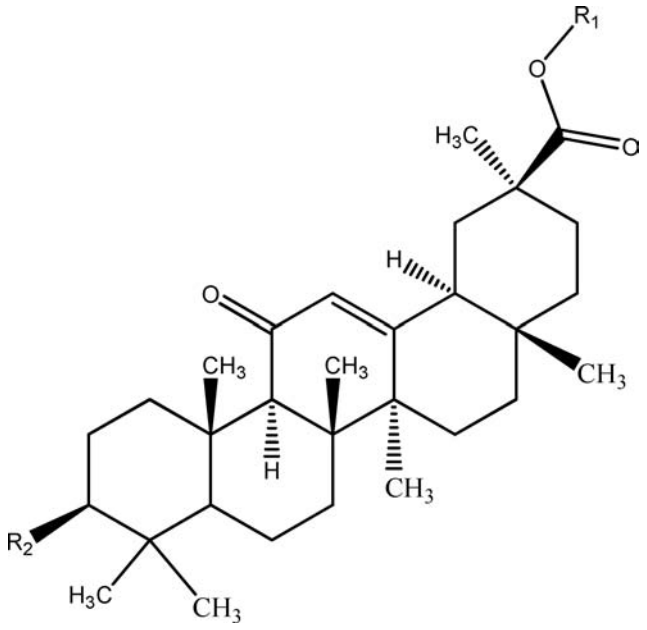

FIGURE 1
Glycyrrhetinic Acid. R_1 and R_2 are OH groups (Gottschalck and McEwen 2004).

Glycyrrhetinyl Stearate (CAS no. 4827-59-2) is the stearic acid ester of Glycyrrhetinic Acid that conforms generally to the structure displayed in Figure 1, with R groups given in Table 1. It is also known as 3-Stearoyloxy Glycyrrhetinic Acid (Gottschalck and McEwen 2004).

Stearyl Glycyrrhetinate (CAS no. 13832-70-7) is the ester of stearyl alcohol and Glycyrrhetinic Acid which conforms to the structure displayed in Figure 1, with R groups given in Table 1. Synonyms include Octadecyl Glycyrrhetinate; Octadecyl 3-Hydroxy-11-Oxoolean-12-En-29-Oate; and Olean-12-En-29-Oic Acid, 3-Hydroxy-11-Oxo-, Octadecyl Ester (Gottschalck and McEwen 2004).

Glycyrrhizic Acid (CAS no. 1405-86-3) is a natural material extracted from the plant *Glycyrrhiza Glabra* (Gottschalck and McEwen 2004). Glycyrrhizic Acid is a conjugate of Glycyrrhetinic Acid and two molecules of glucuronic acid. It conforms generally to the structure in Figure 1, with R groups given in Table 1. Glycyrrhizic Acid is also known as (3β, 20β)-20-Carboxy-11-Oxo-30-Norlean-12-En-3-Yl-2-*O*-β-17-Glucopyranuronosyl-α-D-Glucopyranosiduronic Acid; α-D-Glucopyranosiduronic Acid, (3β, 20β)-20-Carboxy-11-Oxo-30-Norlean-12-En-3-Yl-2-*O*-β-17-Glucopyranuronosyl-; Glycyrrhetinic Acid Glycoside; Glycyrrhizin; and Glycyrrhizinic Acid (Gottschalck and McEwen 2004).

Ammonium Glycyrrhizate (CAS no. 53956-04-0) is the ammonium salt of Glycyrrhizic Acid (q.v.). Synonyms include Ammonium Glycyrrhizinate; α-D-Glucopyranosiduronic Acid, (3β, 20β)-20-Carboxy-11-Oxo-30-Norlean-12-En-3-Yl-2-*O*-β-D-Glucopyranuronosyl-, Ammoniate; Glycyrrhizin Ammonium Salt; Glycyrrhizic Acid Ammonium Salt; Monoammonium Glycyrrhizinate; and Monoammonium α-Glycyrrhizinate (Gottschalck and McEwen 2004).

Dipotassium Glycyrrhizate (CAS no. 68797-35-3) is the dipotassium salt of Glycyrrhizic Acid (q.v.). Synonyms for Dipotassium Glycyrrhizate include Dipotassium Glycyrrhizinate; α-D-Glucopyranosiduronic Acid, (3β, 20β)-20-Carboxy-11-Oxo-30-Norlean-12-En-3-Yl-2-*O*-β-D-Glucopyranuronosyl-, Dipotassium Salt; and 30-Noroleanane, α-D-Glucopyranosiduronic Acid Derivative (Gottschalck and McEwen 2004).

Disodium Glycyrrhizate (CAS no. 71277-79-7) is the disodium salt of Glycyrrhizic Acid. Synonyms for Disodium Glycyrrhizate include Disodium Glycyrrhizinate; α-D-Glucopyranosiduronic Acid, (3β, 20β)-20-Carboxy-11-Oxo-30-Norlean-12-En-3-Yl-2-*O*-β-D-Glucopyranuronosyl-, Disodium Salt; and 30-Noroleanane, α-D-Glucopyranosiduronic Acid Derivative (Gottschalck and McEwen 2004).

Trisodium Glycyrrhizate (no CAS no. available) is the trisodium salt of Glycyrrhizic Acid (q.v.) (Gottschalck and McEwen 2004).

Methyl Glycyrrhizate (no CAS no. available) is the ester of methyl alcohol and Glycyrrhizic Acid (q.v.). It is also known

TABLE 1
R groups in Figure 1 for derivatives of Glycyrrhetinic Acid (Gottschalck and McEwen 2004).

Compound	R_1	R_2
Potassium Glycyrrhetinate	K	OH
Disodium Succinoyl Glycyrrhetinate	Na	O-C(O)-CH_2-CH_2-C(O)-ONa
Glyceryl Glycyrrhetinate	CH_2-CH(OH)-CH_3	OH
Glycyrrhetinyl Stearate	H	O-C(O)-$(CH_2)_{17}$-CH_3
Stearyl Glycyrrhetinate	$(CH_2)_{17}$-CH_3	OH
Glycyrrhizic Acid	H	(β-Glucuronylglucuronic acid)
Ammonium Glycyrrhizate	NH_3	(β-Glucuronylglucuronic acid)
Dipotassium Glycyrrhizate	K	(β-Glucuronylglucuronic acid·K)
Disodium Glycyrrhizate	Na	(β-Glucuronylglucuronic acid·Na)
Methyl Glycyrrhizate	CH_3	(β-Glucuronylglucuronic acid)

TABLE 2
Physical and chemical properties of Glycyrrhetinic Acid and Glycyrrhizic Acid and their derivatives (Lide 1993; CTFA 2004; Cognis 2002).

Compound	Molecular Weight	Boiling point	Melting point	Soluble in:	UV absorbance hrulefill Max.	UV absorbance hrulefill $E^{1\%}_{1cm}$
Glycyrrhetinic Acid	470.69	296°C	—	Ethanol	249	237.6
Disodium Succinoyl Glycyrrhetinate	—	—	—	Water	258.5	186.7
Glycyrrhetinyl Stearate	—	—	—	Ethanol	249	157.1
Stearyl Glycyrrhetinate	—	—	—	Ethanol	248.5	156.3
Glycyrrhizic Acid	882.94	—	220°C	Water	—	—
Ammonium Glycyrrhizate	—	—	—	Ethanol	251.5	134.6
Dipotassium Glycyrrhizate	—	—	—	Water	257.5	119.6

as Glycyrrhizic Acid, Methyl Ester (Gottschalck and McEwen 2004).

Potassium Glycyrrhizinate is an enzymatically produced mixture of potassium salts of Glycyrrhizic Acid (q.v.) and Glycyrrhetinic Acid monoglycoside. It is also known as Glycopyranosiduronic Acid, 20-Carboxy-11-Oxo-30-Norolean-12-En-3-Ly and 2-Glycopyranuronosyl-, Potassium Salt (Gottschalck and McEwen 2004).

Physical and Chemical Properties

The physical and chemical properties of Glycyrrhetinic Acid and Glycyrrhizic Acid are summarized in Table 2. Data were not available for the other ingredients.

Method of Manufacture

Glycyrrhetinic Acid is derived from Glycyrrhizic Acid or isolated from shredded licorice roots (Gottschalck and McEwen 2004). 18β-Glycyrrhetinic Acid is obtained from the licorice root (*Glycyrrhiza glabra L.*) by hydroalcoholic extraction, hydrolysis and crystallization methods (CTFA 2004).

Glycyrrhizin (Glycyrrhizic Acid) and Ammonium Glycyrrhizate are isolated from water-soluble extracts from the dried rhizome and roots of *Glycyrrhiza glabra* or of other *Glycyrrhiza* species yielding a yellow and sweet wood (Informatics, Inc. 1972). Glycyrrhizin and Ammonium Glycyrrhizate may also be isolated from extracts of the roots of *Abrus precatorius*, *Periandra dulcis*, *Periandra mediteranea*, and from the bark of the trees *Lucuma glycophylla*, *Achras sapota*, and *Sideroxylon richarhii* (Informatics, Inc. 1972).

Ammoniated Glycyrrhizin (also known as Ammonium Glycyrrhizate) is prepared from the water extract of licorice root by acid precipitation followed by neutralization with dilute ammonia (21CFR148.1408). It is also reported that Ammonium Glycyrrhizate is obtained from the licorice root (*Glycyrrhiza glabra L.*) by hydroalcoholic extraction, hydrolysis and crystallization methods (CTFA 2004).

Dipotassium Glycyrrhizate is obtained from the licorice root (*Glycyrrhiza glabra L.*) by hydroalcoholic extraction, hydrolysis and crystallization methods (CTFA 2004).

Analytical Methods

Spinks and Fenwick (1990) used high-performance liquid chromatography (HPLC) to determine the content of Glycyrrhizic Acid in licorice-containing confectionary and health products. Okamura et al. (2001) used semi-micro-HPLC to simultaneously detect Glycyrrhizic Acid, Glycyrrhetinic Acid, and glycyrrhetinic acid mono-glucuronide in a combination of licorice root and peony root with rat feces. Liu et al. (2001) used mass spectrometry and nuclear magnetic resonance spectra to isolate and identify Glycyrrhizic Acid, Glycyrrhetinic Acid, and other constituents in a licorice extract.

Impurities

Cognis (2002) reports that its products Plantactiv® GLA18 (Glycyrrhetinic Acid), Plantactiv® AGL (Ammonium Glycyrrhizate), and Plantactiv® PGL (Dipotassium Glycyrrhizate) are a minimum 98%, 98%, and 95% pure, respectively.

Maruzen Pharaceuticals Company, Ltd. (2004) analyzed Glycyrrhetinic Acid. They found 99.4% 18β-Glycyrrhetinic Acid and 0.6% 24-OH-Glycyrrhetinic Acid by the HPLC method. They also found not more than 20 μg/g of heavy metals and not more than 2 μg/g of arsenic. Hill Laboratories (2004) conducted a multiresidue Pesticide Analysis on Glycyrrhetic Acid. There was no residue detected by ethyl acetate extraction, gel permeation chromatography cleanup, gas chromatography-electron capture detector/nitrogen phosphorus detector, and gas chromatography–mass spectrometry (GC-MS).

Cognis reports that Dipotassium Glycyrrhizate, 18β-Glycyrrhetinic Acid, and Ammonium Glycyrrhizate samples are absent of allergens except for *d*-Limonene at <1 ppm (CTFA 2004).

USE

Cosmetic Use

As given in the *International Cosmetic Ingredient Dictionary and Handbook*, **Glycyrrhetinic Acid** is a skin conditioning agent-miscellaneous (Gottschalck and McEwen 2004) and is reportedly used in 65 cosmetic formulations (FDA 2002) at a maximum reported concentration of use of 2% (CTFA 2003).

The frequencies of use and use concentrations of Glycyrrhetinic Acid in specific product categories are listed in Table 3.

Potassium Glycyrrhetinate functions as a flavoring agent and skin-conditioning agent—miscellaneous in cosmetic products (Gottschalck and McEwen 2004). However, there were no reports to FDA (2002) of its use in any cosmetic product categories and no concentration of use data were available.

Disodium Succinoyl Glycyrrhetinate is used in four permanent wave products (FDA 2002) as shown in Table 3. No concentration of use data were available for this compound. The function of this ingredient is not given in the *International Cosmetic Ingredient Dictionary and Handbook* (Gottschalck and McEwen 2004).

Glyceryl Glycyrrhetinate functions as a flavoring agent in cosmetic products (Gottschalck and McEwen 2004). However, there were no reports to FDA (2002) of its use in any cosmetic product categories and no concentration of use data were available.

Glycyrrhetinyl Stearate functions as a flavoring agent in cosmetic products (Gottschalck and McEwen 2004). However, there were no reports to FDA (2002) of its use in any cosmetic product categories and no concentration of use data were available.

Stearyl Glycyrrhetinate functions in cosmetics as a flavoring agent (Gottschalck and McEwen 2004) and is reportedly used in 35 cosmetic products (FDA 2002) at use concentrations up to 0.1% (CTFA 2003) as shown in Table 3.

Glycyrrhizic Acid functions as a flavoring agent and skin-conditioning agent—miscellaneous (Gottschalck and McEwen 2004). The use of Glycyrrhizic Acid in specific product categories (FDA 2002) are listed in Table 3. Glycyrrhizic Acid is reportedly used at 0.1% in many cosmetic product categories (CTFA 2003).

Ammonium Glycyrrhizate is a flavoring agent and skin-conditioning agent—miscellaneous (Gottschalck and McEwen 2004) and is used in 72 cosmetic formulations (FDA 2002) at a maximum concentration of use of 5% (CTFA 2003) as shown in Table 3.

Dipotassium Glycyrrhizate functions as a flavoring agent and skin-conditioning agent—miscellaneous (Gottschalck and McEwen 2004) and is used in 29 cosmetic formulations (FDA 2002) at a maximum concentration of 1% (CTFA 2003). The frequencies of use and use concentrations of Dipotassium Glycyrrhizate in specific product categories are listed in Table 3.

Disodium Glycyrrhizate is a flavoring agent and skin-conditioning agent—miscellaneous in cosmetic products (Gottschalck and McEwen 2004). However, there were no reports to FDA (2002) of its use in any cosmetic product categories. No concentration of use data were available.

Trisodium Glycyrrhizate is a flavoring agent and skin-conditioning agent—miscellaneous (Gottschalck and McEwen 2004). FDA (2002) reports that it is used in one hair conditioner and in one other noncoloring hair preparation as shown in Table 3. No concentration of use data were available.

Methyl Glycyrrhizate is a flavoring agent in cosmetic products (Gottschalck and McEwen 2004). However, there were no reports to FDA (2002) of its use in any cosmetic product categories. No concentration of use data were available.

Potassium Glycyrrhizinate is a skin-conditioning agent—miscellaneous in cosmetic products (Gottschalck and McEwen 2004). Although there were no reports to FDA (2002) of its use in any cosmetic product categories, a concentration of use of 1.0% was given in an industry survey and is shown in Table 3 (CTFA 2003).

Noncosmetic Use

Bombardelli (1991) demonstrated that 18β-Glycyrrhetinic Acid can be used as a vehicle to increase the transdermal absorption of some topical medications.

Although licorice and licorice derivatives are generally recognized as safe (GRAS) as food ingredients, the Food and Drug Administration (FDA) has established the following maximum restrictions on the levels of Glycyrrhizic Acid in certain types of foods: 0.05% in baked goods; 0.1% in alcoholic beverages; 0.15% in nonalcoholic beverages; 1.1% in chewing gum; 16.0% in hard candy; 0.15% in herbs and seasonings and in plant protein products; 3.1% in soft candy; 0.5% in vitamins and dietary supplements; and 0.1% in all other food except sugar substitutes. Glycyrrhizic Acid may not be used as a non-nutritive sweetener in sugar substitutes (12CFR184.1408).

Touitou et al. (1988) described the use of a Glycyrrhizic Acid gel as a vehicle for an idoxuridine topical preparation. The Glycyrrhizic Acid gel facilitated the idoxuridine absorption through hairless mouse skin.

Glycyrrhizic Acid is used in the treatment of chronic hepatitis B and C (Chitturi and Farrell 2001).

The GRAS status of Ammonium Glycyrrhizate was supported by a review performed by the Life Science Research Office (LSRO 1974).

Dipotassium Glycyrrhizate is an anti-inflammatory agent that is used to treat chronic dermatitis (Trotta et al. 2002).

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Overview

Ploeger et al. (2001a) published an extensive review of the pharmacokinetics of Glycyrrhizic Acid in rodents and humans. Glycyrrhizic Acid is poorly absorbed by the gastrointestinal tract. However, Glycyrrhizic Acid is hydrolyzed by a specialized β-glucuronidase produced by intestinal bacteria to make Glycyrrhetinic Acid. The plasma of germ-free rats showed no Glycyrrhetinic Acid after oral administration of Glycyrrhizic Acid. After hydrolysis by bacteria, Glycyrrhetinic Acid is then absorbed into the circulatory system. However, this absorption is saturable at doses above 25 mg/kg in both rats

TABLE 3
Frequency of use and concentration of Ammonium Glycyrrhizate, Dipotassium Glycyrrhizate, Disodium Succinoyl Glycyrrhetinate, Glycyrrhetinic Acid, Glycyrrhizic Acid, and Stearyl Glycyrrhetinate.

Product category (total in category) (FDA 2002)	Frequency of use (FDA 2002)	Concentration of use (%) (CTFA 2003)
	Glycyrrhetinic Acid	
Baby products		
Baby Lotions, Oils, Powders, and Creams (60)	—	0.1–1
Eye Makeup		
Eye Shadow (576)	1	—
Eye Lotion (25)	1	0.2
Other Eye Makeup Preparations (152)	1	—
Fragrance Products		
Other Fragrance Preparations (173)	—	0.1
Noncoloring Hair Products		
Hair Conditioners (651)	—	0.1
Shampoos (noncoloring) (884)	—	0.2
Tonics, Dressings, and Other Hair-Grooming Aids (598)	3	0.05–0.2
Other Hair Preparations (277)	—	0.2
Makeup		
Blushers (All Types) (245)	2	—
Face Powders (305)	4	1
Foundations (324)	3	—
Other Makeup Preparations (201)	1	0.1–1
Personal Hygiene Products		
Underarm Deodorants (247)	1	0.2
Shaving Products		
Aftershave Lotion (231)	2	—
Skin Care Products		
Cleansing Creams, Lotions, and Sprays (775)	2	0.05
Face and Neck Creams, Lotions, and Sprays (301)	6	0.01–2
Body and Hand Creams, Lotions, and Sprays (840)	7	0.001–2
Moisturizers (905)	10	0.01 – 1
Night Creams, Lotions, and Sprays (200)	7	0.3
Skin Fresheners (184)	—	0.01
Paste Masks (Mud Packs) (271)	6	—
Other Skin Care Preparations (725)	3	0.1–2
Suntan Products		
Suntan Gels, Creams, and Liquids (131)	2	0.1
Other Suntan Preparations (38)	3	—
Total	**65**	**0.001–2**
	Disodium Succinoyl Glycyrrhetinate	
Noncoloring Hair Products		
Permanent Waves (207)	4	—
Total	*4*	—
	Stearyl Glycyrrhetinate	
Bath Products		
Other Bath Preparations (196)	—	0.1
Eye Makeup		
Eyeliner (548)	—	0.1
Eye Lotion (25)	1	—
Mascara (195)	—	0.05

(Continued on next page)

TABLE 3
Frequency of use and concentration of Ammonium Glycyrrhizate, Dipotassium Glycyrrhizate, Disodium Succinoyl Glycyrrhetinate, Glycyrrhetinic Acid, Glycyrrhizic Acid, and Stearyl Glycyrrhetinate. *(Continued)*

Product category (total in category) (FDA 2002)	Frequency of use (FDA 2002)	Concentration of use (%) (CTFA 2003)
Fragrance Products		
Other Fragrance Preparations (173)	2	—
Noncoloring Hair Products		
Permanent Waves (207)	1	—
Makeup		
Blushers (245)	—	0.007–0.02
Face Powders (305)	—	0.01
Foundations (324)	—	0.02–0.1
Lipstick (962)	2	0.05–0.2
Makeup Bases (141)	—	0.1
Makeup Fixatives (20)	—	0.04
Other Makeup Preparations (201)	2	—
Nail Care Products		
Basecoats and Undercoats (44)	1	—
Nail Polish and Enamel (123)	4	0.02
Skin Care Products		
Cleansing Creams, Lotions, and Sprays (775)	3	0.05–0.1
Face and Neck Creams, Lotions, and Sprays (310)	1	0.05–0.1
Body and Hand Creams, Lotions, and Sprays (840)	—	0.05–0.1
Moisturizers (905)	13	0.01–0.1
Night Creams, Lotions, and Sprays (200)	1	0.01–0.1
Paste Masks (Mud Packs) (271)	—	0.1–0.2
Other Skin Care Preparations (725)	3	0.05–1
Suntan Products		
Suntan Gels, Creams, and Liquids (131)	—	0.05–0.2
Other Suntan Preparations (38)	—	0.1–0.2
Total	**34**	**0.007–1**
Glycyrrhizic Acid		
Bath Products		
Other Bath Preparations (196)	—	0.1
Bath Soaps and Detergents (421)	—	0.1
Eye Makeup		
Eye Lotion (25)	1	0.1
Noncoloring Hair Products		
Tonics, Dressings, and Other Hair-Grooming Aids (598)	—	0.1
Makeup		
Face Powders (305)	—	0.1
Makeup Fixatives (20)	1	—
Skin Care Products		
Cleansing Creams, Lotions, and Sprays (775)	—	0.1
Face and Neck Creams, Lotions, and Sprays (263)	—	0.1
Night Creams, Lotions, and Sprays (188)	—	0.1
Total	**2**	**0.1**
Ammonium Glycyrrhizate		
Baby Products		
Other Baby Products (34)	—	1
Bath Products		

(Continued on next page)

TABLE 3
Frequency of use and concentration of Ammonium Glycyrrhizate, Dipotassium Glycyrrhizate, Disodium Succinoyl Glycyrrhetinate, Glycyrrhetinic Acid, Glycyrrhizic Acid, and Stearyl Glycyrrhetinate. *(Continued)*

Product category (total in category) (FDA 2002)	Frequency of use (FDA 2002)	Concentration of use (%) (CTFA 2003)
Bath Oils, Tablets and Salts (143)	—	0.01
Bath Soaps and Detergents (421)	—	0.1
Eye Makeup		
Eye Shadow (576)	1	—
Eye Lotion (25)	—	0.5
Noncoloring Hair Products		
Hair Conditioners (651)	—	0.01
Hair Sprays (275)	—	0.0007
Shampoos (884)	—	0.001–0.1
Tonics, Dressings, and Other Hair-Grooming Aids (598)	3	0.05–0.2
Hair-Coloring Products		
Hair Dyes and Colors (1690)	—	0.2
Makeup		
Lipstick (962)	51	1–5
Other Makeup Preparations (201)	2	0.1
Nail Care Products		
Nail Polish and Enamel Removers (36)	—	0.01
Oral Hygiene Products		
Dentifrices (40)	1	0.02–0.3
Mouthwashes and Breath Fresheners (46)	—	0.05–1
Other Oral Hygiene Products (6)	—	0.07
Personal Hygiene Products		
Other Personal Hygiene Products (308)	—	0.2
Shaving Products		
Aftershave Lotion (231)	—	0.02–0.05
Other Shaving Preparation Products (63)	1	—
Skin Care Products		
Cleansing Creams, Lotions, and Sprays (775)	4	0.05–0.3
Face and Neck Creams, Lotions, and Sprays (310)	1	0.1
Body and Hand Creams, Lotions, Powders and Sprays (840)	—	0.05–1
Moisturizing Creams, Lotions, and Sprays (905)	2	0.05–0.1
Night Creams, Lotions, and Sprays (200)	1	0.05–0.1
Paste Masks (Mud Packs) (271)	3	0.05
Other Skin Care Preparations (725)	1	0.1
Suntan Products		
Suntan Gels, Creams and Liquids (131)	—	0.01–0.1
Indoor Tanning Preparations (71)	1	0.01
Other Suntan Products (38)	—	0.1
Total	**72**	**0.0007–5**
Dipotassium Glycyrrhizate		
Bath Products		
Bath Oils, Tablets, and Salts (143)	—	0.1
Bath Soaps and Detergents (421)	—	0.1–0.2
Other Bath Preparations (196)	—	0.1
Eye Makeup		
Eye Lotion (25)	—	0.1
Mascara (195)	—	0.05

(Continued on next page)

TABLE 3
Frequency of use and concentration of Ammonium Glycyrrhizate, Dipotassium Glycyrrhizate, Disodium Succinoyl Glycyrrhetinate, Glycyrrhetinic Acid, Glycyrrhizic Acid, and Stearyl Glycyrrhetinate. *(Continued)*

Product category (total in category) (FDA 2002)	Frequency of use (FDA 2002)	Concentration of use (%) (CTFA 2003)
Eye Makeup Remover (100)	2	—
Noncoloring Hair Products		
Hair Conditioners (651)	1	0.0007–0.1
Rinses (Noncoloring) (42)	—	0.1
Shampoos (Noncoloring) (884)	—	0.0007–0.1
Tonics, Dressings, and Other Hair-Grooming Aids (598)	—	0.00007–0.01
Other Hair Preparations (277)	—	0.01–0.2
Fragrance Products		
Colognes and Toilet Waters (684)	—	0.01
Powders (273)	3	—
Oral Hygiene Products		
Dentrifices (40)	—	0.05
Shaving Products		
Aftershave Lotions (231)	—	0.1
Preshave Lotion (14)	—	0.05
Shaving Cream (134)	—	0.1–0.05
Other Makeup Preparations (201)	—	0.05
Makeup		
Foundations (324)	5	0.01–0.2
Lipstick (962)	—	0.05
Makeup Bases (141)	—	0.05
Other Shaving Preparations (63)	—	0.05
Skin Care Products		
Cleansing Creams, Lotions, and Sprays (775)	3	0.01–1
Depilatories (34)	—	0.05
Face and Neck Creams, Lotions, and Sprays (310)	1	0.05–0.5
Body and Hand Creams, Lotions, Powders and Sprays (840)	—	0.05–0.2
Foot Powders and Sprays (35)	—	0.01
Moisturizing Creams, Lotions, and Sprays (905)	8	0.05–0.2
Night Creams, Lotions, and Sprays (200)	—	0.05–0.2
Paste Masks (Mud Packs) (271)	1	0.05
Skin Fresheners (184)	—	0.01–0.1
Other Skin Care Preparations (725)	5	0.1–0.5
Suntan Products		
Suntan Gels, Creams and Liquids (131)	—	0.02
Other Suntan Preparations (38)	—	0.1
Total	**29**	**0.00007–1**
Potassium Glycyrrhetinate		
Skin Care Products		
Cleansing Creams, Lotions, and Sprays (775)	—	1
Face and Neck Creams, Lotions, and Sprays (310)	—	1
Night Creams, Lotions, and Sprays (200)	—	1
Total	—	**1**

and humans. Both Glycyrrhetinic Acid and Glycyrrhizic Acid bind extensively to rat and human plasma albumin.

This binding is saturable at high plasma concentrations. These compounds do not absorb well into tissues, as the tissue-to-blood partition coefficients for all rat tissues tested are less than 1.

Hepatic metabolism of Glycyrrhetinic Acid produces the conjugates 18-β-glycyrrhetyl-3-*O*-monoglucuronide, 18-β-glycyrrhetyl-3-*O*-hydrogen sulfate, and 18-β-glycyrrhetyl-3-*O*-monoglucuronide. Glycyrrhetinic Acid and Glycyrrhizic Acid and metabolites are mostly excreted in the bile. Once in the gastrointestinal tract, bacterial enzymes can remove the glucuronide and sulfate conjugates to restore absorbable Glycyrrhetinic Acid. Thus, Glycyrrhetinic Acid and its metabolites are subject to enterohepatic cycling in rats and, presumably, in humans. Very little is excreted in the urine (Ploeger et al. 2001a).

Imai et al. (1999) used in vivo (Sprague-Dawley rats) and in vitro (Caco-2 cells) models to evaluate the enhancing effects of Dipotassium Glycyrrizate on the intestinal absorption of drugs. The authors found that it is not Dipotassium Glycyrrizate but its metabolite Glycyrrhetinic Acid that enhances drug absorption in the intestine. Salmon calcitonin was the drug used to demonstrate this effect.

Trotta et al. (2002) studied the ability of elastic liposomes to increase the transepidermal permeation of Dipotassium Glycyrrhizate through pig skin. Full-thickness pig ear skin was prepared and fixed in a Franz-type apparatus for the measurement of transdermal permeation. The diffusion area was 2.05 cm^2, and the receptor chamber was filled with 6 ml of 0.002% aqueous sodium azide. The application side was dry when 100 μg of test material was applied. The test materials were 0.25% Dipotassium Glycyrrhizate in water solution, 1% Dipotassium Glycyrrhizate in a water micelle solution, liposomes of soya lethicin or hydrogenated soya lethicin each with 0.25% Dipotassium Glycyrrhizate, or oil/water emulsions containing 0.25% Dipotassium Glycyrrhizate. Test material was left on the skin surface for 12 h, and samples of fluid from the receptor chamber were collected at regular intervals for ultraviolet (UV)-HPLC analysis. All treatments showed negligible flux of Dipotassium Glycyrrhizate across the pig skin, as concentrations of Dipotassium Glycyrrhizate in the receptor chamber fluid were below the UV-HPLC detection limit.

After the 12-h treatment period, the treatment surface was washed five times with 50% ethanol and then water. The skin was removed from the apparatus and homogenized, and the Dipotassium Glycyrrhizate content of the skin was determined by HPLC. Dipotassium Glycyrrhizate was found in the skin at 12 ± 3 $\mu g/cm^2$ from the 0.25% water solution, 19 ± 5 $\mu g/cm^2$ from the 1% water micelle solution, and 13 ± 4 $\mu g/cm^2$ from the 0.25% oil/water emulsion. The liposomes containing 0.25% Dipotassium Glycyrrhizate increased the skin content from 62 $\pm$ 8 to 71 $\pm$10 $\mu g/cm^2$, approximately four to five times the amount absorbed without liposomes. Thus, although Dipotassium Glycyrrhizate was able to enter the pig skin, it was unable to permeate through all layers of the skin tissue (Trotta et al. 2002).

Animal

Parke et al. (1963) treated six albino rats with a single oral dose of 60 mg/kg of tritium-labeled β-Glycyrrhetinic Acid in aqueous suspension. Two additional rats received a single subdermal injection of 60 mg/kg tritium-labeled β-Glycyrrhetinic Acid in aqueous suspension. Blood, urine, and feces were collected for several time points after the dose was given. Detection of Glycyrrhetinic Acid and its metabolites in the biological samples was accomplished by paper chromatography using different media and scintillation counting of radioactivity.

In rats receiving 60 mg/kg orally, an average of 86% of the radioactivity administered was recovered in 1 to 3 days with 83% in the feces, 1% in the urine, and 4% remained in the liver. In rats given 60 mg/kg subdermally, 74% of the administered radioactivity was recovered with 73% in the feces and 1% in the urine. Of the radioactivity recovered in the feces, 7.4% was unchanged β-Glycyrrhetinic Acid in the orally dosed rats, and 5.2% in subdermally dosed rats. The remaining radioactivity in the feces was attributed to α-Glycyrrhetinic Acid, 3-keto- β-glycyrrhetinic acid, 3-aceto- β-glycyrrhetinic acid, Ammonium Glycyrrhizate, and other metabolites (Parke et al. 1963).

Ishida et al. (1989) investigated the pharmacokinetics of Glycyrrhetinic Acid. Male Wistar rats were given a bolus intravenous injection of 2, 5, or 12 mg/kg Glycyrrhetinic Acid. The sample size per group was not reported. Samples of blood, bile, and lymph were collected by cannulae. The investigators found that Glycyrrhetinic Acid in rats adhered to a two-compartment pharmacokinetic model.

The first-order partition rate from the central compartment to the peripheral compartment was 0.0797 min^{-1}, and from the peripheral back to the central compartment was 0.0817 min^{-1}. The volume of distribution of the central compartment was 72.293 ml/kg. The Michaelis constant and maximum velocity of elimination from the central compartment were 27.970 μg/ml and 15.329 μg/ml/min/kg, respectively. The plasma-to-blood concentration ratio was constant at all doses (1.787), indicating that Glycyrrhetinic Acid was not well absorbed into erythrocytes. Tissue-to-plasma concentration ratios were all less than 1 (maximum was 0.121 in kidney), indicating poor tissue distribution. The tissue-to-plasma concentration ratio of skin was 0.089 ± 0.006. The steady state volume of distribution was statistically similar among the three dose groups. However, the total body clearance decreased in a dose-dependent manner with increasing doses: 8.194, 6.047, and 3.694 ml/min/kg for the 2, 5, and 12 mg/kg groups, respectively. The authors concluded that the elimination of Glycyrrhetinic Acid was saturable (Ishida et al. 1989).

Cantelli-Forti et al. (1997) studied the biliary excretion of Glycyrrhizic Acid and Glycyrrhetinic Acid after oral or intravenous administration of licorice extract or Glycyrrhizic Acid. Male Sprague-Dawley rats received 480 mg/kg Glycyrrhizic

Acid or 6278 mg/kg licorice extract by oral gavage ($n = 6$ rats per treatment group). The Glycyrrhizic Acid dose was selected to give the same amount of Glycyrrhizic Acid found in the licorice extract dose. Six control rats were given 10 ml/kg distilled water. After dosing, animals were anesthetized, and their abdomens were opened. The common bile duct of each animal was tied and cannulated with a polyethylene catheter, through which bile was steadily collected every 120 min for 16 h.

Bile samples were analyzed by HPLC to detect Glycyrrhizic Acid and Glycyrrhetinic Acid. After oral gavage of Glycyrrhizic Acid, the excretion of Glycyrrhizic Acid via bile peaked (18.02 μg/min·kg) at 8 to 10 h and then decreased sharply. After oral gavage with licorice extract (containing the same amount of Glycyrrhizic Acid), the biliary excretion of Glycyrrhizic Acid was significantly reduced and peaked (3.43 μg/min·kg) at 6 to 8 h. Area under the curve analysis indicated a significant sevenfold increase in biliary excretion of Glycyrrhizic Acid after oral Glycyrrhizic Acid, compared to licorice extract. Thus, excretion of Glycyrrhizic Acid is greater after oral Glycyrrhizic Acid than oral licorice extract. Levels of Glycyrrhetinic Acid in the bile were below the detection limits in all bile samples.

In another experiment, rats were anesthetized and their bile ducts were cannulated for bile collection. Baseline bile was collected for 1 h and then 32.7 mg/kg licorice extract or 2.5 mg/kg Glycyrrhizic Acid was injected intravenously over a period of 1 h by means of a peristaltic pump. The Glycyrrhizic Acid dose was selected to give the same amount of Glycyrrhizic Acid found in the licorice extract dose. Bile samples were collected hourly for 6 h. After intravenous administration of Glycyrrhizic Acid, bile flow was increased after extract injection, compared to Glycyrrhizic Acid injection. The authors did not compare the biliary Glycyrrhizic Acid concentrations after intravenous injection of licorice extract and Glycyrrhizic Acid (Cantelli-Forti et al. 1997).

Ichikawa et al. (1986) demonstrated that Glycyrrhizic Acid is excreted in the bile and undergoes enterohepatic cycling in rats. One group of rats was surgically fitted with biliary fistulas, whereas control rats were left intact. The rats received a single intravenous dose of 100 mg/kg Glycyrrhizic Acid. Plasma concentrations of Glycyrrhizic Acid were measured from blood samples taken at several time points after the dose was given. Plasma decay in control rats was biphasic. The area under the curve (AUC) of plasma concentrations for 12 h after dosing was significantly increased ($p < 0.01$) in control rats than in rats with biliary fistulization. Total body clearance of Glycyrrhizic Acid was 0.372 ml/min/kg in control rats and 0.936 ml/min/kg in rats with biliary fistulization, a significant difference ($p < 0.01$). The total cumulative excretion of Glycyrrhizic Acid in rats for 48 h was as follows: 80.6% of administered dose in bile in both control and fistulated rats; 9.8% in urine of control rats and 4.5% in urine of rats with bile fistulas; 5.5% in feces of control rats; and no Glycyrrhizic Acid was detected in the feces of rats with bile fistulas. These findings were attributed to the elimination of Glycyrrhizic Acid primarily in the bile.

Ishida et al. (1992) studied the pharmacokinetics of Glycyrrhizic Acid in rats. Male Wistar rats received 5, 10, 20, or 50 mg/kg Glycyrrhizic Acid via intravenous administration in the femoral vein. Sample size per group was not reported. Blood, bile, and urine were collected by cannulae at several time points up to 48 h. The decrease in mean plasma concentration was biphasic.

The steady-state volume of distribution (Vd_{ss}) for the 20 and 50 mg/kg groups (86.4 and 115.0 ml/kg, respectively) was significantly higher ($p < 0.05$) than in the 5 and 10 mg/kg groups (58.2 and 59.8 ml/kg, respectively). Total body clearance (Cl_{tot}) for the 20 and 50 mg/kg groups (1.54 and 1.20 ml/min/kg, respectively) was significantly lower ($p < 0.05$) than in the 5 and 10 mg/kg groups (1.91 and 1.98 ml/min/kg, respectively). Biliary excretion for 8 h after dosing was similar between all dose levels, ranging from 85.9% to 95.7% of the administered dose, suggesting a high potential of enterohepatic cycling. Calculations of plasma protein concentration and binding suggested that plasma protein binding may play a role in the changes in pharmacokinetic parameters at different dose levels (Ishida et al. 1992).

Yamamura et al. (1995) compared the phamacokinetic behavior of Glycyrrhizic Acid as a function of route of administration. Male Wistar rats were exposed to 2, 10, or 50 mg/kg Glycyrrhizic Acid either by intravenous (i.v.), intraperitoneal (i.p.), or oral (p.o.) routes. The number of rats per group was not reported. Cannulae were surgically placed and used to collect blood from the femoral artery and urine from the bladder. Blood and urine were collected at regular intervals before and after dosing.

After each i.v. administration, the Glycyrrhizic Acid plasma concentration decreased biexponentially, resulting in a biological half-life of 2.1 to 2.7 h. The steady-state volume of distribution was 98.6 to 166.0 ml/kg [p = n.s. (nonsignificant) between doses], and the total body clearance was 77.3 to 93.0 ml/h·kg (p = n.s. between doses). Urinary excretion of Glycyrrhizic Acid over the 24 h after 2, 10, and 50 mg/kg amounted to 1.3%, 1.9%, and 3.2% of the administered dose, respectively. The metabolite Glycyrrhetinic Acid appeared in the urine at 0.03%, 0.04%, and 0.01% of the administered dose of Glycyrrhizic Acid, respectively, to the above doses.

After i.p. injection, Glycyrrhizic Acid appeared rapidly in the plasma and reached maximum concentration within 30 min. The mean maximum concentration for the 2, 10, and 50 mg/kg dose groups was 4.7, 33.0, and 238.9 μg/ml, respectively. The biological half-life ranged from 2.5 to 2.9 h. The respective apparent clearance values for the three doses were 137.3, 108.62, and 85.6 ml/kg·h.

Rats receiving 2 or 10 mg/kg doses orally had Glycyrrhizic Acid plasma concentrations of 0.2 or 0.4 μg/ml, respectively. Glycyrrhizic Acid was eliminated by 2 h after dosing, whereas, it was detected at a concentration of 1.3 μg/ml in rats of the 50 mg/kg p.o. group, and Glycyrrhizic Acid remained in the plasma for over 8 h.

These investigators also studied the intestinal absorption of Glycyrrhizic Acid in an in situ experiment. A closed intestinal

loop of ileum was prepared, and the mesenteric vein was cannulated. A dose of 10 or 50 mg/kg Glycyrrhizic Acid was injected into the ileum, and mesenteric blood was collected for up to 100 min. Only 1.2% of the 10 mg/kg dose and 1.9% of the 50 mg/kg dose was absorbed as Glycyrrhizic Acid by the ileum. From 0.2% to 0.4% of the administered dose was collected in the blood as Glycyrrhetinic Acid. The intestinal contents contained 4.8% of the administered dose as Glycyrrhizic Acid. Two unspecified metabolites of Glycyrrhizic Acid were in the intestinal contents, amounting to 83.8% and 0.45% of the administered dose. Glycyrrhetinic Acid was not detected in the intestinal contents. In another experiment, Glycyrrhizic Acid was stable in gastric juices at 37°C and pH 1.4 for 3 h.

In summary, the pharmacokinetic profiles of i.v.- and i.p.-administered Glycyrrhizic Acid were similar, whereas the oral route had low bioavailability, possibly due to poor absorption from the intestinal tract (Yamamura et al. 1995).

Imai et al. (1999) and Akao (1994, 1997) demonstrated that Dipotassium Glycyrrhizate and Glycyrrhizic Acid, respectively, are metabolized by intestinal microflora into Glycyrrhetinic Acid before absorption can occur.

Wang et al. (1994) found that, in rats, Glycyrrhizic Acid is hydrolyzed to Glycyrrhetinic Acid in the stomach and large intestine, and that most of the Glycyrrhetinic Acid formed was absorbed from the large intestine.

Cantelli-Forti et al. (1994) and Wang et al. (1995) reported that more Glycyrrhizic Acid is absorbed in the intestine after oral administration of pure Glycyrrhizic Acid than after an oral administration of licorice extract with equal Glycyrrhizic Acid content. The authors suggested that other constituents of licorice extract inhibit the intestinal absorption of Glycyrrhizic Acid.

Human

Terasawa et al. (1986) measured the disposition of Glycyrrhetinic Acid in healthy human subjects. Five healthy male subjects (aged 20 to 24 years) each ingested 5 g of licorice dissolved in 100 ml water. The dose was determined by HPLC to contain 133 mg Glycyrrhizic Acid and no detectible Glycyrrhetinic Acid. Volunteers were prohibited from consuming alcohol but were not otherwise restricted in normal diet and activities. Blood was collected daily for 7 days after administration. Urine was collected every 10 h over the 7-day post-dose period. Glycyrrhetinic Acid and Glycyrrhizic Acid concentrations in the serum were measured using an enzyme immunoantibody assay. Urinary levels were measured by HPLC.

The maximum serum Glycyrrhizic Acid occurred at 4 h and levels rapidly decreased afterwards. By the 96th h, Glycyrrhizic Acid was undetectable in the sera of all subjects. Likewise, urinary Glycyrrhizic Acid peaked at the first 10-h collection after dose administration and then rapidly decreased. Serum Glycyrrhetinic Acid concentrations peaked at 30 ng/ml about 24 h after dosing and then declined. Four subjects had low but detectable serum Glycyrrhetinic Acid at 48 h, and two at 96 h. Glycyrrhetinic Acid appeared in the urine at the 20- and 30-h time points and decreased at a rate consistent with serum levels. Total urinary excretion of Glycyrrhetinic Acid was about 2% of the administered dose (Terasawa et al. 1986).

Yamamura et al. (1992) investigated the pharmacokinetics of Glycyrrhetinic Acid in human subjects. Three healthy volunteers received a single oral dose of 100 mg Glycyrrhizic Acid in tablet form. Demographics (sex, age, etc.) of the subjects were not reported. Blood was drawn from a cannula in the forearm 1 h before and 0.5, 1, 2, 4, 6, 8, 10, and 12 h after dosing. Urine was collected for a 24-h period after dosing. Diet and beverage were controlled during the study duration. Glycyrrhizic Acid was not detected in plasma after oral administration. Glycyrrhetinic Acid was detected at <200 ng/ml. Total urinary excretion of Glycyrrhizic Acid and its metabolites over the 24-h post-dose period for each of the subjects was 0.8%, 0.3%, and 0.4% of the administered dose.

The stability of Glycyrrhizic Acid in gastric juices at 37°C and pH 1.78 was examined. After 5 h, the hydrolysis products glycyrrhetinic acid 3-*O*-glucuronide and Glycyrrhetinic Acid were not detected. Thus, Glycyrrhizic Acid does not appear to be hydrolyzed in the stomach and is absorbed as intact Glycyrrhizic Acid.

Three healthy volunteers received either 40, 80, or 120 mg Glycyrrhizic Acid by intravenous infusion in the right forearm. Demographics (sex, age, etc.) of the subjects were not reported. Blood was drawn from a cannula in the forearm 1 h before administration and 5, 15, and 30 min, and 1, 2, 4, 6, 8, 10, and 12 h after dosing. Urine was collected for a 24-h period after dosing. The plasma concentration of Glycyrrhizic Acid decreased in a biphasic manner. The half-life of intravenously administered Glycyrrhizic Acid at the three dose levels was 3.8 to 4.8 h and total body clearance was 16 to 25 ml/kg/h. The steady-state volume of distribution was 78 to 98 ml/kg. Urinary excretion of Glycyrrhizic Acid over 24 h amounted to 1.1% to 2.5% of the administered dose. The metabolites, glycyrrhetininc acid 3-*O*-glucuronide and Glycyrrhetinic Acid, were not detected in plasma or urine (Yamamura et al. 1992).

Ploeger (2001b) compared the pharmacokinetics of Glycyrrhetinic Acid after a single dose and after multiple doses in humans. Twelve healthy male volunteers, aged 19 to 29 years with a mean body weight of 70 ± 6.6 kg, ingested 130 mg/day Glycyrrhetinic Acid in a propylene glycol and water vehicle for 5 days. Blood samples were collected prior to dosing and at several time points after dosing on the 1st and 5th days and again 5 days after the last dose. Urine was also collected throughout the study. After the first dose, the maximum plasma concentration (C_{max}) of Glycyrrhetinic Acid was achieved within 4 h post-dose, C_{max} was 1.11 ± 0.94 mg/L. After the fifth dose, the C_{max} was 1.80 ± 0.95 mg/L. Time to C_{max} after the fifth dose was not reported. The half-life of Glycyrrhetinic Acid was 23.6 h. Glycyrrhetinic Acid (0.028 ± 0.044 mg/L) was still detectable in the subjects' plasma 219 h after the last dose.

Tanaka et al. (1993) studied the phamacokinetics of Glycyrrhizic Acid in patients with chronic hepatitis. Hepatitis patients, aged 38 to 69, received an intravenous injection of 120 mg Glycyrrhizic Acid. Plasma concentrations of Glycyrrhizic Acid were monitored for 10 h after dosing and declined in a monophasic manner. The mean biological half-life of Glycyrrhizic Acid was 6.0 ± 2.1 h, the mean total clearance was 7.9 ± 3.0 ml/h/kg, and the mean volume of distribution was 61.8 ± 18.3 ml/kg.

GENERAL BIOLOGY

Systemic Effects Overview

Moderate chronic or high acute exposure to Glycyrrhizic Acid, Ammonium Glycyrrhizate, and their metabolites have been demonstrated to cause several transient systemic alterations, including increased potassium excretion, sodium and water retention, body weight gain, alkalosis, suppression of the renin-angiotensis-aldosterone system, hypertension, and muscular paralysis. Most of these effects are the consequence of the Glycyrrhizic Acid inhibition of the enzyme 11β-hydroxysteroid dehydrogenase-2 (11β-OHSD2) in the kidney. This enzyme protects mineralocorticoid (aldosterone) receptors from being stimulated by cortisol. Stimulation of mineralocorticoid receptors in the distal renal tubules causes the excretion of potassium and hydrogen ions in the urine and the reabsorption of sodium bicarbonate and water. Retention of sodium and water causes hypertension. Reabsorption of bicarbonate causes alkalosis. Hypokalemia resulted in myoglobinuria, which caused muscular weakness and paralysis. Ataxia has been observed after a very high acute exposure to Glycyrrhizic Acid. These effects dissipate when exposure to Glycyrrhizic Acid is ceased, and these effects can be blocked by the mineralocorticoid receptor antagonists spironolactone and eplerenone (Olukoga and Donaldson 2000; Davis and Morris 1991; Conn et al. 1968).

Animal

Finney et al. (1958) observed a marked antidiuretic effect in rats within 30 min after a single i.p. dose of 125 mg/kg Glycyrrhetinic Acid. Sodium retention and increased urinary excretion of potassium were also observed.

Rossi et al. (1994) compared the pharmacological effects of Glycyrrhizic Acid and 18β-Glycyrrhetinic Acid in rats. Normotensive Sprague-Dawley rats received 30 mg/kg/day Glycyrrhizic Acid or 15 mg/kg/day 18β-Glycyrrhetinic Acid by oral intubation for 30 days (exact sample size not reported, $n \geq 10$ per dose group). A control group of unspecified size received a comparable volume of water on the same dosing schedule. Systolic blood pressure was measured prior to the first dose and on the 7th, 15th, and 30th day of treatment. In order to reduce stress-induced effects while measurements were made during the study, all animals were acclimated to the blood pressure measuring procedure prior to study initiation. The day prior to the first dose and on days 6, 14, and 29 of dosing, rats were placed in metabolism cages, and 24-h urine samples were collected. The urine volume was recorded and the urine was analyzed for electrolyte content. Additional systolic blood pressure measurements and urine samples were collected on day 60 (30 days after the last dose).

Systolic blood pressure was significantly increased at 15 days in the Glycyrrhizic Acid and the 18β-Glycyrrhetinic Acid groups ($p < 0.05$) and at 30 days in the 18β-Glycyrrhetinic Acid group only ($p < 0.01$). Urine volume was decreased and urine Ca^{2+} concentration was increased in the 18β-Glycyrrhetinic Acid group at 15 and 30 days. All parameters returned to normal by day 60. The authors concluded that 18β-Glycyrrhetinic Acid is an active agent in the hypertensive effects seen in excessive licorice consumption, and that these effects are reversible (Rossi et al. 1994).

Rossi et al. (1999) studied the effects of Glycyrrhizic Acid and the 18α- and 18β- isomers of Glycyrrhetinic Acid on heart function, cardiac tissues, and apoptosis. This study had two parts, acute treatments and subchronic treatments. In the acute treatment, Sprague-Dawley rats were given a single intraperitoneal injection of 0.5, 1.0, or 1.5 mg/kg of Glycyrrhizic Acid, 18α-Glycyrrhetinic Acid, or 18β-Glycyrrhetinic Acid ($n = 4$ rats per dose per test material). An additional group of four rats received 2.0 mg/kg 18α-Glycyrrhetinic Acid, of which all died within 140 min of dosing due to atrioventricular block, as detected by electrocardiogram (ECG). The ECG profiles of all other animals were similar to controls. Microscopic examination showed no abnormalities in the surviving rats. However, edema in the brain, cerebellum, and lungs, and hematic stasis in the kidneys occurred in the 2.0 mg/kg 18α-Glycyrrhetinic Acid group. Calculi of calcium salt occurred in the renal tubules of two of the dead rats. Focal changes in the papillary muscles of the heart included edema, myolysis, and cell distortion. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) analysis of the papillary muscles of the heart indicated evidence of apoptosis in the 18α-Glycyrrhetinic Acid group.

In the subchronic treatment, Sprague-Dawley rats were assigned to four treatment groups and received daily oral doses of 30 mg/kg Glycyrrhizic Acid, 15 mg/kg 18β-Glycyrrhetinic Acid, 15 mg/kg 18α-Glycyrrhetinic Acid, or an equal volume of water (control) for 30 days ($n = 40$ rats per group on day 0 of dosing). Ten rats per group were killed prior to the first dose. Ten rats per group were killed on day 15 of dosing and another 10 on day 30 of dosing. The remaining 10 animals per group were kept alive an additional 30 days following the treatment period, at which time they were killed. Twenty-four hours prior to scheduled deaths, animals were placed in metabolism cages for urine collection. Blood was collected at death, and tissues and organs were examined microscopically. Certain tissues were set aside for apoptosis analysis by the TUNEL assay.

At 15 days urine electrolyte analysis had potassium (K^+) increased in the Glycyrrhizic Acid group and sodium (Na^+), and calcium (Ca^{2+}) increased in the 18α-Glycyrrhetinic Acid group; at 30 days, K^+ and Na^+ in the 18α-Glycyrrhetinic Acid group, and K^+ and Ca^{2+} increased in the 18β-Glycyrrhetinic Acid

group; and at 60 days (30 days after last dose), Na^+ increased in the Glycyrrhizic Acid group, and K^+ and Ca^{2+} increased in the 18α-Glycyrrhetinic Acid group (all urine electrolyte differences reported were significant at $p < 0.05$). Na^+ levels were elevated in serum of all three treatments at 15 and 30 days but returned to normal by day 60. Myolysis of the papillary muscles appeared in the Glycyrrhizic Acid and 18α-Glycyrrhetinic Acid group at day 30 and did not regress over the 30-day recovery period. Animals in these two treatment groups also had tubular caliculi in the bronchus-associated lymphoid tissue at day 15 and thereafter. Renal calculi occurred in concert with increased urinary Ca^{2+} (see above). In this study 18α-Glycyrrhetinic Acid was the most potent cardiotoxin of the three compounds studied, and while some effects occurred and ceased with exposure, other damage (e.g., myolysis) persisted after exposure had ended (Rossi et al. 1999).

Human

Ploeger et al. (2001b) studied the inhibitory effect of Glycyrrhetinic Acid on 11β-hydroxysteroid dehydrogenase-2 (11β-OHSD2) by measuring the cortisol cortisone ratio in urine before and after human exposure to Glycyrrhetinic Acid. Twelve healthy male volunteers, aged 19 to 29 years with a mean body weight of 70 $\pm$ 6.6 kg, refrained from eating any licorice or Glycyrrhetinic Acid-containing foods for 72 h prior to and throughout the study. The subjects did not eat or drink anything except water for 10 h before dose administration. Urine was collected for 24 h before the first dose was given. Subjects ingested 130 mg/day Glycyrrhetinic Acid in a propylene glycol and water vehicle for 5 days. Blood samples were collected prior to dosing and at several time points after dosing on the 1st and 5th days and again 5 days after the last dose. Urine was also collected throughout the study.

The ratio of cortisol/cortisone was significantly increased on days 2, 3, 4, and 5 of dosing, compared to baseline data. Also, the ratio of cortisol/cortisone increased with each successive day of dosing. Because 11β-OHSD2 is the enzyme responsible for converting active cortisol to inactive cortisone ("active" here is in reference to activity as an aldosterone receptor antagonist), the inhibition of 11β-OHSD2 by Glycyrrhetinic Acid (or its metabolite Glycyrrhizic Acid) caused the changes in cortisol/cortisone ratio. The median concentration of inhibition (IC_{50}) of 11β-OHSD2 was between 325 and 394 μg/L Glycyrrhetinic Acid in plasma (Ploeger et al. 2001b).

Molhuysen et al. (1950) stated that licorice extract was found to be an effective treatment for gastric ulcers during World War II, but edema occurred in about 20% of the patients receiving this treatment. To investigate possible causes of edema, these authors administered a licorice extract (succus liquiritiæ) containing 15% Glycyrrhizic Acid to seven patients who had persistent gastric ulcers and to three patients who did not. Subjects received 20 to 45 g of the licorice extract, administered in eight equal parts (2-h intervals) throughout the day and night (duration of treatment was not specified, but seemed to vary with each patient). Urine was collected at each dose interval. Subjects were on a strictly controlled diet during the experiment.

Urine output volume decreased within the first 3 days of treatment, as did chloride (Cl^-) excretion. Excretion of urea plus ammonia was not affected, nor was urea clearance. There was no albumin or abnormal urinary sediment detected in the urine. Blood hemoglobin level decreased with dosing. Venous blood pressure, pulse pressure, and systolic pressure all increased significantly. Patients also gained several kilograms in body weight due to edema. The authors attributed this combination of effects to "stimulation of the renal tubules to an excessive reabsorption of water and chlorides, and probably also of sodium." Another patient received an unspecified amount of pharmaceutical Ammonium Glycyrrhizate, and the effects were similar to those receiving the licorice extract (Molhuysen et al. 1950).

Louis and Conn (1956) administered seven normal human subjects 6 g/day Ammonium Glycyrrhizate for three days. Compared to baseline data before dosing, sodium (Na^+) and chloride (Cl^-) excretion in the urine was significantly decreased while potassium (K^+) excretion was slightly increased during the dosing period. Withdrawal of the Ammonium Glycyrrhizate caused an immediate above-normal increase in renal excretion of Na^+ and Cl^- and retention of K^+. During the exposure period, four subjects gained weight attributed to water retention. Body weights returned to normal 5 to 7 days after withdrawal of Ammonium Glycyrrhizate treatment. Details of other effects are described in Clinical Experimental Studies.

Hormonal Effects

Quaschning et al. (2001) demonstrated that inhibiting the aldosterone (mineralocorticoid) receptor prevents the hypertension normally induced by Glycyrrhizic Acid in rats. Glycyrrhizic Acid (3 g/L) was added to the drinking water of Wistar-Kyoto rats for 21 days. From days 8 to 21, 5.8 mg/kg/day spironolactone, 182 mg/kg/day eplerenone, or a placebo was added to the diet ($n = 7$ animals/group). Glycyrrhizic Acid increased systolic blood pressure from 142 to 185 mm Hg. Spironolactone and eplerenone, known antagonists of the aldosterone receptor, normalized blood pressure in animals also given Glycyrrhizic Acid.

Takii et al. (2001) demonstrated an anti-diabetic effect of Glycyrrhizic Acid in genetically diabetic mice. Male 4-week-old KK-A^y/Ta Jcl mice with genetic type II non–insulin-dependent diabetes were given control diet, diet containing 0.27% Glycyrrhizic Acid, or diet containing 0.41% Glycyrrhizic Acid for 9 weeks ($n = 8$ mice/group, matched for body weight and blood glucose concentration). Animals were fasted for 5 h, and blood was collected for analysis before, weekly during, and after the treatment period. Animals were killed for necropsy after the treatment period.

Food consumption and body weight gain did not differ between treatment groups. Water consumption was decreased at week 7 and thereafter. Five-hour fasting blood glucose

levels were similar between groups until week 7 of treatment, when the control group had increased blood glucose levels, whereas Glycyrrhizic Acid–treated mice maintained steady blood glucose levels. At week 8, the blood glucose of the 0.27% Glycyrrhizic Acid group increased to match that of the control group. At weeks 8 and 9, the blood glucose of the 0.41% Glycyrrhizic Acid group was significantly less than that of the other two groups. At week 9, the blood insulin level of the 0.41% Glycyrrhizic Acid group was significantly less ($p < 0.05$) than that of the other two groups.

After week 9, an oral glucose tolerance test was performed in which the mice received an oral dose of 200 μl 20% glucose solution (1 g/kg glucose) after an 18-h fast. Blood samples were taken before the glucose dose and 30, 60, and 120 min after. Blood glucose levels of the 0.41% Glycyrrhizic Acid group at 60 and 120 min were significantly less ($p < 0.05$) than the blood glucose levels of the control group and 0.27% Glycyrrhizic Acid group, which were similar. Necropsy did not reveal any remarkable effects of Glycyrrhizic Acid treatment. The authors proposed that Glycyrrhizic Acid inhibits the Na^+-glucose active transport system in the small intestine, but further studies would be required to investigate the mechanism (Takii et al. 2001).

Cardiac Effects

Glycyrrhizic Acid and related compounds can increase blood pressure by promoting the retention of sodium and water from the kidneys and suppression of the aldosterone-renin-angiotensin system as described above.

Kilgore et al. (1998) discovered that treatment with Glycyrrhizic Acid but not Glycyrrhetinic Acid can reduce the size of myocardial infarcts in rabbits. New Zealand white rabbits were anesthetized, and the left jugular vein was cannulated for drug administration and blood sampling. The left carotid artery was cannulated and connected to a transducer to monitor systolic blood pressure. Thoracotomy and pericardiotomy were performed on each rabbit. A silk suture was placed around the left coronary artery to occlude blood flow for 30 min. Ischemia was confirmed by cyanosis distal to the site of occlusion. At the end of the occlusion period, the suture was released to allow reperfusion of the cardiac tissue. Animals received an i.v. bolus infusion of 10 mg/kg/h Glycyrrhizic Acid or 10 mg/kg/h Glycyrrhetinic Acid immediately before reperfusion and every hour for 5 h ($n = 6$ rabbits/group). Eleven control animals received saline infusions on the same schedule. Blood was collected at hourly intervals. After the 5-h reperfusion and dosing period the infarcted area was determined by perfusion of the heart with dyes that are metabolized by healthy tissue. The dyes differentiated between tissue unaffected by the occlusion of the left coronary artery (red), noninfarcted tissues that became ischemic during the occlusion (purple-black), and infarcted tissue (pale yellow).

There was no difference between treatment groups in the area affected by the occlusion. However, 59.3% of the occlusion-affected area was infarcted in the control group, whereas that percentage was only 30.3% in the Glycyrrhizic Acid group, a statistically significant difference ($p < 0.05$). When expressed as percent of the left ventricle, 27.5% was infarcted in control rabbits while 13.0% was infarcted in the Glycyrrhizic Acid group, also a significant difference ($p < 0.05$). The infarcted area of the Glycyrrhetinic Acid group was similar to that of control animals. The myeloperoxidase activity in left ventricle tissue of the Glycyrrhizic Acid group was lower than that of control animals. The authors suggest that Glycyrrhizic Acid is effective in reducing the degree of myocardial injury after an acute period of ischemia and reperfusion (Kilgore et al. 1998).

Effects on Enzyme Activities

Whitehouse et al. (1967) found that Glycyrrhetinic Acid at 100 mM is a potent inhibitor of oxidative phosphorylation coupled to succinate oxidation in rat liver mitochondria.

O'Brian et al. (1990) demonstrated that Glycyrrhetinic Acid inhibits the calcium- and phospholipid-dependent phosphotransferase activity of protein kinase C (PKC). The IC_{50} of PKC inhibition was approximately 450 μM Glycyrrhetinic Acid. Glycyrrhizic Acid was a much weaker inhibitor of PKC activity.

Jung et al. (2001) exposed B16/F10 murine melanoma cells to Glycyrrhizic Acid or Glycyrrhetinic Acid for three days and then determined the melanin contents and tyrosinase activities in the cells. Cellular melanin content and tyrosinase activity were dose dependently increased by Glycyrrhizic Acid. However, the rise in melanin content reached a plateau of 160% of control at 0.50 mM Glycyrrhizic Acid, whereas tyrosinase activity continued to rise 220% of control at the highest concentration tested, 1.00 mM Glycyrrhizic Acid. Additionally, the intracellular levels of tyrosinase mRNA and expression of tyrosine-related protein-2 (TRP-2), but not TRP-1, were also dose dependently increased by Glycyrrhizic Acid. These effects were not seen when the cells were treated with Glycyrrhetinic Acid. Rather, Glycyrrhetinic Acid caused decreases in tyrosinase activity and melanin content. These data indicate that the glycoside structure is important in the Glycyrrhizic Acid–induced stimulation of melanogenesis in this cell line.

Noda (1964) found that 0.13 to 4 mmol/L Glycyrrhizic Acid inhibited the enzyme activity of proteinase in an in vitro system. This inhibition was greater at higher Glycyrrhizic Acid concentrations.

Paolini et al. (1998) studied the effects Glycyrrhizate on the cytochrome P450 monooxygenase activities in Swiss Albino CD1 mice. The mice received daily oral doses of 480 mg/kg Glycyrrhizic Acid for 1, 4, or 10 days. Controls received vehicle only. Animals were fasted for 16 h after the last dose and then killed. The livers were rapidly removed and prepared for enzymatic assays, electrophoresis, Western immunoblot, RNA isolation, and Northern hybridization. The single dose of Glycyrrhizic Acid did not induce any enzymes. However, hepatic cytochrome P450 (CYP) 3A-, 2B1-, and 1A2-dependent

microsomal monooxygenase were induced by the multiple doses of Glycyrrhizic Acid. The following testosterone hydroxylase (TH) enzymes in the liver were also induced by multiple doses of the two test materials: 6β-TH, 2α-TH, 6α-TH, 7α-TH, and 16β-TH. The authors suggested that these results indicate that the induction of cytochrome P450-dependent activities by the prolonged intake of Glycyrrhizic Acid at high doses may cause accelerated metabolism of coadministered drugs, and that the adverse effects associated with cytochrome P450 changes may also have clinical consequences.

Sakamoto et al. (2001) found that Glycyrrhizic Acid binds to high mobility group proteins 1 and 2 (HMGP1/2), causing a conformational change in the proteins, and thus inhibiting the phosphorylation of HMPG1/2 by protein kinases. Glycyrrhetinic Acid had 10 times the binding affinity of Glycyrrhizic Acid to HMPG1/2. HMPG1/2 requires phosphorylation for its DNA-binding affinity; however, the physiological significance of this DNA-binding was unclear. Blockade of HMPG1/2 by Glycyrrhizic Acid or Glycyrrhetinic Acid may be involved in the anti-inflammatory activity of these compounds.

Gap Junction Inhibition

As described by Rozental et al. (2001), gap junctions are pores connecting the cytoplasm of adjacent cells and are important for intercellular communication and normal function in several tissue types. Glycyrrhetinic Acid and its derivatives reversibly block gap junction communication. 18α-Glycyrrhetinic Acid and 18β-Glycyrrhetinic Acid mediate a concentration-dependent inhibition of junctional conductance by 60%. Complete blockade of the gap junction channels does not occur even at concentrations as high as 100 μM. Concentrations above 75 μM Glycyrrhetinic Acid become cytotoxic and irreversible damage occurred. The mechanism for action is unknown. The inhibitory effect of Glycyrrhetinic Acid and its derivatives is used as a tool in electrophysiology experiments and other areas of cellular research.

Davidson and Baumgarten (1988) studied the structure-activity relationship of Glycyrrhetinic Acid derivatives as gap junction inhibitors in human fibroblasts. The authors reported the relative potencies of the test compounds in terms of median concentration of inhibition of gap junction channels (IC_{50}) and median toxic concentration (TC_{50}). For 18α-Glycyrrhetinic Acid, the IC_{50} was 1.5 μM, and the TC_{50} was $>$100 μM. For 18β-Glycyrrhetinic Acid, the IC_{50} was 2 μM, and the TC_{50} was 25 μM.

Gou et al. (1999) found that 18α-Glycyrrhetinic Acid inhibits communication between alveolar epithelial cells at concentrations as low as 5 μM. Extended exposure of alveolar epithelial cells to higher concentrations of 18α-Glycyrrhetinic Acid caused inhibition of gap junction intracellular communication and time- and concentration-dependent reductions in Cx43 protein and mRNA expression. Cx43 is one of the proteins responsible for gap junction formation.

Schiller et al. (2001) found that gap junction intercellular communication between cultured MC3T3-E1 osteoblastic cells was concentration-dependently inhibited by 100 μM 18α-Glycyrrhetinic Acid. Likewise, the blockade of gap junctions by 18α-Glycyrrhetinic Acid interfered with the maturation of the osteoblastic cells, possibly by affecting signal regulating the expression of genes involved in maturation and/or differentiation.

Bou-Flores and Berger (2001) used 50 μM 18α- and 18β-Glycyrrhetinic Acid in neuronal slice preparations from neonatal Swiss Webster mice to demonstrate the importance of gap junction coupling in interneuronal communication in the central respiratory rhythm-generating system.

Böhmer et al. (2001) measured the effects of 18β-Glycyrrhetinic Acid on electrolyte transmission between confluent primary cultures of rat hepatocytes. 18β-Glycyrrhetinic Acid at 40 μM did not affect Na^+ and K^+ conductance, but it did block the conductance of Cl^-.

Tabernero et al. (2001) found that gap junction communication is important in the metabolic syncytium of sharing energetic intermediates among astrocytes. Inhibition of gap junctions by 100 μM 18α-Glycyrrhetinic Acid caused increased glucose uptake in individual astrocyte cells and induced astrocyte proliferation.

Cytotoxicity

Babich et al. (1993) studied the cytotoxicities of the 18α- and 18β- stereoisomers of Glycyrrhetinic Acid among a list of dietary non-nutrients with chemopreventive properties. Cytotoxicity was measured with the neutral red assay, using BALB/c mouse 3T3 fibroblasts as indicators. The neutral red assay quantifies the number of viable, uninjured cells after incubation with a test compound and is based on the uptake and lysosomal accumulation of neutral red dye. Relative toxicity was reported as the concentration at which the count of dead cells was 50% of control cells (NR_{50}). The range of concentrations used was not reported. The NR_{50} values for 18α-Glycyrrhetinic Acid and 18β-Glycyrrhetinic Acid were 0.26 mM and 0.13 mM, respectively. For comparison, 18α-Glycyrrhetinic Acid and 18β-Glycyrrhetinic Acid were more cytotoxic than caffeic acid (NR_{50} = 1.1 mM), vanillin (8.0 mM), and D-saccharic acid 1,4-lactone (18.6 mM), and less toxic than benzyl isothiocyanate (0.019 mM) and tamoxifen (0.016 mM).

Hepatotoxin Protection

Kiso et al. (1984), noting that Glycyrrhizic Acid and Glycyrrhetinic Acid have been shown to protect liver tissue from known hepatotoxins such as carbon tetrachloride (CCl_4) and galactosamine, exposed rat hepatocytes in primary culture to medium containing 10 mM CCl_4 or 0.5 mM D-galactosamine with 0.01, 0.1, or 1.0 mg/ml Glycyrrhizic Acid or 0.01, 0.1, or 1.0 mg/ml Glycyrrhetinic Acid. The concentration of glutamic-pyruvic transaminase (GPT) in the medium was used as a measure of cytotoxicity. Glycyrrhizic Acid and Glycyrrhetinic Acid

at the 0.1 and 1.0 mg/ml concentrations each showed a dose dependent decrease in the GPT levels, compared to hepatotoxin treatment without Glycyrrhizic Acid or Glycyrrhetinic Acid. The 0.01 mg/ml concentrations of Glycyrrhizic Acid or Glycyrrhetinic Acid did not protect the cells from cytotoxicity. Glycyrrhetinic Acid at 1.0 mM also decreased the generation of free radicals induced by CCl_4 in rat liver microsomes. Glycyrrhetinic Acid at 0.01, 0.1, and 1.0 mg/ml reduced the lipid peroxidation induced by ADP/Fe^{3+} or ascorbate/Fe^{3+} in rat liver microsomes. The authors suggested that Glycyrrhizic Acid or Glycyrrhetinic Acid may be hepatoprotective by preventing lipid peroxidation and free radical generation.

Shibayama (1989) treated male Wistar rats with 200 mg/kg Glycyrrhizic Acid in saline intraperitoneally (i.p.) or by injection into the tail vein. Two or 20 h after the Glycyrrhizic Acid injection, the rats received a hepatotoxin: 0.15 ml/kg CCl_4 in olive oil injected i.p.; 0.1 ml/kg allyl formate in saline injected i.p.; or 3 mg/kg endotoxin in saline injected i.p. or via the tail vein ($n = 3$ rats/treatment condition). Appropriate vehicle controls were used for each combination of Glycyrrhizic Acid and hepatotoxin. The rats were anesthetized 24 h after the hepatotoxin injection, and blood was collected from the inferior vena cava for liver function tests. The livers were fixed and stained for microscopic examinations.

All groups of rats receiving hepatotoxin without Glycyrrhizic Acid had elevated serum glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) levels, an indicator of hepatotoxicity. Pretreatment with Glycyrrhizic Acid for 2 h had no effect on the serum GOT and GPT levels given hepatotoxins. Pretreatment with Glycyrrhizic Acid for 24 h significantly reduced ($p < 0.001$) the serum GOT and GPT levels in rats given CCl_4 or allyl formate. Glycyrrhizic Acid did not affect the rise in serum GOT or GPT in rats given endotoxin. There were close relationships between the serum GPT and GOT level and the degree of cellular necrosis seen in the microscopic examinations. Thus, pretreatment with Glycyrrhizic Acid protected the liver from CCl_4 and allyl formate toxicity, but apparently did not affect the hepatotoxicity of endotoxin (lipopolysaccharide B, derived from *Escherichia coli*) (Shibayama 1989).

Nakamura et al. (1985) exposed primary cultures of rat hepatocytes to 0, 1, 2, 3, 4, or 5 mM CCl_4 for 24 h to demonstrate hepatoxocity as measured by the cellular leakage of the cytosolic enzymes lactic dehydrogenase (LDH), GOT, and GPT. CCl_4 at lower doses caused concentration dependent leakage of the enzymes without cytolysis, but cytolysis was seen with 5 mM CCl_4. When the hepatocytes were exposed to 5 mM CCl_4 with 25, 50, 100, or 200 μg/ml Glycyrrhizic Acid, there was a concentration-dependent decrease in the LDH and GOT leakage from the cells, and cytolysis was reduced. These data support the ability of Glycyrrhizic Acid to protect the liver from hepatotoxic injury.

Nose et al. (1994) compared the in vivo and in vitro antihepatotoxic activities of Glycyrrhizic Acid and Glycyrrhetinic Acid. Male Wistar rats received 100 or 300 mg/kg of 18β-Glycyrrhetinic Acid or Glycyrrhizic Acid orally 1, 24, and 48 h (three injections) prior to an i.p. dose of 500 mg/kg D-galactosamine. Additional groups of rats received 18β-Glycyrrhetinic Acid or Glycyrrhizic Acid i.p. 1 h prior to an i.p. dose of 500 mg/kg D-galactosamine. Blood was collected from the hearts of the rats 24 h after the D-galactosamine dose. Serum GOT and GPT activities were determined using transaminase kits. Rats pretreated with 100 or 300 mg/kg 18β-Glycyrrhetinic Acid had significantly decreased ($p < 0.05$) serum GOT and GPT activities than rats given D-galactosamine without the pretreatment. Glycyrrhizic Acid at 100 and 300 mg/kg resulted in a slight but nonsignificant reduction in the enzyme activities.

Rat parenchymal cells in primary culture monolayers were treated with 5, 10 or 50 μg/ml 18β-Glycyrrhetinic Acid or 5, 50, or 1000 μg/ml Glycyrrhizic Acid for 24 h. The cells were then washed and exposed to medium containing 5 mM CCl_4 for 6 h, when transaminase activities in the medium were assayed. All three concentrations of 18β-Glycyrrhetinic Acid reduced the GOT and GPT activities, compared to cultures exposed only to 5 mM CCl_4. Glycyrrhizic Acid only reduced transaminase activities at the 1000 μg/ml concentration.

The authors concluded that these studies demonstrated that 18β-Glycyrrhetinic Acid is a more potent protector against hepatotoxicity than Glycyrrhizic Acid. The authors suggested that the difference in antihepatotoxicity of these two compounds may lie in the transformation rate of Glycyrrhizic Acid to Glycyrrhetinic Acid (Nose et al. 1994).

Lin et al. (1999) used an in vivo study design similar those described above to demonstrate that pretreatment with 200 mg/kg/day Glycyrrhizic Acid or 10 mg/kg/day Glycyrrhetinic Acid i.p. for 3 days protected rats from hepatotoxicity induced by 35 mg/kg i.p. of retrorsine. A single pretreatment with 200 mg/kg/day Glycyrrhizic Acid or 10 mg/kg/day Glycyrrhetinic Acid i.p. did not protect against retrorsine-induced hepatotoxicity.

Hu et al. (2001) demonstrated that 50 μM Glycyrrhizic Acid improved the viability of V79 cells also exposed to 5 mM acetaldehyde or 10 μM cadmium. Glycyrrhizic Acid at up to 200 μM, however, did not prevent cadmium-induced lipid peroxidation in these cells.

Antihepatitis Activity

Crance et al. (1994) demonstrated that Glycyrrhizic Acid at 1000 and 2000 μg/ml concentration dependently inhibited the replication of hepatitis A virus in PLC/PRF/5 cells in vitro in a dose-dependant manner. Glycyrrhizic Acid was not virucidal, but it did inhibit an early stage of replication of the virus. Glycyrrhizic Acid inhibited the penetration of hepatitis A virus through the plasma membranes of the PLC/PRF/5 cells.

In a summary of presentations at a symposium on chronic active liver disease, Fujisawa and Tandon (1994) reported that Glycyrrhizic Acid has been known as a treatment for chronic hepatitis in Japan for over 20 years. The author suggested that Glycyrrhizic Acid appears to inhibit the penetration of hepatitis

virus into hepatocytes and it may also work as a free radical scavenge and modulate the immune system through interferon mechanisms and T cells (Crance et al. 1994).

Iino et al. (2001) randomly selected 100 subjects from a pool of 178 chronic hepatitis patients whose liver function did not improve with 2 weeks of 40 ml/day stronger neo-minophagen C (SNMC), a hepatitis treatment containing 0.2% Glycyrrhizic Acid, 0.1% cysteine, and 20% aminoacetic acid as active ingredients. The 100 patients received 40 ml/day or 100 ml/day SNMC for 3 weeks ($n = 50$/group). Hepatic enzyme function was recorded 4 and 8 weeks prior to the first dose, weekly during the dosing period and weekly for 3 weeks after the last dose. Serum alanine transaminase (ALT) activity was used as a measure to rate improvement in liver function. Six patients in the 100 ml/day SNMC group were unable to complete the experiment, but of those 44 remaining, 23 (52.3%) had improved liver function. Of the 46 patients in the 40 ml/day group that completed the trials, 12 (26.1%) had improvement in liver function. Thus, the 100 ml/day group had significantly more improvement ($p = 0.017$) in liver function than patients in the 40 ml/day group. The authors proposed that patients who do not respond to moderate doses of SNMC might respond better to a higher dose.

van Rossum et al. (2001) gave Glycyrrhizic Acid to patients with chronic hepatitis C who did not respond to interferon therapy. Forty-one patients received 80, 160, or 240 mg Glycyrrhizic Acid by drip infusion three times per week for 4 weeks (number of patients per dose was not reported). Thirteen patients received a placebo three times per week for 4 weeks. In another experiment, 15 patients received 200 mg Glycyrrhizic Acid by intravenous injection six times per week for 4 weeks. Hematological and biochemical assessments were performed weekly during the study. Virological assessments by enzyme-linked immunosorbent assay (ELISA) were done prior to and after treatment. Serum ALT was used to measure hepatic health before and after treatments and was reported in terms of upper limit of normal (ULN), which is 41 IU/L for men and 31 IU/L for women.

Serum ALT levels after placebo treatments were similar to those before the study. In the placebo group, 92% of patients had ALT levels $>1.5\times$ ULN and 8% $<1.5\times$ ULN but higher than normal. Ten percent of the patients that received Glycyrrhizic Acid three times per week had normal ALT levels, whereas 27% and 63% of the patients in that group had ALT levels $<1.5\times$ ULN and $>1.5\times$ ULN, respectively. Of the patients receiving 200 mg Glycyrrhizic Acid six times per week, 20% had normal ALT, 27% had ALT levels $<1.5\times$ ULN, and 53% had ALT levels $>1.5\times$ ULN. Serum ALT levels increased in both Glycyrrhizic Acid groups after treatment ended, and by 4 weeks after the end of treatment, the ALT levels in the dosed groups were similar to the placebo group.

The levels of hepatitis C virus RNA after treatment with Glycyrrhizic Acid were similar to those before treatment. Thus, in chronic hepatitis C patients who do not respond to interferon, hepatic function as measured by serum ALT improves with Glycyrrhizic Acid treament, but the virus appears to be unaffected (van Rossum et al. 2001).

Other Antiviral Activities

Pompei et al. (1979) demonstrated that Glycyrrhizic Acid inhibits the growth of several virus types. Human aneuploid HEp2 cells were prepared into monolayers for 24 h and then infected with vaccinia, herpes simplex 1, Newcastle disease, vesicular stomatitis, or polio type 1 for 1 h. Cells were then washed, and 1, 2, 4, or 8 mM Glycyrrhizic Acid or control medium was applied to the cells for 18 h, when the cells were stained for microscopic examination. Glycyrrhizic Acid at 8 mM completely inhibited the growth of and cell damage caused by vaccinia, herpes simplex 1, Newcastle disease, and vesicular stomatitis. The 2 and 4 mM Glycyrrhizic Acid treatments caused concentration dependent reductions in virus load. Glycyrrhizic Acid at 1 mM did not inhibit any virus growth. The growth of polio type 1 virus was unaffected by any of the Glycyrrhizic Acid concentrations tested.

Badam (1997) tested the anti-viral activities of Glycyrrhizic Acid and Ammonium Glycyrrhizate on the Japanese encephalitis virus (JEV). Porcine stable kidney cells in culture were infected with JEV strains Nakayama, P-20778, or 821564 XY48. One hour after infection, the cells were exposed to various (unspecified) concentrations of Glycyrrhizic Acid, licorice root, or Ammonium Glycyrrhizate for 96 h. Infected control cells received no treatment with licorice-related compounds. The cells were then stained with Amido black, rinsed, dried, and the plaques were counted. The experiments were performed in triplicate. Glycyrrhizic Acid at 500 μg/ml completely inhibited plaque formation in all strains, whereas this effect was seen with Ammonium Glycyrrhizate at 1000 μg/ml. No signs of cytotoxicty were observed at these concentrations.

Additional groups of cells infected with the three strains of JEV were exposed to 500, 1000, or 2000 μg/ml Glycyrrhizic Acid for only 2 h. Cells were stained, and plaques were counted. After 2 h of exposure to Glycyrrhizic Acid, only the 2000 μg/ml treatment caused inhibition of plaque formation, and the inhibition was complete. The two lower concentrations had no effect on plaque formation.

Pretreatment of the cells with 1000 μg/ml Glycyrrhizic Acid for 24 h prior to JEV infection did not affect subsequent plaque formation (Badam 1997).

Utsunomiya et al. (1997) infected 8-week-old BALB/c mice with the mouse-adapted Kumamoto strain of influenza virus A_2 (H_2 N_2) by inhalation with a nebulizer. Viral doses were 1, 10, 20, and 100 times the median lethal dose (LD_{50}) of virus, as determined in preliminary tests. Mice received doses of 1.25 to 80 mg/kg Glycyrrhizic Acid i.p. the day before viral infection and 1 and 4 days after infection. Mice receiving 1, 10, and 20 times the viral LD_{50} and 10 mg/kg or higher doses of Glycyrrhizic Acid had greater survival rate (10 to 20 mice/dose; 100% survival), compared to similarly infected control mice given saline (15 mice; 50% survival). Mean survival time in Glycyrrhizic

Acid–treated mice was greater than that of infected control mice. The grade of pulmonary consolidations and the virus titers in lung tissues were less in treated mice than in infected control mice. Glycyrrhizic Acid had no protective impact on the mice given 100 times the LD_{50} of the virus.

In additional experiments, healthy mice were treated with 0 or 10 mg/kg/day Glycyrrhizic Acid i.p. 1, 3, and 5 days before their spleens were removed. The spleens were processed into single-cell suspensions of intact cells. Whole spleen cells (WSCs) or macrophages, T cells, or B cells derived from WSCs were then injected i.v. into recipient mice at 5×10^6 cells per mouse. Two hours later, the mice were infected with 10 times the LD_{50} of influenza virus. Infected mice that had been given WSCs or T cells from Glycyrrhizic Acid–treated donor mice had a 100% survival rate 21 days after infection. Infected mice given B cells or macrophages from Glycyrrhizic Acid–treated donor mice all died by day 15 after infection. When the donor mice had been given saline and no Glycyrrhizic Acid, the infected recipient mice all died by day 15, regardless of the donor cell type. The authors suggested that Glycyrrhizic Acid protected the mice from the virus through a function of T cells, but not through B cells or macrophages (Utsunomiya et al. 1997).

Sekizawa et al. (2001) applied herpes simplex virus type 1 (HSV1) to the abraded corneas of 66 young female SJL mice to infect the mice with herpetic encephalitis. Fifty mice were given i.p. injections of 50 μg/g/day Glycyrrhizic Acid on post-infection days 3, 4, and 5. Fifty control mice received the vehicle physiological saline without Glycyrrhizic Acid. Mice were monitored until post-infection day 14. The survival rates at post-infection day 14 were 81.8% for the mice given Glycyrrhizic Acid and 37.5% in infected control mice. The brains from 26 Glycyrrhizic Acid–treated mice and 25 control mice were removed on post-infection day 6 and processed for a plaque assay to determine propagation of the HSV1 virus in the mouse brains. The brains of mice treated with Glycyrrhizic Acid had 45.6% of the number of plaque forming units (PFU) found in the control mice. Thus, the survivability of HSV1-infected mice was improved with Glycyrrhizic Acid treatment, which also decreased the propagation of the virus in the brain.

Anti-Inflammatory Effects

Tangri et al. (1965) induced arthritis in albino rats by injection of formalin into the ankle joints. Normal and arthritic rats received 0 or 2 mg/100 g/day Glycyrrhetinic Acid i.p. for 10 days. The degree of inflammation was determined by the anteroposterior diameter of the feet. The inflammation was significantly decreased ($p < 0.01$) in the ankles of rats treated with Glycyrrhetinic Acid. Without Glycyrrhetinic Acid treatment, the serum ALT and aspartate aminotransferase (AST) of arthritic rats was 57.4% and 33.2% higher, respectively, than of normal rats. Glycyrrhetinic Acid reduced the serum ALT and AST in arthritic rats by 37.4% and 25.4%, respectively. Glycyrrhetinic Acid did not affect the serum AST or ALT level in normal rats.

Amagaya et al. (1984) found that Glycyrrhetinic Acid (3 to 30 mg/kg p.o.) inhibits edema caused by an injection of λ-carrageenan in the hind feet of mice. Glycyrrhetinic Acid (30 mg/kg p.o.) also reduced the granuloma formation on dorsally implanted cotton pellets in mice. The 18α-isomer of Glycyrrhetinic Acid was more potent in these effects than the 18β-isomer.

Rui (1997) found that Glycyrrhetinic Acid inhibited ear edema induced by croton oil in mice. No other details of this study were given.

Gujral et al. (1961) induced arthritis in bilaterally adrenalectomized albino rats by injecting a fomaldehyde solution into the ankle joints daily for five days. In one experiment, the rats received 250 μg/100 g of deoxycorticosterone acetate (DOCA) subcutaneously, 250 μg/100 g DOCA plus 0.5 mg/100 g hydrocortisone orally, or 250 μg/100 g DOCA plus 20 mg/100 g Glycyrrhizic Acid orally. Doses were administered daily for 10 days after the adrenalectomy surgery. Inflammation of the ankle joint was measured by a micrometer screw gauge to determine the linear cross section of the ankle joint. The DOCA-plus-Glycyrrhizic Acid group showed an anti-arthritic effect by having a significantly smaller ($p < 0.01$) ankle diameter than the other groups.

In a second experiment using the same arthritis model, rats received 0.5 mg/100 g hydrocortisone orally or 20 mg/100 g Glycyrrhizic Acid plus 0.5 mg/100 g hydrocortisone orally. Doses were administered daily for 10 days after the adrenalectomy surgery. Ankle diameter was measured with a micrometer screw gauge as above. The Glycyrrhizic Acid with hydrocortisone group had significantly smaller diameter ankles ($p < 0.005$) than the group that only received hydrocortisone (Gajral et al. 1961).

Ohuchi et al. (1981) found that in rats Glycyrrhizic Acid inhibits prostaglandin E_2 in activated peritoneal macrophages. Sprague-Dawley rats received 5 ml/kg i.p. injections of 5% bacto peptone and 5% soluble starch in solution in order to stimulate macrophage migration. The rats were killed and peritoneal macrophages were isolated and seeded in culture at 6×10^6 cells/60 mm dish and incubated for 2 h. Non-adherent cells were washed away, and the replacement medium contained 0.1, 1, 10, 100, or 1000 μg/ml Glycyrrhizic Acid or Glycyrrhetinic Acid in ethanol for 20 h. Control plates contained only medium with the ethanol vehicle. Sheep red blood cells were added to the medium, and 95% of them had been ingested within 2 h. Prostaglandin E_2 in the medium was determined by radioimmunoassay. Glycyrrhizic Acid and Glycyrrhetinic Acid each concentration dependently inhibited prostaglandin synthesis at 100 and 1000 μg/ml. However, the Glycyrrhetinic Acid caused detachment of 30% of the cells after just 8 h of exposure, which was seen as a cytotoxic effect. In an additional experiment, 3000 μg/ml Glycyrrhizic Acid also inhibited the release of arachidonic acid from macrophages. The authors proposed that the known anti-inflammatory effect of Glycyrrhizic Acid may be partially due to inhibition of prostaglandin E_2.

OTHER CELLULAR EFFECTS

Horigome et al. (2001) found that Glycyrrhetinic Acid caused apoptosis in splenocytes and thymocytes in C57BL/6 mice that had received an i.p. injection of 2.5 mg/animal. Additional in vitro studies indicated that the apoptosis of splenocytes was a result of the inhibition of 11β-hydroxysteroid dehydrogenase by Glycyrrhetinic Acid, which increased the levels of corticosterone.

Glycyrrhizic Acid has also been reported to enhance the production of interleukin-12, interleukin-2, and interferon (Dai et al. 2001; Utsunomiya et al. 2001; Abe et al. 1982; Zhang et al. 1993).

Interactions with Other Chemicals

Hydrocortisone

Teelucksingh et al. (1990) conducted several experiments to study the ability of Glycyrrhetinic Acid to potentiate the activity of hydrocortisone in the skin. Healthy female and male volunteers, aged 21 to 50 years, were treated with test materials on a 7 $\times$ 7-mm site on the flexor side of the forearm. The treatments included 0.1, 0.3, 1.0, 3.0, and 10 mg/ml hydrocortisone acetate, 20 mg/ml Glycyrrhetinic Acid only, and 20 mg/ml Glycyrrhetinic Acid with each of the hydrocortisone concentrations listed above (four to six subjects per treatment). A positive-control group received 0.1, 0.3, 1.0, 3.0, or 10 mg/ml beclomethasone diproprionate. Solutions were prepared in 95% ethanol and made within 24 h before application. After the applied solution had dried, the test area was covered with polyester film for 16 to 18 h. The dose sites were scored for degree of blanching 1, 2, 3, and 6 h after removal of the occlusive dressing. Scoring was performed by observers unaware of the doses applied, and the scale was 0 = none, 1 = mild, 2 = definite, and 3 = intense blanching.

The degree of blanching was presumed to be associated with cutaneous vasoconstriction. Hydrocortisone acetate alone and Glycyrrhetinic Acid alone each had no effect on degree of blanching (vasoconstriction). However, hydrocortisone and Glycyrrhetinic Acid combined produced significant dose-dependent increases ($p < 0.01$–0.001) in the degree of blanching of the skin at the application site, compared to the vehicle control 95% ethanol. Beclomethasone diproprionate, the positive control, produced the expected dose-dependent increase in degree of blanching. The authors concluded that Glycyrrhetinic Acid appeared to potentiate the activity of hydrocortisone in the skin.

Dorsal skin from freshly killed nude mice was removed, homogenized, and proteins were separated and measured. The proteins on suspension were diluted to match the protein content per weight in the skin. The protein suspension was incubated in Krebs-Ringer with 12 nmol/L tritiated corticosterone (82 Ci/mmol) added. This preparation was treated with 0, 0.01, 1.0, or 100 μmol/L Glycyrrhetinic Acid (duration of treatment was not reported). After centrifugation, corticosteroids were extracted from the supernatant, and ^{3}H-11-dehydrocorticosterone was separated by thin-layer chromatography. The percent of corticosterone converted into ^{3}H-11-dehydrocorticosterone was determined.

Glycyrrhetinic Acid significantly inhibited the production of ^{3}H-11-dehydrocorticosterone in a concentration dependent manner ($p < 0.0001$–0.05), thus verifying the inhibition of 11β-OHSD in the skin by Glycyrrhetinic Acid.

Samples of normal, psoriatic, and eczematous human skin were collected from patients. In immunolabeling assays, 11β-OHSD was found in the epidermis with the exception of the basal layer. Distribution of 11β-OHSD was similar in all three skin types, but the activity of the enzyme seemed higher in the psoriatic and eczamatous skin samples.

Given the results of these studies, the authors suggested that by inhibiting the metabolism of hydrocortisone by 11β-OHSD in the dermis, Glycyrrhetinic Acid allowed greater access of hydrocortisone to glucocorticoid receptors (Teelucksingh et al. 1990).

In a commentary in response to the Teelucksingh et al. (1990) study described above, Greaves (1990) suggested that the potentiation of hydrocortisone by Glycyrrhetinic Acid could be due to the ability of Glycyrrhetinic Acid to increase the percutaneous absorption of hydrocortisone.

Diclofenac Sodium

Nokhodchi (2002) applied formulations of 1% diclofenac sodium (nonsteroidal anti-inflammatory) with or without 0.1% to 0.5% Glycyrrhizic Acid to excised abdominal rat skin in Franz-type diffusion cells. Flux of diclofenac sodium into the receptor compartment was measured. The formulation containing 0.1% Glycyrrhizic Acid produced a 10-fold increase in the flux of diclofenac sodium across the rat skin. The formulation containing 0.5% Glycyrrhizic Acid increased the flux of diclofenac sodium by twofold. The author concluded that Glycyrrhizic Acid enhanced the absorption of diclofenac sodium across rat skin.

ANIMAL TOXICOLOGY

Acute Toxicity

Glycyrrhetinic Acid

The median acute lethal dose (LD_{50}) of intraperitoneal (i.p.) injection of Glycyrrhetinic Acid in mice is 308 mg/kg (Informatics, Inc. 1972).

Finney et al. (1958) found that no albino mice died after acute oral or subcutaneous doses of up to 610 mg/kg Glycyrrhetinic Acid. The LD_{50} of Glycyrrhetinic Acid via intraperitoneal route was 308 mg/kg. Of the animals that died, most did so on the second day after dose administration.

Cognis (2002) reported an oral LD_{50} of 610 mg/kg for Glycyrrhetinic Acid in rats.

Ammonium Glycyrrhizate

The LD_{50} of crude Ammonium Glycyrrhizate in mice via oral (p.o.) and i.p. routes are 12,700 and 1050 mg/kg, respectively.

The i.p. LD_{50} of Monoammonium Glycyrrhizate in mice is 1070 mg/kg. Diammonium Glycyrrhizate has p.o. and i.p. LD_{50} values in mice of 9600 and 1250 mg/kg, respectively (Informatics, Inc. 1972).

Food and Drug Research Laboratories (FDRL) conducted acute oral toxicity studies of Ammonium Glycyrrhizate in several species. The LD_{50} values in rats, mice, hamsters, and rabbits were 7.1, 8.6, 8.8 and 10.0 g/kg, respectively (FDRL 1971a).

Inverni della Beffa (1970) studied the acute oral toxicity of Ammonium Glycyrrhizate (also known as Glycamil) in the mouse, rat, and guinea pig. The range of doses used was not reported, but the LD_{50} values for rats and mice were each >5000 mg/kg. The LD_{50} for guinea pigs was >3000 mg/kg.

Fujimura (no date) reported an LD_{50} of 1050 mg/kg for crude Ammonium Glycyrrhizate i.p. in mice. The oral LD_{50} was 12,700 mg/kg. The LD_{50} of refined Ammonium Glycyrrhizate was 1070 mg/kg i.p. The oral LD_{50} of refined Ammonium Glycyrrhizate was >10,000 mg/kg.

Potassium Glycyrrhizinate

The LD_{50} values of Potassium Glycyrrhizinate via p.o., subcutaneous (s.c.), intravenous (i.v.), and intramuscular (i.m.) routes in mice are 1220, 697, 412, and 695 mg/kg, respectively (Informatics, Inc. 1972).

Fujimura (no date) reported an LD_{50} of 1260 mg/kg for crude Potassium Glycyrrhizinate i.p. in mice. The oral LD_{50} was 12,400 mg/kg.

Short-Term Toxicity

Finney et al. (1958) administered intramuscular injections of 10 or 20 mg/kg Glycyrrhetinic Acid to young rats three times a week for four weeks. Rats were killed for examination at the end of the dosing period. There were no clinical effects observed while the animals were alive. At necropsy all tissues were normal except for a slight thinning of the lipid in the zona glomerulosa of the adrenal glands.

Inverni della Beffa (1970) treated eight Wistar rats with 700 mg/kg/day Ammonium Glycyrrizate via oral gavage for 8 weeks. The author described this dose as 150 times greater than the dose used in man as a sweetening agent with respect to body weight. Eight non-treated rats served as a control group.

All animals survived to the end of the treatment period, and there were no indications of intoxication. Weight gain of treated animals was similar to controls. At the end of treatment, examinations of glycemia, hepatic function, diuresis, and urinary excretion of Na^+, K^+, and Cl^- in treated rats were similar to those of control rats. Systolic blood pressure measured in the tail was similar between treated and untreated rats at weeks 4 and 8 of treatment. Hematology and blood chemistry parameters were similar between the control rats and those treated with 700 mg/kg/day.

There were no appreciable differences between treated and control rats in the histological examination of the heart, liver, kidneys, adrenal glands, spleen, and gonads. Of these organs only the liver had a reduced organ weight in treated rats. In this study, reduced liver weight was the only clinical effect noted after 8 weeks of oral treatment with 700 mg/kg/day Ammonium Glycyrrizate (Inverni della Beffa 1970).

In an unpublished report described by Informatics, Inc. (1972), Fujimura and Okamoto fed Dipotassium Glycyrrhizate or Diammonium Glycyrrhizinate to Wistar rats for 90 days. Doses were 0%, 0.1%, and 0.5% test material mixed in feed ($n = 5$ rats/sex/dose). After the 90-day exposure period, the rats were killed and examined. Male rats in the 0.5% groups for each test material had smaller increases in body weight than control rats; females were not affected. No other findings were reported.

Subchronic Toxicity

Inverni della Beffa (1970) tested male mice with 0, 18, or 90 mg/kg/day Ammonium Glycyrrhizate by oral gavage six days a week for 16 weeks ($n = 10$ mice per group). All mice survived the dosing period, and there were no clinical signs of toxicity. The body weight gain of treated mice was similar to control mice. There were no remarkable observations during necropsy examinations.

Rats were exposed using the same protocol. All rats survived the dosing period, and there were no clinical signs of toxicity.

Male rats of the 90 mg/kg/day group had reduced body weights at weeks 9, 10, and 11, but after this period, body weights for all groups were not statistically significant. Female body weights were similar for all groups. Hepatic function, as determined by the measurable degradation of a 50 mg/kg i.v. injection of bromsulphalein, was similar between all groups. There were no treatment-related effects on glycemia or blood pressure. By urinalysis males of the 90 mg/kg/day Ammonium Glycyrrhizate group had increased Na^+ and Cl^- excretion and diuresis at week 8 but not at week 16. Males of the lower dose had diuresis only at week 8 but not at week 16. Female rats had a decrease in Cl^- excretion in the urine at week 16 only. No groups had a difference in renal excretion of K^+.

Analysis of the blood showed a decrease in lymphocytes and increases in neutrophilic granulocytes in males of the higher dose group at week 8, and at week 16 there was a decrease in the total white blood cell count. Female rats of the higher dose had decreased hemoglobin and eosinophilic granulocytes at week 16. Females of the lower dose group had increased hemoglobin and lymphocytes and decreased neutrophylis at week 8 and decreased eosinophilis at week 16.

At necropsy, histological and gross examinations and weights of the major organs did not differ between treatment and control groups (Inverni della Beffa 1970).

Chronic Toxicity

Monlux (1974) fed Ammonium Glycyrrhizate to Osborn-Mendel rats for 2 years. All rats were F_1 offspring of rats in a subacute Ammonium Glycyrrhizate feeding study in which

the F_0 generation were exposed to concentrations of Ammonium Glycyrrhizate (0%, 0.5%, 1.0%, 2.0%, and 4.0%) mixed in powdered rat diet during breeding. No results were available for the F_0 generation. The F_1 rats were fed the same dose as their respective parents' group or control diet for 102 weeks ($n = 10$ rats/group). At the end of the treatment period, all rats were killed and necropsied. Tissues were fixed in formalin and examined microscopically for lesions. The feeding of Ammonium Glycyrrhizate over a period of 2 years did not produce any lesions or anatomic alterations in this study.

Dermal Irritation and Sensitization

Finney et al. (1958) applied cotton-wool pellets saturated with 0.5 ml of a suspension of 100 mg/ml Glycyrrhetinic Acid to the shaved back of albino rabbits. Three pellets were fixed with an adhesive plaster and the trunk of the rabbit was wrapped in a plastic film for 24 h. Another rabbit was similarly treated, except the three exposure sites were abraded before the pellets were applied. There was no evidence of edema or erythema on the intact or abraded exposure sites upon removal of the pellets or 2 days later. Glycyrrhetinic Acid had no apparent primary irritant activity on the skin of the albino rabbit.

Ocular Irritation

Cognis (2002) reported that 6% Glycyrrhetinic Acid in water was classified as "slightly irritating" in an in vitro test using the chorioallantoic membrane of a chicken embryo in hen's egg (HET-CAM) assay. In the assay, 0.3 ml of the test material was applied to the test system (details of the assay were not provided). The irritation potential was evaluated as a function of reaction time and reaction intensity of effects such as hemorrhage, lysis of vessels, and protein coagulation. No reaction to the test material was observed.

GENOTOXICITY

Wang et al. (1991) showed that 0.025, 0.1, and 0.5 μg/plate18β-Glycyrrhetinic Acid concentration-dependently inhibited the mutagenic activities of 10 μg/plate benzo[a]-pyrene, 20 μg/plate 2-aminoflourene, and 0.5 μg/plate aflatoxin B_1 in *Salmonella typhimurium* strains T98 and T100. 18α-Glycyrrhetinic Acid also inhibited the mutagenicity of these compounds but was less potent, as the only effective concentration was 0.5 μg/plate.

Litton Bionetics, Inc. (1972) investigated the mutagenicity of Ammonium Glycyrrhizate, using the host-mediated assay, in vivo and in vitro cytogenetics studies, and the dominant lethal assay. In the host-mediated assay, ICR male rats received Ammonium Glycyrrhizate (dose not specified), which was considered a possible mutagen at the dose levels used in the host-mediated assay. Oral doses of up to 5000 mg/kg Ammonium Glycyrrhizate produced no detectable significant aberration of the bone marrow metaphase chromosomes of rats. Ammonium Glycyrrhizate did not produce significant aberration in the anaphase chromosomes of human tissue culture cells when tested at concentrations up to 1000 μg/ml. Ammonium Glycyrrhizate was considered to be non-mutagenic in rats in the dominant lethal assay at oral doses of up to 5000 mg/kg.

Green (1977) reported that Ammonium Glycyrrhizate was not mutagenic in screening assays in human WI-38 embryonic lung cells, *Saccharomyces cerevisiae* strain D-3, and in the *Salmonella typhimurium* strains G46 and TA1530.

Stanford Research Institute (SRI) evaluated the mutagenic potential of Ammonium Glycyrrhizate with the dominant lethal assay in rats (SRI 1977). Male Sprague-Dawley rats were given 0, 4000, 13,333, or 40,000 ppm Ammonium Glycyrrhizate mixed in the diet for 10 weeks ($n = 20$ male rats/group). After the dosing period, each male was then caged with two virgin females for 7 days for a first mating period, and then the 2nd week with a different pair of virgin females for the second mating. Fourteen days after the midweek of mating, each female was killed and the uteri were removed for fetal examinations.

The male rats of the 40,000 ppm group had significantly reduced ($p < .01$) body weight gain over the 10-week dosing period. The two lower doses of Ammonium Glycyrrhizate did not cause consistent mutagenic effects in the fetuses. Offspring of the 40,000 ppm group had significantly higher ($p < 0.05$) number of dead implants and dead implants/total implants. The authors stated that Ammonium Glycyrrhizate may be a mutagen in the rat at 40,000 ppm (2000 mg/kg/day) in the diet (SRI 1977).

Generoso et al. (1983) evaluated the mutagenic potential of Ammonium Glycyrrhizate in male germ cells, using a dominant lethal assay and a heritable translocation test. In the heritable translocation test, 100 male (101 $\times$ CH3)F_1 mice were each caged with three female (SEC $\times$ C57BL)F_1 mice for 1 week prior to exposure to Ammonium Glycyrrhizate. Then the male mice were separated from the females and fed powdered feed containing 0 or 2.25% Ammonium Glycyrrhizate for 8 weeks ($n = 50$ treated and 50 control). After the exposure period, the males were caged with the same females for 1 week. Male offspring from the two mating periods (before and after exposure) were mated to hybrid stock (SEC $\times$ C57BL)F_1 females. The outcome of these pregnancies were used to measure any inherited effects of Ammonium Glycyrrhizate on male fertility.

Based on data from 475 experimental and 507 control male progeny tested, the authors determined that exposure to Ammonium Glycyrrhizate in this study did not produce any heritable effects on male fertility.

In the dominant lethal assay, male mice were fed powdered feed containing 0% or 2.25% Ammonium Glycyrrhizate for 8 weeks ($n = 36$ treated and 36 control). Beginning immediately after the treatment period each male was caged with one (C3H $\times$ C57BL)F_1 and one (SEC $\times$ C57BL)F_1 virgin females. Females were examined daily for the presence of vaginal plugs for 1 week. Mated females were replaced with new virgins. All matings occurred $1/2$ to $7 1/2$ days after the end of treatment. Pregnant mice were killed 12 to 15 days after appearance of the vaginal plugs. The uteri were examined to determine frequency of pre- and

post-implantation loss. In this study, Ammonium Glycyrrhizate did not induce any dominant lethal effects (Generoso et al. 1983).

Sheu et al. (1986) performed dominant lethal assays of Ammonium Glycyrrhizate in rats and mice and a heritable translocation test in mice. Male rats were fed diet containing 0.4%, 1.3%, or 4.0% Ammonium Glycyrrhizate or corn oil for 10 weeks (n = 20 rats/group). The males were mated with virgin females in the 1st and 2nd weeks after treatment ended. Male fertility was not affected by treatment. However, analysis of the fetuses at day 14 of gestation resulted in a statistically significant ($p < 0.05$) increase in dead implants in females mated to males treated with 4.0% Ammonium Glycyrrhizate. The authors stated that the biological significance of this result is unknown.

Male mice were fed diet containing 2.0% or 2.5% Ammonium Glycyrrhizate or corn oil for 8 weeks (n = 36 mice per group). Immediately after the treatment period, males were mated with virgin females. Females were killed for uterine analysis on gestation days 12 to 15. There was no evidence of infertility or dominant lethal effect in mice.

In the heritable translocation test, groups of 50 to 75 untreated male mice were each caged with three females for 1 week. The females were then caged individually through gestation, whereas the males were fed diet containing 2.25% Ammonium Glycyrrhizate or 2% corn oil (n = 50/group). At the end of the treatment period, the males were paired with the same females for another week of mating. There was no reduction in litter size after treatment with Ammonium Glycyrrhizate. There was no evidence of heritable genetic toxicity in this test (Sheu et al. 1986).

Sasaki et al. (1980) evaluated the in vitro mutagenic potential of Trisodium Glycyrrhizate in Chinese hamster Don-6 cells and in human HE-2144 fibroblastic cells. Treatment of the cells with 0.49 to 2.46 mg/ml Trisodium Glycyrrhizate in the nutrient medium was nonmutagenic.

CARCINOGENICITY

Kobuke et al. (1985) studied the tumorigenicity of Disodium Glycyrrhizate in B6C3F$_1$ mice. Male mice received drinking water containing 0.04%, 0.08%, or 0.15% Disodium Glycyrrhizate (n = 50 to 70 mice per group). The respective Disodium Glycyrrhizate exposure levels in the three dose groups were 2.5, 5.8, and 8.0 mg/kg/day. Female mice received drinking water containing 0.08%, 0.15%, or 0.3% Disodium Glycyrrhizate (n = 50 mice per group). Female exposure levels were 3.5, 6.5, and 12.2 mg/kg/day, respectively. Control groups of 60 male and 50 female mice received distilled drinking water only. The exposure period was 96 weeks, and treated drinking water was replenished every 3 days. After the 96-week treatment period, surviving mice received distilled drinking water without Disodium Glycyrrhizate. Animals that died during the study and those that were killed at the end of the study were examined. Any tumors found were weighed and described. There was no difference between treated and control groups in the tumor incidence, in the latency period before tumor appearance, or in the types of tumors found. The authors suggested that this study provided no evidence that chronic exposure to Disodium Glycyrrhizate was tumorigenic.

ANTICANCER AND TUMOR SUPPRESSION

Wang et al. (1991) reported tht Glycyrrhetinic Acid also inhibited the tumor initiation and tumor promotion by 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) in mice. Shaved female SENCAR mice were treated with 10 μmol 18α- or 18β-Glycyrrhetinic Acid in dimethyl sulfoxide (DMSO) or 0.2 ml DMSO vehicle daily for seven days. One hour after the last treatment, 40 nmol DMBA was applied to the same site on all mice. Seven days later, 4 nmol TPA was applied to the same site, and this treatment was continued daily for 16 weeks. In two additional groups, mice were treated with DMSO, DMBA, and TPA as described above, but they were given 10 μmol 18α- or 18β-Glycyrrhetinic Acid topically 30 min before each TPA treatment. Each combination of treatments was performed on 20 mice. Tumors larger than 1 mm that persisted for 2 weeks were counted.

Mice treated with 18α- or 18β-Glycyrrhetinic Acid prior to the tumor initiator DMBA had 20% or 50% fewer tumors, respectively, than control mice. Likewise, mice given 18α- or 18β-Glycyrrhetinic Acid prior to the tumor promotor TPA had 80% and 60% fewer tumors, respectively, than control mice. The authors concluded that topical application of either stereoisomer of Glycyrrhetinic Acid inhibited the binding of radiolabeled DMBA and benzo[a]pyrene (B[a]P) to epidermal DNA. 18α- or 18β-Glycyrrhetinic Acid also inhibited the TPA-induced increases in ornithine decarboxylase activity and lipoxygenase activity in vitro (Wang et al. 1991).

Other studies by Nishino et al. (1984, 1986) and Takasaki et al. (1995) demonstrated that Glycyrrhetinic Acid inhibits the tumor-promoting activity of TPA and teleocidin.

Kitagawa et al. (1986) found that Glycyrrhetinic Acid dose-dependently inhibited the binding of TPA to the TPA receptor in mouse skin. Kinetic analysis suggested that Glycyrrhetinic Acid competitively binds directly to the TPA receptor with an inhibition constant (K_i) of 2.2×10^{-4} M. The concentration required to inhibit 50% of TPA binding was around 500 μM Glycyrrhetinic Acid. The authors proposed that this competitive binding of Glycyrrhetinic Acid to the TPA receptor may play a role in the anti–tumor-promoting effect of Glycyrrhetinic Acid. Glycyrrhizic Acid did not show any binding affinity to the TPA receptor.

Inoue et al. (1989) found that pretreatment of a mouse ear with 1 mg/ear Glycyrrhetinic Acid inhibited TPA-induced ear edema by 81%. When 1 mg/ear Glycyrrhetinic Acid was applied after 2 μg TPA, the ear edema was reduced by 23%, compared to mouse ears that received TPA only.

Rossi et al. (1995) transplanted Erlich ascites tumor cells into healthy female Swiss mice. The mice also received oral doses of 60 mg/kg/day Glycyrrhizic Acid or 30 mg/kg/day Glycyrrhetinic Acid for 10 days before infection, 10 days after infection, or for 10 days before and 10 days after infection with the tumor cells. Control animals received the tumor infection without Glycyrrhizic Acid or Glycyrrhetinic Acid. The rate of mouse deaths was used to determine the extent of protection provided by the test materials. Pretreatment or post-treatment with either test material did not protect the mice from the lethal tumors. However, the combination of pretreatment and post-treatment of Glycyrrhizic Acid delayed the 50% mortality rate from days 15 to 18–19. Glycyrrhetinic Acid on any dose schedule tested did not affect the mortality rate.

Kelloff et al. (1994) and Wang and Nixon (2001), in review articles, state that Glycyrrhizic Acid and related compounds have been used as an herbal remedy to prevent and to treat cancer for centuries and that several animal and in vitro studies suggest that these compounds have antimutagenic, tumor-suppressive, and anticarcinogenic properties.

Agarwal et al. (1991) treated SENCAR mice with 0% or 0.05% Glycyrrhizic Acid in drinking water for 50 days. The mice were then shaved and 40 nmol DMBA was applied to the bare skin. Twenty control group and 20 Glycyrrhizic Acid—treated animals were given untreated drinking water for the remainder of the experiment. Twenty Glycyrrhizic Acid–treated mice continued to receive drinking water with 0.05% Glycyrrhizic Acid. Seven days after the DMBA application, all mice were treated with a topical dose of 4 nmol TPA twice weekly for 16 weeks. Tumors were counted.

The two Glycyrrhizic Acid groups had similar outcomes in the number of tumors produced, which was significantly fewer ($p < 0.05$) than incidence of tumors in control mice. The latent period prior to the onset of tumor appearance was later in the Glycyrrhizic Acid–treated mice than in the control mice. The authors suggested that Glycyrrhizic Acid inhibited the tumor initiation by DMBA, but not by TPA (Agarwal et al. 1991).

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

FDRL (1971b) studied the developmental toxicity potential of Ammonium Glycyrrhizate in four species of mammals. Pregnant CD-1 outbred mice received 0, 27, 90, 300, or 1000 mg/kg/day Ammonium Glycyrrhizate by oral gavage on gestation days 6 through 15 ($n = 19$ to 21 pregnant dams/dose group). The fetuses were removed by caesarian section on gestation day 17 and examined. There was no effect on maternal or fetal survival and no remarkable observation upon visceral and skeletal examinations of the fetuses.

Pregnant Wistar rats received 0, 27, 90, 300, or 1000 mg/kg/day Ammonium Glycyrrhizate by oral gavage on gestation days 6 through 15 ($n = 21$ to 22 pregnant dams/dose group). The fetuses were removed by caesarian section on gestation day 20 and examined. There was no effect on maternal or fetal survival and no remarkable observation upon visceral and skeletal examinations of the fetuses.

Pregnant golden hamsters received 0, 27, 90, 300, or 1000 mg/kg/day Ammonium Glycyrrhizate by oral gavage on gestation days 6 through 10 ($n = 21$ to 23 pregnant dams/dose group). The fetuses were removed by caesarian section on gestation day 15 and examined. There was no effect on maternal or fetal survival. Aside from dose-dependent delayed cranial ossification, no remarkable observation upon visceral and skeletal examinations of the fetuses was noted.

Pregnant Dutch-belted rabbits received 0, 27, 90, 300, or 1000 mg/kg/day Ammonium Glycyrrhizate by oral gavage on gestation days 6 through 18 ($n = 10$ to 12 pregnant dams/dose group). The fetuses were removed by caesarian section on gestation day 29 and examined. There was no effect on maternal or fetal survival and no remarkable observation upon visceral and skeletal examinations of the fetuses (FDRL 1971b).

In the previously mentioned Monlux (1974) study where rats were fed Glycyrrhizate, exposure to up to 4% Ammonium Glycyrrhizate in utero did not cause any observed developmental defects.

Mantovani et al. (1988) studied the teratogenicity of Ammonium Glycyrrhizate in Sprague-Dawley rats. Pregnant rats received drinking water containing 0, 10, 100, or 250 mg/ml Ammonium Glycyrrhizate on gestation days 7 through 17. The actual exposure levels, based on water consumption calculations, were 0, 21.33, 238.8, and 679.9 mg/kg/day, respectively. Dams were killed on gestation day 20. Dams and fetuses were examined. Water consumption was increased in dams of the 238.8 and 679.9 mg/kg/day groups. Fetuses exposed to Ammonium Glycyrrhizate did not have changes in the rate of external malformations, body weight, or degree of ossification, compared to controls. The incidence of external hemorrhages was increased in fetuses of the low- (21.33 mg/kg/day) and high- (679.9 mg/kg/day) dose groups ($p < 0.01$), but not in the middle- (238.8 mg/kg/day) dose group. Renal variants and ectopic kidneys were observed in fetuses of the lowest-dose group ($p < 0.05$). High-dose fetuses also had ectopic kidneys. Skeletal examinations revealed a dose-dependent increase in sternebral variants, significant ($p < 0.01$) in the middle- and high-dose groups. The lowest observed effect level in this study was 21.33 mg/kg/day.

Itami et al. (1985) gave pregnant Wistar rats 0%, 0.08%, 0.4%, or 2% Disodium Glycyrrhizate mixed in feed on gestation days 0 through 20 ($n = 8$ to 11 pregnant dams/dose group). There was no treatment-related effect on any of the following parameters: number of corpora lutea, number of implants, number of live fetuses, number of intrauterine dead fetuses per litter, sex ratio, fetal body weights, placental weights, degree of ossification, live birth index, survival rate, and pup body weight gain up to 8 weeks postpartum. The authors concluded that Disodium Glycyrrhizate was not teratogenic in rats under the treatment conditions of this study.

NEUROTOXICITY

Finney et al. (1958) found that 1250 mg/kg Glycyrrhetinic Acid given intraperitoneally to mice caused sedation, hypnosis, hypothermia, and respiratory depression. Glycyrrhetinic Acid given i.p. (1250 mg/kg) or subcutaneously (625 mg/kg) did not stimulate or depress either the sympathetic or parasympathetic branches of the autonomic nervous system of mice. A cat given 125 mg/kg Glycyrrhetinic Acid i.p. did not exhibit any changes in blood pressure and had normal responses to stimulation of sympathetic and parasympathetic nerves.

Sobotka et al. (1981) studied the neurobehavioral toxicity of Ammoniated Glycyrrhizin (Ammonium Glycyrrhizate) in male Sprague-Dawley rats. The rats were fed powdered diet containing 0%, 2%, 3%, or 4% Ammonium Glycyrrhizate for 4 to 6 months ($n = 40$ rats per dose group). Based on food consumption rates, the actual exposure levels were 0, 1.2, 1.9, and 2.6 mg/kg/day, respectively. Parameters evaluated included physiology (blood pressure, heart rate, temperature), motor functions (open-field exploration, Rotorod performance, general motility), and cognitive abilities (passive avoidance, active avoidance, and operant conditioning with incremental shock and incremental fixed-interval food reinforcement).

Hypertension (4% group), increased relative kidney weights (all treatment groups), decreased body weights and weight gain (4% group), bradycardia (4% group), and polydipsia (excessive thirst; 3% and 4% groups) were observed. Rectal temperatures were not affected by Ammonium Glycyrrhizate treatment. There were no treatment-related effects on motor function tests. Performance on active avoidance tests was facilitated in the 4% group, unaffected in the 3% group, and depressed in the 2% group. The authors suggested that the observations in this study are consistent with the reported hormonal effects of Ammonium Glycyrrhizate (Sobotka et al. 1981).

CLINICAL ASSESSMENT OF SAFETY

Clinical Studies

Mori et al. (1989) administered 200 to 800 mg/day Glycyrrhizic Acid to nine human immunodeficiency virus (HIV) patients (asymptomatic carrier) with hemophilia for 8 weeks. Lymphocyte counts increased in all nine patients. OKT4-positive lymphocyte numbers increased in eight patients, and the ratio of OKT4 to OKT8 cells increased in six patients. Four patients who previously had signs of liver dysfunction had clear improvements in hepatic function parameters such as serum GOT and GPT. Serum electrolytes, lipids, and protein, along with kidney function, were not affected by the Glycyrrhizic Acid.

van Gelderen et al. (2000) conducted a study using human subjects in the Netherlands to determine a no-effect level for Glycyrrhizic Acid. Thirty-nine healthy female volunteers aged 19 to 40 years and weighing 60 to 70 kg were selected after medical histories, physical examinations, and blood and urine tests. The volunteers were divided into four Glycyrrhizic Acid dose groups based on a randomized double blind treatment scheme: 0 mg/kg control ($n = 10$), 1 mg/kg/day ($n = 9$), 2 mg/kg/day ($n = 9$), and 4 mg/kg/day ($n = 11$). Glycyrrhizic Acid doses were administered orally in capsules. The experiment lasted 12 weeks: initial 2 weeks without treatment, 8 weeks of dosing with Glycyrrhizic Acid, and 2 weeks of post-treatment observations. During the 12-week period, all volunteers were required to abstain from smoking, using drugs, or using products containing Glycyrrhizic Acid. Parameters included physical examinations (at beginning and end of experimental period), questionnaire on physical condition (daily), body weight (weekly), blood pressure (weekly), edema scoring (weekly), blood potassium (weekly), blood plasma renin and aldosterone (every 2 weeks), dietary questionnaire (every 2 weeks), and blood atrial natriuretic peptide (ANP) (at weeks 8 and 10).

The aldosterone concentration and renin activity in the serum of the 4 mg/kg/day group were significantly lower ($p < 0.001$) than control after 2, 4, 6, and 8 weeks of dosing. Aldosterone levels and renin activities in the 1 and 2 mg/kg/day groups were similar to controls. The concentration of ANP was significantly decreased ($p < 0.001$) in the 4 mg/kg/day group, but not in the 1 and 2 mg/kg/day groups. Systolic and diastolic blood pressure measurements remained the same throughout the study in the 2 and 4 mg/kg/day groups but decreased slightly in the control group. Thus, relative to the control group, the 4 mg/kg/day group had significantly higher ($p = 0.018$) blood pressure than the control group. Body weight was similar between all treatment groups. Volunteers in the 4 mg/kg/day group had a significantly lower ($p < 0.01$) blood potassium concentration than those in the control group at 2 and 4 weeks into the dosing period.

In daily questionnaires of physical conditions, volunteers in the 4 mg/kg/day group described headache, nausea, vomiting, change in defecating pattern, swollen face, and tickling in the arms and legs. However, the number of complaints decreased as the study progressed.

The authors determined that the no effect level of Glycyrrhizic Acid in this study was 2 mg/kg/day. They proposed an acceptable daily intake of 0.2 mg/kg/day, using a safety factor of 10. This corresponds to 6 g of licorice per day would be safe for a 60-kg person, assuming that licorice contains 0.2% Glycyrrhizic Acid. They noted that the Dutch Nutritional Council advises a limit of 200 mg Glycyrrhizic Acid per day (van Gelderen et al. 2000).

Sigurjónsdóttir et al. (2001) demonstrated a linear dose-response relationship between Glycyrrhizic Acid and increases in blood pressure. Healthy Swedish and Icelandic volunteers aged 23 to 37 years ingested licorice amounting to 75, 270, or 540 mg/day Glycyrrhizic Acid for 2 to 4 weeks ($n = 24$, 30, or 10/dose group, respectively). After 2 weeks of dosing, mean systolic blood pressure rose 3.1, 5.2, and 14.4 mm Hg for the low-, middle-, and high-dose groups, respectively, compared to blood pressure measured prior to treatments. All rises in systolic blood pressure were statistically significant at $p$values of .03 to .000 [sic]. Increases in blood pressure were of similar magnitude after four weeks of dosing. Thus, a dose-response was demonstrated,

but a time-response was not observed. There did not seem to be a special demographic group that was especially sensitive to the effects of licorice or Glycyrrhizic Acid.

Louis and Conn (1956) administered seven normal human subjects 6 g/day Ammonium Glycyrrhizate for 3 days. Subjects maintained normal diets during the experiment. During the exposure period, renal excretion of Na^+ and Cl^- was significantly decreased, K^+ excretion was slightly increased, and water retention caused a 1.5- to 2-lb increase in body weight. Treatment also caused a reduction in excretion of Na^+ and Cl^- in thermal sweat. Withdrawal of the Ammonium Glycyrrhizate caused an immediate above-normal increase in renal excretion of Na^+ and Cl^- and retention of K^+. Body weights returned to normal five to seven days after withdrawal of Ammonium Glycyrrhizate treatment. Renal excretion of 17-ketosteroids was decreased during the exposure period, which the authors attributed to inhibited production of ACTH. Parameters of carbohydrate metabolism and protein metabolism were not affected by Ammonium Glycyrrhizate exposure.

Dermal Irritation and Sensitization

Universita' Delgi Studi Di Urbino (1990) conducted a human dermal sensitization study of an ointment containing 3% Glycyrrhetinic Acid. The subjects in this study were six male and nine female volunteers, ages 22 to 41. A 4 cm^2 occlusive pad with an unspecified amount of the ointment was applied to the skin of the arm of each volunteer. The pad remained in place for 3 days. The treatment site was scored following removal of the pad. After 7 days without treatment, the procedure was repeated, and the treatment site was examined after the second 3-day exposure. All 15 subjects had scores of "no reaction" after each treatment. The researchers concluded that the ointment (3% Glycyrrhetinic Acid) did not produce irritation or sensitization in human subjects.

Hilltop Research Inc. (1994) conducted a repeated insult patch test of a moisturizer product containing 0.3% Glycyrrhetinic Acid on 10 male and 98 female human subjects. In the induction phase, 0.15 g of the product was applied to a 2 $\times$ 2-cm area of the lateral surface of the upper arm, under an occlusive patch. Details of the protocol were not reported, but there were nine induction applications, an unspecified rest period, and a challenge patch. None of the 108 subjects had a response during the challenge phase. The authors concluded that, under the conditions of this study, there was no evidence that the test material induced a delayed contact hypersensitivity response.

Consumer Product Testing Company (2002) conducted a repeated insult patch test of Glycyrrhetinic Acid on 112 human subjects. Subjects included males and females, ranging in age from 16 to 79 years. One hundred six subjects completed the study. Those subjects who did not complete the study discontinued for reasons not related to the test material. In the induction phase, 0.2 ml of a formulation of 6% Glycyrrhetinic Acid in glycerine was applied to a 1 $\times$ 1-inch area of the scapular region under a gauze occlusive patch. Patches were applied three times per week for 3 weeks for a total of nine applications. Treatment sites were evaluated for signs of irritation 24 h after each application.

After a 2-week rest period, a challenge patch was applied to an untreated site, following the same formulation and application procedure performed in the induction phase. The challenge site was evaluated 24 and 72 h after application. None of the subjects had any sign of erythema or edema during the induction or challenge phase in this study. The authors concluded that 6% Glycyrrhetinic Acid in glycerine did not indicate a potential for dermal irritation or allergic contact sensitization (Consumer Product Testing Company 2002).

Allergisa Pesquisa Dermato-Cosmética Ltd. (2004a) conducted a human dermal sensitization study of 0.6% Glycyrrhetic Acid (also known as Glycyrrhetinic Acid). The subjects in this study were 51 females and 5 males between the ages of 18 and 66. Samples of 0.05 g/cm^2 were spread on filter paper and attached to the right or left part of the back then covered with semi-permeable hypoallergenic tape. Applications were conducted every other day, totaling 15 applications with each patch being removed after 24 h. The same was done with the saline control. After a 14-day rest period, two sample patches were applied in a fresh area. These patches were removed after 48 h of skin contact. These were scored for reaction after 30 min and 24 h. No adverse reactions were detected in the sites where the product was applied.

Allergisa Pesquisa Dermato-Cosmética Ltd. (2004b) conducted a human dermal irritation study of 0.6% Glycyhrrhetinic Acid. The subjects in this study were 52 females and 4 males between the ages of 18 and 62. Samples of 0.05 g/cm^2 were spread onto filter paper and attached to the back then covered with semipermeable hypoallergenic tape. The same was done with a control of saline. The patches were removed after 48 h and reactions were recorded after 30 min and 24 h of removal. No adverse reactions were detected in the sites where the product was applied.

Uemura (no date) used a patch test method to evaluate the skin irritation potential of 0.5% or 1.5% Stearyl Glycyrrhetinate in olive oil. The treatment sites were examined 24 h after application. There was no sign of skin irritation in any of the 89 subjects tested.

Lachartre Laboratories (1992a) evaluated the cutaneous tolerance to an eye gel containing 0.1% Glycyrrhizic Acid in 20 women. The gel was applied to the face in the area around the eyes with a soft digital massaging once or twice daily after washing for 21 days. Amounts of gel product applied ranged from 0.10 to 0.57 g per day. Each subject maintained her usual habits of makeup and hygiene, except for products similar to the test material. The eyes and the skin around the eyes were examined for signs of irritation on the 1st and 21st days of application. After the first day of application, there were no reactions. Twenty-four hours after the 20th application, two subjects had desquamation patches on the eyelids. The researchers did not think this event was related to the treatment. Eight subjects reported discomfort

sensations, as stretching. No other observations were noted. The authors concluded that repeated use of the eye gel (containing 0.1% Glycyrrhizic Acid) was well tolerated.

Lechartre Laboratories (1992b) conducted a repeated insult patch test with an eye gel containing 0.1% Glycyrrhizic Acid on 232 women, aged 18 to 62 years. In the induction phase, 0.1 ml of the test material was applied to the left shoulder of each subject, under occlusive patch, three times a week, for 3 weeks. After a 2-week period of no application, the challenge phase involved a similar application to each the induction site and a naive site. Three subjects had slight and transient erythema during the induction phase. One subject had well-defined erythema with pruritus after the first application. No other signs of irritation were reported in the induction phase. After the challenge application, one volunteer had a well-defined erythema with pruritus. No other treatment-related observations were reported.

Hilltop Research, Inc. (1995) conducted a human repeated insult patch test of an eye gel containing 0.1% Glycyrrhizic Acid on 57 male and 43 female subjects. In the induction phase, 0.1 ml of the test material was applied to a 2 × 2-cm patch pad. The pad was then applied to either the lateral surface of the upper arm or one side of the back. The pads were secured with hypoallergenic tape. Patch pads were applied for 24 h, three times per week, for 3 weeks. Twelve to 20 days after the last induction patch, a challenge patch was applied to the induction site and a naive site on the body for 24 h. The induction and challenge sites were examined 24 h after each application. The report did not give details of the study results. Six subjects had mild erythema with or without edema at the challenge phase. These observations were diminished 48 to 96 h after the challenge application and were considered irritant in nature. The authors concluded that the test material (eye gel containing 0.1% Glycyrrhizic Acid) did not produce evidence of induced contact hypersensitivity.

Quintiles (1999) conducted a human repeated insult patch test of a moisturizer containing 0.1% Glycyrrhizic Acid on 92 subjects. Induction consisted of application of 0.3 ml of the test material to the outer upper arm under occlusive patch for 24 h, three times per week, for 3 weeks. Eleven to 16 days after the last induction patch, the challenge phase involved a 24-h occluded application of the test material at the induction site and a naive site. Treatment sites were evaluated after each application. During induction, 35 subjects had moderate erythema and 42 subjects had mild erythema. There was no evidence of contact sensitization.

Phototoxicity and Photosensitization

Yamamoto (1976a) evaluated Glycyrrhizic Acid, Ammonium Glycyrrhizate, and Dipotassium Glycyrrhizate in human patch tests. In a double-blind method, the test articles (each at 5%) were applied to the forearms of 21 female volunteers for 48 h. The sites were observed for signs of irritation or inflammation. Forty-eight hours later, the dose sites were exposed to irradiation from a Dermaray Model 1 (BLB; 15 cm, 3 min), and the sites were evaluated again 48 h later. None of the exposure sites showed observable irritation reactions before or after irradiation.

Clinical Science Research International, Ltd. (CSRI) conducted a photosensitization study of an eye gel containing 0.1% Glycyrrhizic Acid in nine human subjects (CSRI 1992a). Induction treatments consisted of 24-h occluded applications of 0.2 ml of the test material, followed by UV exposure (1000-W xenon arc solar simulator) of the bare induction site. Induction treatments were performed six times over a period of 3 weeks. About 10 days after the last induction treatment, the challenge treatment consisted of a 24-h application of the test material at the induction site and two naive sites followed by exposure to 4 J/cm^2 of UVA irradiation. There was no evidence of photosensitization.

CSRI (1992b) conducted a phototoxicity study of an eye gel containing 0.1% Glycyrrhizic Acid in nine human subjects. Approximately 0.2 ml of the test material was applied to each of two circular test sites (4.5 cm^2 each) under occlusive pads for 24 h. After the application period, one of the sites was exposed to 10 J/cm^2 of UVA irradiation. The treatment sites were evaluated for up to 72 h. There was no evidence of phototoxicity to the eye gel.

Yamamoto (1976b) applied 5% Glycyrrhizic Acid, 5% Ammonium Glycyrrhizate, or 5% Dipotassium Glycyrrhizate in distilled water to the skin of 21 healthy female volunteers. The treatment area was irradiated with UV (UV dose not specified), and the irradiated treatment site was examined 48 h later. There was no sign of phototoxicity.

St. Marianna University (1995) repeated the above study (Yamamoto 1976b) and found the exact same results of no phototoxicity to 5% Glycyrrhizic Acid, 5% Ammonium Glycyrrhizate, or 5% Dipotassium Glycyrrhizate in distilled water.

Epidemiology Studies

Strandberg et al. (2001) gave questionnaires on Glycyrrhizic Acid (licorice) consumption to a sample of 1049 Finnish women with young infants. Glycyrrhizic Acid consumption was grouped into three levels: low (< 250 mg/week; $n = 751$), moderate (250–499 mg/week; $n = 145$), and heavy (≥ 500 mg/week; $n = 110$). Hospital birth records were analyzed and compared to Glycyrrhizic Acid exposure. Birth weight and maternal blood pressure were not affected by Glycyrrhizic Acid consumption. Babies with heavy exposure were significantly more likely to be born before 38 weeks ($p < 0.03$).

Case Reports

Donaldson and Duthie (1956) treated 30 patients with eczematous skin lesions with an ointment made of wool fat, liquid paraffin, and white soft paraffin with or without 2% Glycyrrhetinic Acid. There was no significant difference in the degree of healing of the lesions between the two ointment types. In this study, 2% Glycyrrhetinic Acid did not improve the condition of eczema patients.

Colin-Jones (1957) reported the results of Glycyrrhetinic Acid treatments on several cases of different skin disorders.

Treatment with 2% Glycyrrhetinic Acid in a water-miscible base markedly improved or cleared 11/13 cases of infantile eczema, 6/8 cases of flexural eczema, 3/4 cases of nummular eczema, 4/4 cases of traumatic dermatitis, 3/3 cases of dermatitis, 3/4 cases of neurodermatitis of the nape of the neck, 2/2 cases of disseminated neurodermatitis, and 3/4 cases of pruritis vulvæ et ani. Treatment with Glycyrrhetinic Acid (% not given) in 0.5% neomycin sulphate markedly improved or cleared 8/8 cases of impetigo, 2/2 cases of impetigo with associated penicillin sensitivity, 3/4 cases of impetiginized eczema, 3/4 cases of impetiginized seborrhœa, 4/5 cases of pustular psoriasis, and 2/2 cases of acne varioliformis.

Evans et al. (1958) reported the outcomes of 124 cases of various skin disorders (e.g., contact and allergic dermatitis, anogenital pruritus, and several types of eczema) after treatment with Glycyrrhetinic Acid (doses not specified). Of the 124 cases, 91 showed marked improvement or cleared, and 25 cases showed improvement. Eight of the reported cases showed no improvement. The author stated that in many cases Glycyrrhetinic Acid was more effective than hydrocortisone in the treatment of subacute, chronic, and intractable skin conditions.

Watanabe et al. (2001) described a case of a woman with chronic hepatitis C who did not initially respond to interferon therapy. She was treated with Glycyrrhizic Acid (60 ml Stronger Neo-minophagen C) three times per week for 2 years in conjunction with interferon-β therapy. After the treatment period, the patient's aminotransferase levels returned to normal, and the hepatitis virus C RNA level was negative. The authors commented that the interferon therapy was successful only after the liver function had improved with Glycyrrhizic Acid treatment.

Conn et al. (1968) reported a case of a 58-year-old man who was admitted to the University of Michigan Hospital with complaints of severe muscular weakness. Tests revealed that the patient had hypertension, hypokalemic alkalosis, suppressed renin activity, and aldosteronopenia. Chlorothiazide (0.5 g twice daily) and chlorthalidone (100 mg/day) failed to correct the hypertension. A careful history revealed that he had ingested 72 to 108 g of licorice candy daily for 6 to 7 years. The candy's manufacturer indicated that amount to contain about 0.5 g/day Ammonium Glycyrrhizate. The patient was put on a strict licorice-free diet with controlled sodium and supplements to replace lost potassium. The second day after admission, the patient began to regain muscular strength. Diuresis and weight loss began 8 days later. Aldosterone excretion was first measured on the 9th day after admission and was 0.8 μg/day, whereas renin activity was below detectable limits. On day 10, the 24-h secretion rate of aldosterone was 30.8 μg/day, compared to the normal mean rate of 109 μg/day. By 28 days after admission, the patient was recovering, with aldosterone excretion at 5.1 μg/day and renin activity at 222 μg/100 ml, values within normal range, and blood pressure was 120/80 to 130/85 mm Hg.

After recovery, the patient agreed to participate in an investigation to reproduce the clinical effects of Ammonium Glycyrrhizate. The patient was given 0.5 g every 12 h for 2 days, followed by 1 g every 12 h for 3 days, and then 2 g every 12 h for 5 days. There was a prompt increase in body weight. Serum sodium and blood pressure increased, and serum potassium steadily declined. Aldosterone excretion fell from 5.1 μg/day before dosing to 1.7 μg/day by day 4 and to 0.3 μg/day by day 9. Plasma renin activity fell from normal 222 μg/100 ml to undetectable levels by day 8. Ten months of a licorice-free diet after discharge, the patient felt generally healthy, and all aldosterone, renin, electrolyte and blood pressure parameters were normal. The data supported the investigators' suspicions that Ammonium Glycyrrhizate in the licorice candy that the patient had eaten for years caused a pseudoaldosteronism that contributed to electrolyte imbalance and hypertension. The patient's initial muscle weakness was attributed to hypokalemia (Conn et al. 1968).

EUROPEAN COMMISSION EVALUATION

The European Commission's Scientific Committee on Food (SCF) prepared an opinion on the safety of Glycyrrhizic Acid and Ammonium Glycyrrhizate in foods (SCF 2003). The opinion noted that the Committee had previously reviewed Glycyrrhizic Acid in 1991 and concluded that the data were inadequate to derive an acceptable daily intake (ADI), but they did advise that regular daily ingestion from all food products should not exceed an upper use level (UUL) of 100 mg/day.

In 2003, the SCF was asked to review new data on Glycyrrhizic Acid and to consider the safety of Ammonium Glycyrrhizate as a flavoring substance. After reviewing the new data, the SCF concluded that there is a stronger basis for the limit of 100 mg/day, but they still did not feel that the human toxicity data were sufficient to develop an ADI. The committee noted that there are subgroups of people for whom a limit of 100 mg/day may not provide sufficient protection. These groups include people with decreased 11-β-hydroxysteroid dehydrogenase-2 activity, people with prolonged gastrointestinal transit time, and people with hypertension or electrolyte-related or water homeostasis-related medical conditions (SCF 2003).

SUMMARY

This review considers the safety of specific organic compounds that may be isolated from licorice plants—Glycyrrhetinic Acid and its salts and esters, and Glyrrhizic Acid and its salts and esters used in cosmetics as considered by the Cosmetic Ingredient Review (CIR) Expert Panel. It does not address the plant extracts from the licorice plant—those will appear in a separate report.

The ingredients in this safety assessment are Glycyrrhetinic Acid, Potassium Glycyrrhetinate, Disodium Succinoyl Glycyrrhetinate, Glyceryl Glycyrrhetinate, Glycyrrhetinyl Stearate, Stearyl Glycyrrhetinate, Glycyrrhizic Acid, Ammonium Glycyrrhizate, Dipotassium Glycyrrhizate, Disodium Glycyrrhizate, Trisodium Glycyrrhizate, Methyl Glycyrrhizate, and

Potassium Glycyrrhizinate. All these compounds have the basic core structure of Glycyrrhetinic Acid with varying R groups.

These ingredients are ethanol or water soluble. Ultraviolet radiation absorption is in the UVC region. Semi-micro-HPLC, mass spectrometry, and nuclear magnetic resonance spectra have been used to detect these ingredients.

Glycyrrhetinic Acid has been found to be at least 98% pure. It has been found to contain 0.6% 24-OH-Glycyrrhetinic Acid, not more than 20 μg/g of heavy metals and not more than 2 μg/g of arsenic. No residue was detected in Glycyrrhetic Acid. Ammonium Glycyrrhizate has been found to be at least 98% pure and Dipotassium Glycyrrhizate has been found to be at least 95% pure.

Glycyrrhetinic Acid and its relatives function in cosmetics as either a flavoring agent, a skin-conditioning agent—miscellaneous or both. Glycyrrhetinic Acid is used at concentrations of up to 2%; Stearyl Glycyrrhetinate, 1%; Glycyrrhizic Acid, 0.1%; Ammonium Glycyrrhizate, 5%; Dipotassium Glycyrrhizate, 1%; and Potassium Glycyrretinate, 1%.

Glycyrrhizic Acid is poorly absorbed by the intestinal tract. However, it may be hydrolyzed by a specialized β-glucuronidase produced by intestinal bacteria into Glycyrrhetinic Acid. Glycyrrhetinic Acid and Glycyrrhizic Acid bind extensively to rat and human albumin but do not absorb well into tissues. Glycyrrhetinic Acid and Glycyrrhizic Acid and metabolites are mostly excreted in the bile and are subject to enterohepatic cycling in rats, and presumably humans. Very little is excreted in urine.

In rats given radioactive Glycyrrhetinic Acid orally, 86% of the radioactivity was recovered in 1 to 3 days with 83% in the feces, 1% in the urine and 4% remained in the liver. In rats given radioactive Glycyrrhetinic Acid subdermally, 74% of the radioactivity was recovered with 73% in the feces and 1% in the urine. Of the radioactivity recovered in the feces, only 7.4% was unchanged Glycyrretinic Acid. In humans Glycyrrhizic Acid was not detected in blood plasma after oral administration, but Glycyrrhetinic Acid was detected at $<$200 ng/ml. In the urine, 0.3% to 0.8% of the Glycyrrhizic Acid was recovered.

Dipotassium Glycyrrhizate was undetectable in the receptor chamber when tested for transepidermal permeation through pig skin.

Glycyrrhizic Acid increased the dermal penetration of diclofenac sodium in rat skin. Dipotassium Glycyrrhizate increased the intestinal absorption of calcitonin in rats. In humans, Glycyrrhetinic Acid potentiated the effects of hydrocortisone in the skin, possibly by increasing the percutaneous absorption of hydrocortisone.

Moderate chronic or high acute exposure to Glycyrrhizic Acid, Ammonium Glycyrrhizate, and their metabolites have been demonstrated to cause several transient systemic alterations including increased potassium excretion, sodium and water retention, body weight gain, alkalosis, suppression of the renin-angiotensis-aldosterone system, hypertension, and muscular paralysis. Many of these effects result from Glycyrrhizic Acid inhibition of 11β-OHSD2 in the kidney. Other enzyme activities affected include proteinases, cytochrome P450 monooxygenase, protein kinases, and tyrosinase.

Glycyrrhetinic Acid and its derivatives block gap junction intracellular communication in a dose-dependent manner in animal and human cells, including epithelial cells, fibroblasts, osteoblasts, hepatocytes, and astrocytes. At high concentrations, Glycyrrhetinic Acid is cytotoxic.

Glycyrrhetinic Acid and Glycyrrhizic Acid have been shown to protect liver tissue from known hepatotoxins such as carbon tetrachloride.

Glycyrrhizic Acid has been used to treat chronic hepatitis, inhibiting the penetration of the hepatitis A virus into hepatocytes. Other anti-viral activity included influenza, vaccinia, herpes simplex 1, Newcastle disease, and vesicular stomatitis, but not polio type 1.

Glycyrrhetinic Acid and Glycyrrhizic Acid have antiinflammatory effects in rats and mice. Other cellular effects include apoptosis and enhanced interleukin and interferon production.

The acute intraperitoneal LD_{50} for Glycyrrhetinic Acid in mice was 308 mg/kg and the oral LD_{50} was $>$610 mg/kg. The oral LD_{50} in rats was reported to be 610 mg/kg. Higher LD_{50} values were generally reported for salts.

Little short-term, subchronic, or chronic toxicity was seen in rats given ammonium, dipotassium, or disodium salts of Glycyrrhizic Acid.

Glycyrrhetinic Acid at 100 mg/ml was not irritating to shaved rabbit skin. In an in vitro irritation assay, Glycyrrhetinic Acid was considered slightly irritating.

Glycyrrhetinic Acid inhibited the mutagenic activity of benzo[a]pyrene in Ames strains TA-98 and TA-100. Glycyrrhetinic Acid inhibited DMBA and TPA tumor initiation and promotion in mice. Glycyrrhizic Acid inhibited tumor initiation by DMBA but not TPA tumor promotion in mice. Glycyrrhizic Acid delayed mortality in mice injected with Erlich ascites tumor cells, but did not reduce the mortality rate. Ammonium Glycyrrhizate was not genotoxic in in vivo and in vitro cytogenetics assays, the dominant lethal assay, Ames strain TA1530 and *Salmonella* strain G46, and heritable translocation tests, except for possible increase in dominant lethal mutations in rats given 2000 mg/kg day^{-1} in their diet. Disodium Glycyrrhizate was not carcinogenic in a mouse drinking water study at exposure levels up to 12.2 mg/kg day^{-1} for 96 weeks.

Glycyrrhizate salts produced no reproductive or developmental toxicity in rats, mice, golden hamsters, or Dutch-belted rabbits, except for a dose-dependent increase (at 238.8 and 679.9 mg/kg day^{-1}) in sternebral variants in study using rats.

Sedation, hypnosis, hypothermia, and respiratory depression were seen in mice given 1250 mg/kg Glycyrrhetinic Acid intraperitoneally. Rats fed a powdered diet containing up to 4% Ammonium Glycyrrhizate had no treatment related effects in motor function tests, but active avoidance was facilitated at 4%, unaffected at 3%, and depressed at 2%.

In a study of 39 healthy volunteers, a no effect of an oral dosage of 2 mg/kg/day was determined for Glycyrrhizic Acid. Glycyrrhizic Acid increased the lymphocyte count in HIV patients with hemophilia, and increased blood pressure in healthy individuals. Clinical tests in six normal individuals using Ammonium Glycyrrhizate at 6g/day revealed reduced renal and thermal sweat excretion of Na^+ and K^+. Carbohydrate and protein metabolism were not affected.

Glycyrrhetinic Acid at concentrations up to 6% was not an irritant or a sensitizer. Neither Glycyrrhizic Acid, Ammonium Glycyrrhizate, nor Dipotassium Glycyrrhizate at 5% was phototoxic agents or photosensitizers. One case reports linked licorice candy consumption (72 to 108 g/day) with muscular weakness, hypertension, alkalosis, suppressed renin activity, and aldosteronopenia. Various uses of these licorice ingredients in clinical treatment have also been reported.

Birth weight and maternal blood pressure were unrelated to the level of consumption of Glycyrrhizic Acid in 1049 Finnish women with infants, but babies whose mothers consumed >500 mg/wk were more likely to be born before 38 weeks.

A European Commission Scientific Committee on Food opinion did not establish an acceptable daily intake of Glycyrrhizic Acid and Ammonium Glycyrrhizate in foods, but did recommend an upper use level of 100 mg/day and identified decreased 11-β-hydroxysteroid dehydrogenase-2 activity, prolonged gastrointestinal transit time, or hypertension or electrolyte-related or water homeostasis-related medical conditions as conditions for which the upper use level may be too high.

DISCUSSION

The CIR Expert Panel noted that the ingredients in this safety assessment are not plant extracts, powders, or juices, but rather are specific chemical species that may be isolated from the licorice plant. Because these chemicals may be isolated from plant sources, however, steps should be taken to assure that pesticide and toxic metal residues are below acceptable levels. The Panel advised the industry that total polychlorobiphenyl (PCB)/pesticide contamination should be limited to not more than 40 ppm, with not more than 10 ppm for any specific residue, and that toxic metal levels must not contain more than 3 mg/kg of arsenic (as As), not more than 0.002% heavy metals, and not more than 1 mg/kg of lead (as Pb).

No published information was identified for Disodium Succinoyl Glycyrrhetinate, Glyceryl Glycyrrhetinate, Glycyrrhetinyl Stearate, Methyl Glycyrrhizate, Potassium Glycyrrhetinate, Potassium Glycyrrhizinate, or Trisodium Glycyrrhizate, but they are considered to be sufficiently structurally related to the rest of the ingredients that the data may be extrapolated to address their safety.

Although Glycyrrhizic Acid is cytotoxic at high doses and ingestion can have physiological effects, there is little acute, short-term, subchronic, or chronic toxicity and Dipotassium Glycyrrhizate is poorly absorbed through the skin. Glycyrrhizate salts were not genotoxic in all but one assay at a high exposure level, were not carcinogenic in animals tests, and inhibited tumor initiation and promotion by known tumor producing chemicals. Glycyrrhizate salts produced no reproductive or developmental toxicity in several animal species, except for one finding of sternebral variants in a rat drinking water study. Because of the poor dermal absorption, this one finding did not suggest a safety concern regarding use in cosmetic formulations.

These ingredients are not considered to be irritants, sensitizers, phototoxic agents, or photosensitizers at the current maximum concentration of use. Accordingly, the CIR Expert Panel concluded that these ingredients are safe in the current practices of use and concentration.

The Panel recognizes that certain ingredients in this group are reportedly used in a given product category, but the concentration of use is not available. For other ingredients in this group, information regarding use concentration for specific product categories is provided, but the number of such products is not known. In still other cases, an ingredient is not in current use, but may be used in the future.

Although there are gaps in knowledge about product use, the overall information available on the types of products in which these ingredients are used and at what concentration indicate a pattern of use. Within this overall pattern of use, the Expert Panel considers all ingredients in this group to be safe.

CONCLUSION

Ammonium Glycyrrhizate, Dipotassium Glycyrrhizate, Disodium Glycyrrhizate, Disodium Succinoyl Glycyrrhetinate, Glyceryl Glycyrrhetinate, Glycyrrhetinic Acid, Glycyrrhetinyl Stearate, Glycyrrhizic Acid, Methyl Glycyrrhizate, Potassium Glycyrrhetinate, Potassium Glycyrrhizinate, Stearyl Glycyrrhetinate, and Trisodium Glycyrrhizate are safe for use in cosmetic formulations in the practices of use and concentration as described in this safety assessment.

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A Study of Biochemical and Hematological Markers in Alcoholic Liver Cirrhosis

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Abstract Progressive fibrosis and cirrhosis, clinically presenting as end-stage liver disease are common outcomes in alcoholic Liver disease (ALD) patients. A variety of laboratory tests are available to assist in the progression and diagnosis of cirrhosis to end stage liver disease. The aim of this study is to identify potential novel biomarkers for progression of cirrhosis to end-stage liver cirrhosis. The biomarkers evaluated in this study included liver function indicators including serum ferritin, prothrombin time, albumin, total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), renal parameters (urea and creatinine) and red blood cell counts, hemoglobin and blood glucose. The study included two groups based on severity of cirrhosis of liver; categorized as compensated and decompensated liver cirrhotic patients based on child Pugh criteria. All decompensated cirrhotic patients in the study group had significantly elevated biomarkers levels ($P<0.001$) than those with compensated cirrhotic patients and control group who were not suffering from liver cirrhosis. Thus these results suggest that elevated and altered liver and hematological biomarkers are associated with pathogenesis and progression of liver cirrhosis.

Keywords: *alcohol, Biochemical marker, haematological markers, γ-Glutamyltransferase, Aminotransferases*

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1. Introduction

Alcoholism is condition resulting from excess drinking of beverages that contain alcohol. The major health risk of alcoholism includes liver disease, heart disease, pancreatitis, central nervous system disorders and certain forms of cancer [1]. Alcohol can be manifested in liver damage from fibrosis to end stage of cirrhosis and may eventually lead to carcinoma of liver. The liver is particularly vulnerable to disease related to heavy drinking, most commonly termed as alcoholic hepatitis or cirrhosis. The progression of alcoholic liver disease is characterized by steatosis, inflammation, necrosis and cirrohosis. When severe Cirrohosis occurs, death is the outcome [2]. Chronic consumption of alcoholic beverages is a primary cause of liver injury. Chronic and excessive consumption of alcoholic beverages provokes membrane lipid-peroxidation due to triglyceride accumulation in hepatocytes [3]. The study underway can serve as potential diagnostic tools for more specific biomarkers of ethanol-induced diseases. Hence, an attempt has been made to evaluate the effect of chronic alcohol consumption on blood, renal and hepatic biomarkers against worsening child pugh criteria.

2. Materials and Methods

2.1. Design and Subjects

A comparative study was carried out in a sample of 85 cirrhotic patients with chronic alcoholism of 31-54 years of age, and a mean consumption of ethanol 153.4 ± 36.9 g/d during the past 10 years (without alcohol ingestion in the past 30 days) and without additional diseases,

GROUP 1: (n=35)

Patients who were having with clinical, biochemical and ultrasonographic evidence of cirrhosis of liver without evidence of ascites, hepatic encephalopathy, GI bleed were classified as compensated cirrhotic patients with a mean age of 42.1 ± 10.19 years.

GROUP 2: (n=50)

Patients with clinical, biochemical, ultrasonographic evidence of cirrhosis of liver with evidence of GI bleed, hepatic encephalopathy & ascites were classified as decompensated cirrhotic patients with mean age of 42.5 ± 8.1 [4].

An equal number of aged matched normal healthy adults of with a mean age of 41.0 ± 9.4 years, with chronic alcoholism. All subjects had lived in central India for the past 10 years at the time of the study. The subjects agreed

to participate in the study after giving their informed consent.

2.2. Blood and Biochemical Analyses

Patients were obtained from gastroenterology ward of super specialty hospital with proven history of liver cirrhosis on the basis of clinical, biochemical and imaging methods and endoscopic signs. Cirrhosis was related to chronic alcohol intake. The severity of the disease was evaluated according to the Child-Pugh classification [4]. Heparin was the anticoagulant agent used. Blood samples containing heparin were analyzed using complete hemoglobin test protocol and ferritin [5,6]. The serum obtained from samples containing no anticoagulant agent was subjected to the following tests: urea, creatinine, albumin, total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT) [6-13]. All reagents employed in biochemical tests were obtained from Randox Laboratories, Ltd. These tests were used as screening measurements for diagnosis of liver injury prevalent in different stages of cirrhotic patients.

2.3. Statistical Analysis

Data were processed by use of standard statistical software Open epi. The results are presented as mean ±SD. The exact measures of associations in results between patients and control were compared using chi square test and fisher statistics. The significance was taken at $P < 0.001$.

3. Results

3.1. Effect of Alcohol on Serum Levels of Liver Chemistries.

Table 1 indicates the levels of serum bilirubin, albumin, GGT and ALT/AST ratio of compensated and decompensated cirrhotic groups as compared to that of control. The serum concentration of bilirubin was found to be significantly altered in patients with decompensation (group 2) as compared to compensated (group 1) and controls**.** The serum bilirubin levels were significantly elevated in patients consuming alcohol for the past 10 years Group 2 as compared to Group 1 and the control subjects ($P<0.001$). The albumin concentration was significantly altered in compensated and decompensated patients (group 1 & 2) as compared to control group. The serum concentration of albumin was significantly decreased ($P<0.001$) and the serum GGT Levels was significantly elevated ($P<0.001$) and gradually declined with progression of cirrhosis in patients consuming alcohol for the past 28- 45 years. Also the serum levels of ALT, AST and AST/ALT ratio was significantly altered in patients consuming alcohol with more pronounced in decompensated subjects (Group 2) as compared to control subjects.

3.2. Effect of Alcohol Intake on Renal Markers

Table 2 shows the activities of serum urea and creatinine compared to control subjects respectively. There was significant change in serum urea and creatinine levels of patients consuming alcohol for the past 10 years in decompensated (Group 2) as compared to the compensated and control subjects. The serum levels of urea and creinatine showed pronounced elevation in decompensated cirrhotic patient as compared to compensated and control subjects.

3.3. Effect of Alcohol Intake on Haemoglobin RBC Count and Serum Ferritin

Table 3 shows the level of total RBC count, Hb concentration, PT values and Serum Ferritin of control compensated and decompensated respectively as compared to that of control. The levels of total RBC count, Hb content showed depleted values against serum ferritin levels which showed steep elevation in decompensated, as against compensated and control groups. The two groups of patients (i.e. compensated and decompensated) were consuming alcohol for the past 10 years as compared to the control subjects. The prothrombin time was significantly altered in decompensated patients than in compensated and decompensated patients. The serum concentrations of cirrhotic patients were reflected according to progression and gradation of cirrhosis and significantly elevated as compared to control subjects.

Table 1. Serum ALT, AST, T.Bilirubin, Albumin, GTT levels of patients

Patients	ALT (mean±SD)	AST (mean±SD)	T.Bilurubin (mean±SD)	Albumin (mean±SD)	GGT (mean ± SD)
Control	29.18±8.23	34.04 ± 6.88	0.62 ± 0.16	3.65 ± 0.23	22.47 ± 3.30
Deompensated	79.52±14.69	132.3 ± 42.88	6.1 ± 3.22	2.70 ± 0.29	34.48 ± 19.52
Compensated	56.32±10.09	120.49 ± 29.54	1.32 ± 0.36	*3.20 ± 0.20*	44.90 ± 10.59

ALT=Alanine aminotransferase; AST=Aspartate aminotransferase; GGT=gamma glutamyl transpeptidase

Table 2. Values of serum urea, creatinine in cirrhosis groups

KIDNEY FUNCTION TESTS	CONTROLS n =90	COMPENSATED CIRRHOSIS n =35	DECOMPENSATED CIRRHOSIS n =50
UREA	23.43 ± 7.27	40.17 ± 8.41	59.51± 32.09
CREATININE	0.90 ± 0.22	1.20 ± 0.34	1.80 ± 1.18
GLUCOSE	89.09 ± 9.19	82.52 ± 9.12	70.11 ± 5.63

Table 3. Values of RBC counts, Ferritin and Hemoglobin in cirrhotic patients

Patients	RBC Count 10^{-6} (mean ± SD)	Ferritin (ng/ml) (mean ± SD)	Hb (g/dl) (mean ± SD)	PT (mean±SD)
Control	5.27 ± 1.54	68.85 ± 19.39	15.12 ± 0.51	12.66 ± 0.75
Compensated	4.12 ± 0.42	154.82 ± 19.67	14.11 ± 0.36	14.00 ± 0.51
Decompensated	3.10 ± 0.70	432.61 ± 71.57	8.82±1.39	19.94 ± 4.59

RBC counts=red blood cell counts; Hb=Hemoglobin; PT=Prothrombin time

4. Disscusion

Excessive chronic consumption of alcohol results in profound alterations in the blood chemistries which may be associated with alterations in metabolic activities of cell resulting in several clinical and/or biochemical changes.

4.1. Effect of Chronic Alcohol Consumption on Liver Chemistries

The serum gamma glutamyl transferase (γGT), aspartate aminotransferase, bilirubin and albumin are considered to be well known markers of cirrhosis [14]. We have measured liver function tests, albumin and gamma glutamyl transferase, with worsening child Pugh classification in the present study, considering γGT as the most sensitive markers for acute hepatocellular damage. Our results revealed that levels of γGT were high in patients with severe alcoholic liver disease and low in the later stages of cirrhosis (Table 1). This pattern of rise in γ-GT levels were in congruence with the earlier study showing rise in compensated cirrhosis and fall in decompensated cirrhosis [15]. However some researches depicted controversial results [16]. The rise in the levels of γGT as also concluded by certain other studies [15] in alcoholic liver diseases may probably be due to inductive action of alcohol. Hyperbilirubinemia and hypoalbuminemia were also observed to be common features with alcoholics in our study. The decrease in serum albumin level can be attributed to nutritional status of the subjects as also reported by Das et. al. earlier [14]. Albumin has been known as a potential subject for the formation of adduct by acetaldehyde, an alcohol metabolite. Ethanol consumption also slows down the rate of hepatic protein catabolism and may be related to degree of ethanol-induced Liver injury [14]. The serum transaminases viz, aspartate aminotransferase (AST) and alanine amino-transferase (ALT) are significantly elevated in decompensated cirrhosis as compared to controls and compensated cirrhosis. The ratio of AST to ALT may help in the differential diagnosis of alcoholic liver disease. The ratio is generally 1 or less with acute liver injury [4]. Following heavy alcohol consumption there has been evidence of mitochondrial damage. In Mitochondrial damage, the elevation in ratio of mitochondrial AST to total AST (mAST: tAST) has also been reported in heavy alcohol consuming individuals. This rise is proportionately higher in alcoholic individuals than healthy individuals which contribute only 10% of mitochondrial AST activity in serum [14]. The pattern of aberration in AST and ALT observed in our study (Table 1) has been correlated well with the reports in the past [16]. Ozenirler et. al. [16] reported rise in values of ALT in decompensated cirrhosis than that of compensated cirrhosis. The values of total bilirubin as reported by Agnieszka-Szuster-Ciesieka et. al. in compensated cirrhosis showed upward trend with progression of cirrhosis [15]. The Bilirubin levels observed by us were low in compensated cirrhosis than that of decompensated cirrhosis. Ozenirler et. al. [16] showed similar pattern of bilirubin values in compensated cirrhosis. Serum albumin as reported by Agnieszka-Szuster-Ciesieka et. al. [15] and Ozenirler et. al. [16] was found to be lowered with progression from compensated to decompensated cirrhosis which matched with our study as evident from Table 1.

4.2. Effect of Chronic Alcohol Consumption on Renal Chemistries and Glucose Levels

Our present findings of kidney profile comprising slightly elevated blood urea and serum creatinine in later stages of decompensation as compared to controls and compensated cirrhosis are quite similar to earlier reports of Das et al [14]. The values of bilirubin are associated with urea and creatinine as observed by us may be used as markers in combination for diagnosis for ALD. It has been reported that liver disease has been associated with renal disorders [17]. We also have noticed hypoglycemia in patients associated with liver cirrhosis with increasing Child –Pugh score (Table 2). The finding is well supported by SQ Siler et al [18] who suggested that inhibition of gluconeogenesis and low intake of carbohydrates and other nutrients, associated with alcohol intake results in hypoglycemia.

4.3. Effect of Chronic Alcohol Consumption on Haematological Values

Alcohol has a variety of pathologic effects [17] in general including hematolological abnormalities. On hematopoiesis, it has been found that alcohol has direct action on erythroid precursors, thereby contributing to macrocytosis and the anemic state of chronic alcoholics [19]. Ethanol induces sideroblastic anemia due to interference with heme synthesis. The hematological study in the present work showed Prothrombin time (PT) elevated with increasing Child Pugh score thereby showing clear sign of liver injury (Table 3). The percentage of hemoglobin and the total number of RBC were found to be significantly decreased with heavy alcohol intake as a result of hemodilution [18]. Iqbal et. al [20] Reported decrease in hemoglobin values in decompensation which increased on liver transplantation. RBC count showed marked decrease with growing decompensation as compared to control and compensated cirrhosis (Table 3). The degree of liver impairment showed direct relation with decrease in RBC count. Das et. al [18] suggested that chronic ingestion of alcohol causes alterations in erythrocyte membrane lipids which occur with the progression of alcoholic liver disease. Thus this leads to hemolytic anemia.

In our study we also made it a point to note that with the increase in consumption of alcohol and the level of liver injury in cirrhotic patients was profoundly more severe which was evident with elevated serum transaminases levels and other biomarkers (table1,2,3).

5. Conclusion

In conclusion, it is evident from the results of this study and the existing literature that there was a compromise of Liver function system with variation in other related biomarkers of injury with respect to different organs and body systems. The two groups based on child-Pugh classification also suggested compromise of liver function with increase in alcohol consumption. The variation in

liver functions with elevated Ferretin levels and corresponding reduction in RBC'S and hemoglobin values also revealed risk to liver injury and renal functions related to excessive alcohol intake when compared with control group. Glucose levels were decreasing from control to compensated through decompensated phases reiterating the role of alcohol in glucose metabolism. Our results reemphasize the fact that with rising levels of transaminases and variation in other biomarkers of injury reflecting iron as central cofactor in producing injury, alcohol consumption and iron levels play a key role in the progression of liver disease and pathogenecity. Regular monitoring of these markers and iron overload indicators in alcoholic patients is necessary for better patient management and to minimize the morbidity and mortality related to liver injury.

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TOPIC HIGHLIGHT

2015 Advances in Alcoholic liver disease

Immune dysfunction in acute alcoholic hepatitis

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Abstract

Acute alcoholic hepatitis (AAH) is a serious complication of alcohol misuse and has high short term mortality. It is a clinical syndrome characterised by jaundice and coagulopathy in a patient with a history of recent heavy alcohol use and is associated with profound immune dysfunction with a primed but ineffective immune response against pathogens. Here, we review the current knowledge of the pathogenesis and immune defects of AAH and identify areas requiring further study. Alcohol activates the immune system primarily through the disruption of gut tight junction integrity allowing the escape of pathogen-associated molecular particles (PAMPs) into the portal venous system. PAMPs stimulate cells expressing toll-like receptors (mainly myeloid derived cells) and initiate a network of intercellular signalling by secretion of many soluble mediators including cytokines and chemokines. The latter coordinates the infiltration of neutrophils, monocytes and T cells and results in hepatic stellate cell activation, cellular damage and hepatocyte death by necrosis or apoptosis. On the converse of this immune activation is the growing evidence of impaired microbial defence. Neutrophils have reduced phagocytic capacity and oxidative burst and there is recent evidence that T cell exhaustion plays a role in this.

Key words: Alcoholic hepatitis; Alcoholic liver disease; Toll-like receptors; Gut dysbiosis; T cell exhaustion

Core tip: Acute alcoholic hepatitis (AAH) has high short-term mortality and is challenging to treat with

only glucocorticoids demonstrating proven survival benefit. Development of other effective treatment requires a clear understanding of the mechanisms of immune dysfunction in AAH. Here, we review recent progress in the field and identify areas in need of further research; particularly the role of gut dysbiosis in allowing presentation of pathogen associated molecular patterns to innate receptors on myeloid cells and the subsequent recruitment of immune cell subsets. Recent data demonstrating that T cells have an exhausted phenotype and result in impaired antimicrobial defence is also discussed.

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INTRODUCTION

The United Kingdom has seen an increasing burden of liver related mortality over recent decades with rates increasing 4-fold since the 1970s and 5-fold in the under-65s[1]. This is closely mirrored by the relative affordability of alcohol over this time[2], which is 61% more affordable in 2013 than 1980[3] suggesting that alcohol is an important driving factor for liver disease in the United Kingdom. However, this is not a problem unique to the United Kingdom, similar changes in alcohol consumption and alcohol related mortality have been observed in Northern and Eastern Europe[1] as well as in sub-Saharan Africa, South America and Asia[4].

Given the increasing global consumption of alcohol, it is not surprising that the incidence of acute alcoholic hepatitis (AAH), a serious complication of harmful alcohol use, has also been rising over recent years[5]. AAH is a clinical syndrome characterised by jaundice and coagulopathy in a patient with a recent history of heavy alcohol consumption[6] and has a high short term mortality of up to 40%[7]. It should be clearly differentiated from alcoholic steatohepatitis, a histological diagnosis, which can occur outside the context of current alcohol misuse[8-10].

AAH is increasingly recognised as a systemic inflammatory condition, leading to progressive organ dysfunction and the presence of a systemic inflammatory response syndrome confers a poor prognosis[9]. As well as marked immune activation, there is severe impairment of immune protection against pathogens[11,12]. The discordance between the primed state of the immune system and its failure in microbial defence is yet to be fully explained and an understanding of this dysfunction would certainly help in identifying novel therapeutic targets. To date, therapy has focused on the suppression of an activated immune system and numerous clinical trials have been conducted to evaluate immuno-modulatory (for example, glucocorticoids) or anti-inflammatory therapies [for example, tumour necrosis factor (TNF) alpha antagonists] which target systemic immune activation. Initial promising results from an open label study of infliximab[13] were not confirmed in a randomised controlled trial which was stopped due to excess mortality and infection[14]. Similarly, etanercept treatment was associated with increased mortality[15]. Pentoxifylline, a non-selective phosphodiesterase inhibitor with anti-TNF properties, has also shown no benefit either in combination with glucocorticoids[16,17] or alone[18]. To date only glucocorticoids have a proven short term survival benefit[19,20]. The challenge is how to strike the correct balance between supressing an overactive immune system without further impairing its protective role since death through sepsis remains a significant issue with immunosuppressive treatments[13,21].

The rising incidence of AAH together with its high mortality and limited treatment options has resulted in the European Association for the Study of the Liver identifying AAH as a priority area for research with a specific aim to investigate molecular signals which may predict clinical outcome[22]. Here, we review the current knowledge of the immune mechanisms involved in the pathogenesis of AAH and focus on areas in need of future study. We have not discussed the direct toxic effects on the liver of alcohol and its metabolites including oxidative stress and acetaldehyde adducts which have been reviewed in detail elsewhere[23,24].

THE GUT-LIVER AXIS

The mechanisms by which alcohol activates the immune system were first conclusively elucidated by Thurman *et al*[25] in 1999. The presence of alcohol allowed the presentation of pathogen associated molecular patterns (PAMPs) to hepatic macrophages (Kupffer cells) by modulating intestinal permeability[25]. Subsequent studies have highlighted the importance of the effects of alcohol on the gut microbiome itself with alterations in both the number and balance of organisms which contribute to the breakdown of the intestinal barrier[26]. In a murine model of alcohol related liver disease (ALD), intestinal bacterial overgrowth occurs with a corresponding reduction in probiotic species such as *Lactobacillus*[26]. In humans, bacterial overgrowth has been found in jejunal aspirates from chronic alcohol misusers[27] but the species of bacteria is also important. In a randomised controlled trial (RCT) of *Lactobacillus* and *Bifidobacterium* probiotic therapy in patients with alcoholic psychosis, baseline levels of intestinal probiotic species were lower than healthy controls and short term probiotic treatment significantly improved biochemical indices[28]. Improvement in clinical disease score has also been demonstrated in a small RCT by *Escherichia coli Nissle* treatment of patients

with stable cirrhosis[29]. There is also preliminary data that treatment with probiotics can improve neutrophil phagocytic function in stable cirrhosis with normalisation of phagocytosis after 7 d of treatment with *Lactobacillus casei Shirota*[30].

To date, the most detailed human study of the effects of alcohol on gut dysbiosis compared patients with ALD cirrhosis, patients with alcohol dependence and healthy controls using next generation sequencing techniques to analyse the 16S ribosomal RNA (rRNA)[31]. Compared to the control group a subset of patients displayed gut dysbiosis with significantly lower levels of *Bacteroides* and higher levels of *Proteobacteria*, which was associated with increased systemic endotoxin. Next generation sequencing rRNA studies have yet to be performed in patients with AAH.

Gut dysbiosis alters the intestinal lumen integrity through mechanisms that are incompletely understood. It is clear that the microbiota are key in increasing gut permeability through murine experiments in which the sterilised gut protects against alcohol-induced intestinal barrier leakage[32]. Disruption of tight junctions is probably mediated by microbial metabolism of alcohol to acetaldehyde[33]. However, systemic TNFα and IL-1β, which are increased in patients with AAH, also reduce tight junction integrity so there may be a positive feedback loop in these patients[34]. Furthermore, the loss of probiotic bacterial species may reduce barrier protection since transfer of *Lactobacillus* ameliorates ALD in a mouse model[35]. In a more acute murine model of alcoholic steatohepatitis *Lactobacillus* treatment restored intestinal integrity, reduced oxidative stress and improved histological liver damage[36].

The leaky gut seen in patients with AAH results in presentation of PAMPs to hepatic innate immune cells, particularly Kupffer cells. Chronic alcohol misusers have higher levels of endotoxin [lipopolysaccharide (LPS)] systemically[37] as well as in the portal vein[38] suggesting that there is greater exposure of the liver to microbial components. Interestingly, this defect may be rapidly reversible: in a study of alcohol dependent patients, both intestinal permeability and LPS levels were elevated compared to normal controls but returned to normality after 3 wk of abstinence[39].

Most of our understanding of gut dysbiosis in AAH comes from animal models and patients with chronic ALD. Future study should be directed at analysing the gut microbiome of patients with AAH compared to healthy controls and patients with cirrhosis. A well powered RCT should then be conducted to test the efficacy of specific probiotic therapy designed to restore the microbiome. Work in this area is underway as demonstrated by a single trial registered at Clinicaltrials.gov which is investigating 7 d of treatment with several probiotic regimes but this is not powered to detect survival differences[40]. Prevention of the disruption of gut tight junctions is also an appealing therapeutic target but this is likely to be due to a complex interplay between gut bacteria and innate immunity and a more detailed understanding of the mechanisms of disruption is first required.

TOLL-LIKE RECEPTORS

Toll-like receptors (TLRs) are innate pattern recognition receptors for a wide variety of PAMPs such as microbial components, endogenous molecules and danger signals[41]. The TLR family consists of 10 receptors principally expressed by granulocytes and cells of myeloid lineage. The net action of ligand binding is the activation of the nuclear factor kappa B (NF-κB), activating protein 1 (AP1) and interferon regulatory factor (IRF) families and rapid and robust transcription of pro-inflammatory mediators[42]. In animal models of ALD, increased expression of TLR1, 2, 4, 6, 7, 8, and 9 is reported with increased sensitivity to their respective ligands[43] while in humans, TLR2, 4 and 9 are upregulated in neutrophils from AAH patients[44] suggesting that the TLR signalling pathway is important in the pathogenesis of the disease.

LPS acting *via* TLR4 appears to be the most important interaction in the pathogenesis of AAH. Mice with non-functional mutant TLR4 are protected from alcoholic liver injury[45] as are those with inactivated Kupffer cells[46]. However, both Kupffer cells and non-bone marrow derived liver cells are involved in TLR4-mediated alcoholic liver injury shown by development of ALD in TLR4$^{-/-}$ mice transferred with wildtype bone marrow cells[47]. Furthermore, deficiency in IL-1 receptor associated kinase (IRAK)-M (the negative regulator of TLR4) conferred more severe ALD[48].

The action of LPS and other PAMPs *via* innate receptors on intrahepatic cells initiates a sequence of pro-inflammatory responses. Patients with AAH have elevated levels of pro-inflammatory cytokines mostly produced by myeloid cells including IL-1, IL-6, IL-8 and TNFα (the latter is also related to disease severity)[49-51]. This results in hepatocellular damage *via* TNF Receptor 1 and intrinsic death pathways[52]. In addition, alcohol sensitises Kupffer cells to the effects of LPS[53] and hepatic macrophages and Kupffer cells produce reactive oxygen species in response to chronic alcohol exposure or LPS[54], driving further liver damage.

The TLR system has the potential to be modulated to reduce pro-inflammatory signalling in AAH but still requires more thorough evaluation. TLR expression has been studied in detail in neutrophils from patients with AAH but blockade of the overexpressed TLRs did not result in restoration of normal neutrophil function[44]. The function of other immune subsets with high TLR expression (especially monocytes, macrophages and Kupffer cells) should also be examined.

CHEMOKINES

Stimulation of both immune and non-immune intrahepatic cells results in the secretion of an array of soluble mediators including cytokines and chemokines which co-ordinate the subsequent immune response and determine the balance between liver damage and resolution of inflammation. Chemokines and their respective receptors control the influx of leucocyte subsets into the liver and have been shown to play important roles in shaping the immune response in a variety of liver diseases[55]. Chemokines are low molecular weight proteins which bind to transmembrane receptors triggering a signalling cascade which alters integrin expression allowing interaction with endothelial adhesion molecules. The gradient of chemokine expression increases near the site of inflammation, which ensures the leucocyte is attracted to the appropriate site before migrating through the vascular endothelium.

Interest in chemokines and their receptors as therapeutic targets has increased over recent years since they can control the ingress of specific pro-inflammatory leucocyte subsets into sites of inflammation or injury. Currently, a number of clinical trials evaluating several chemokine and chemokine receptor antagonists for the treatment of inflammatory diseases including asthma, inflammatory bowel diseases and primary biliary cirrhosis have been registered at clinicaltrials.gov. In the context of AAH, many different chemokines have been implicated and the challenge is to determine which pathway to block. Neutrophils may be the most appropriate target since liver tissue from patients with AAH demonstrates a significant neutrophilic infiltration, the degree of which correlates with disease severity[56].

Ischaemic models of acute liver injury demonstrate that neutrophils are activated by TNFα, IL-1β and IL-17 and recruited by CXC chemokines such as CXCL1 (GROα) and CXCL8 (IL-8)[57]. Elevated levels of these chemokines among others have been confirmed in transcriptome microarray and PCR analysis of homogenised liver biopsy material from patients with AAH[58]. Levels of CXCL1, 5, 6 and 8 were all elevated in AAH *vs* normal liver and correlated with neutrophil infiltration and degree of portal hypertension and were associated with a poor prognosis at 90 d[58]. However, the exact role of the neutrophil in the pathogenesis of AAH is unclear[11] and the control of their entry into the liver is not fully understood. Moreover, chemokines that are known to attract neutrophils will also attract other immune cell types. CXCL1, 5, 6, and 8 specifically attract both neutrophils and monocytes which both express the relevant CXCR1 and CXCR2 receptors for these chemokines. Therefore, a clearer understanding of the complex pathways of leucocyte trafficking in AAH is required and blockade of a single component of the pathway may not translate into a clinical benefit[59].

The same transcriptome study also identified CCL20 as being the third most upregulated gene expressed in AAH liver tissue compared to controls[58]. CCL20 binds to CCR6 which is expressed on the Th17 subset of T cells as well as on hepatic stellate cells (HSCs) and $\gamma\delta$T cells and is likely to play an important role in the adaptive immune response.

TH17 CELLS

The Th17 cell subset, defined by its production of IL-17 and expression of RORγt, is derived from naïve $CD4^+$ T cells under the influence of cytokines IL-1β and IL-6[60]. As well as being important in the clearance of extracellular pathogens, it is also implicated in the pathogenesis of several autoimmune[61] and inflammatory diseases including ALD and AAH[62,63]. IL-17 enhances the inflammatory response by stimulating a wide variety of cells including monocytes, endothelial cells and fibroblasts, to secrete CXCL8, a neutrophil chemoattractant[64]. In a positive feedback loop, IL-17 also stimulates CCL20 secretion, itself a Th17 chemoattractant, with high levels of its receptor CCR6 expressed by Th17 cells[65,66].

Th17 cells have been implicated in the pathogenesis of AAH[63]. IL-17 protein in serum from patients with AAH was elevated as was peripheral $CD4^+$ T cell capacity to produce IL-17 on stimulation compared to healthy controls. In AAH liver tissue, there was an enrichment of IL-17^+ cells which were T cells and neutrophils and numbers correlated with degree of fibrosis. Furthermore, it was shown that HSCs have the IL-17 receptor and their secretion of important fibrotic mediators was dependent on IL-17[34].

The liver transcriptome study[58] suggests that in AAH, the high expression of CCL20 results in the infiltration of Th17 cells. Further work has shown that CCL20 levels correlate with clinical severity score, degree of portal hypertension and survival in patients with AAH and, using an animal model of acute on chronic ALD [mice treated with carbon tetrachloride (CCl_4), ethanol and LPS], that macrophages and HSCs are the primary source of CCL20[67]. In addition, exposure of primary HSCs *in vitro* to CCL20 promotes fibrogenesis[67].

Therefore, it is likely that CCL20 mediates hepatic inflammation and fibrosis in AAH by direct effects on HSCs and *via* recruitment of Th17 cells. The Th17 cytokine IL-22 has also been shown to be upregulated in peripheral blood of patients with AH and increased levels subsequently predicted better patient outcome[68]. Plasma IL-17 (but not IL-21 or IL-23) was also elevated compared to healthy controls but not related to outcome. Data was not presented to determine whether IL-17 and IL-22 were co-expressed so it is possible that IL-22 may be from the novel Th22 cells rather than pathogenic Th17 cells and hence may have a hepatoprotective effect. The protective

effects of IL-22 have been demonstrated in a chronic/binge ethanol feeding model where administration of exogenous IL-22 ameliorated liver injury and oxidative stress *via* a STAT3 mechanism[69]. A phase 2 clinical trial of recombinant human IL-22 is currently underway in patients with AAH.

Interestingly, a recent study extensively characterised pathogenic Th17 cells from healthy controls and then confirmed that they were enriched in the peripheral blood and inflamed gut of patients with Crohn's disease[70]. These pathogenic Th17 cells were resistant to steroid-mediated T cell suppression in terms of pro-inflammatory cytokine production and proliferation[70]. An animal model of airways inflammation has also suggested that Th17 cells are steroid resistant[71]. This has been further assessed by gene profile analysis, cytokine expression and proliferation in Th17 cells derived from patients with autoimmune uveitis as well as murine Th17 cells in an experimental model of uveitis, which were shown to be resistant to steroid treatment[72]. The latter study also demonstrated that both human Th17 cells *in vitro* and murine Th17 cells *in vivo* were selectively inhibited by the calcineurin inhibitor, ciclosporin A (CsA)[72].

The observation that Th17 cells are enriched in AAH but may be resistant to steroid treatment may help to explain why some patients do not respond clinically to steroid treatment. Further research is needed to clarify whether these cells are indeed steroid resistant in the context of AAH and whether there are different characteristics in this T cell subset between steroid responders and non-responders. As suggested by the recent *in vitro* and *in vivo* data discussed above[72], rescue therapy with CsA in patients with steroid resistant AAH may be efficacious. It is already well-established therapy for the treatment of steroid resistant acute severe ulcerative colitis albeit with significant toxicities and side-effects[73] but the risk of sepsis with such a potent immunosuppressive agent would be too high to justify a clinical trial in AAH patients unless a method to specifically target Th17 cells could be found. Alternatively, if steroid non-responders could be accurately identified, for example by the bioassay recently reported by our group[74], CsA could be selectively offered to these patients with the highest risk of death from AAH. Another perhaps less toxic approach is to prevent Th17 cell ingress to the liver with anti-CCL20 antibody, which may be particularly efficacious in patients who do not respond to steroid treatment.

T CELL EXHAUSTION

The phenomenon of a primed immune system but with failure of pathogen defence may be caused by a defect in effector cell negative regulatory signalling. Inhibitory pathways exist to maintain immune homeostasis to prevent over-activation and exhaustion of immune cells but allow appropriate clearance of pathogens and tumours. Several pathways exist; the best studied involves the cytotoxic T-lymphocyte associated protein 4 (CTLA-4) family. CTLA-4, a T cell surface receptor, by binding to the same molecules on antigen presenting cells, competitively antagonises CD28, the T cell co-stimulation receptor and prevents T cell activation[75]. Deletion of CTLA-4 in murine models leads to the development of fatal multiorgan autoimmune disease[76], probably as a result of unchecked CD28-mediated T cell stimulation, demonstrating its importance in immune control. A member of the CTLA-4 family, programmed death 1 (PD-1) serves a similar purpose to maintain balance of effector T cell function[77] while T-cell immunoglobulin and mucin domain 3 (TIM-3) also has potent inhibitory functions on both T cells and innate immune cells[78]. It has recently been proposed that inappropriate expression of PD-1 and TIM-3 plays a role in the immune paresis seen in AAH[79].

Impairment in both innate and adaptive immunity was seen in patients with AAH; poor neutrophil antimicrobial function and reduced T cell interferon-γ (IFNγ) production was demonstrated. In addition, PD-1, TIM-3 and their ligands were overexpressed on T cells from the peripheral blood of patients with AAH compared to patients with ALD or healthy controls but when these receptors were blocked the immune defect was overcome. It was shown that the overexpression of these inhibitory molecules was mediated by LPS binding to TLR4 on $CD14^+$ monocytes[79].

These intriguing data inform new paradigms of how an active immune system exposed to many PAMPs can remain impaired at pathogen clearance and suggest that gut dysbiosis is central to the pathogenesis of the disease. However, the effect of other TLR ligands, cytokines and the direct effect of alcohol on negative regulatory molecule expression (PD-1, TIM-3 and others) have not been investigated. It is important to note that this study was conducted using peripheral blood derived immune cells which may not accurately reflect what occurs within the liver and therefore a similar effect needs to be demonstrated on intrahepatic immune cell subsets. Finally, careful consideration of how to translate these findings to a possible therapy is required. Restoration of immune homeostasis involves the rebalancing of pro- and anti-inflammatory pathways. Improving host defence by blockade of these regulatory pathways may result in the tipping of the balance too far in favour of immune activation which may drive further liver damage. An experimental model of AAH should be first employed to assess whether this strategy would have a beneficial effect.

GRANULOCYTE COLONY-STIMULATING FACTOR

The cytokine granulocyte colony-stimulating factor

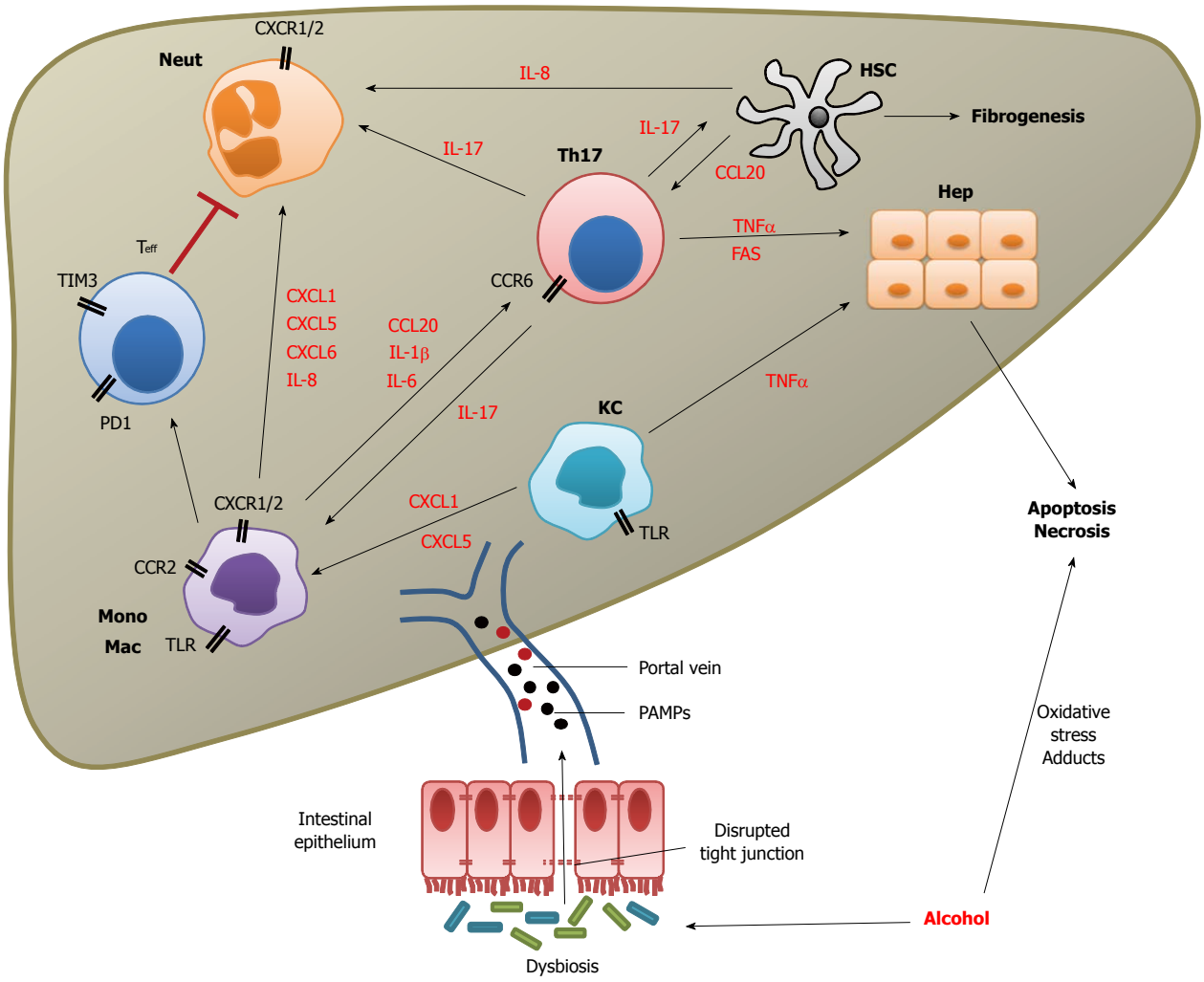

Figure 1 Immune dysfunction in acute alcoholic hepatitis. Alcohol has a direct effect on hepatocytes by production of reactive oxygen species causing oxidative stress. It also results in the production of acetaldehyde adducts which can cause DNA damage, mutagenesis and direct cell death. Alcohol consumption also leads to intestinal bacterial overgrowth and gut dysbiosis with a loss of *lactobacillus* and increase in *proteobacteria* species. Dysbiosis together with the direct effect of acetaldehyde (a metabolite of alcohol) and pro-inflammatory cytokines disrupts epithelial tight junctions and allows the escape of pathogen-associated molecular patterns (PAMPs) into the portal circulation. Within the liver PAMPs are presented to Toll-like receptors (TLRs) on myeloid cells including monocytes (Mono), macrophages (Mac) and Kupffer cells (KC) stimulating release of cytokines and chemokines. In addition, TLR4 activation on monocytes leads to upregulation of negative inhibitory molecules programmed death 1 (PD-1) and T-cell immunoglobulin and mucin domain 3 (TIM-3) on effector T cells (T_{eff}), which in turn inhibit neutrophil (Neut) anti-microbial functions. Chemokines and cytokines coordinate the infiltration and stimulation of other immune cells in particular neutrophils, monocytes (both by CXCL1, 5, 6 and IL-8) and Th17 cells (by CCL20). Th17 cells further increase neutrophil infiltration and also stimulate hepatic stellate cells (HSCs) to produce fibrogenic mediators. TNFα and Fas produced by T cells also leads to hepatocyte cell death by apoptosis through the Fas and TNF receptor pathways.

(G-CSF) stimulates bone marrow production of granulocytes and haematopoietic stem cells and is involved in the proliferation and differentiation of neutrophils but may also play a role in hepatic regeneration[80]. G-CSF treatment enhances the bactericidal and phagocytic capacity of human neutrophils from healthy subjects as well as impaired neutrophils from HIV-1 infected individuals[81]. It is therefore an appealing therapy for AAH which has the potential to both enhance neutrophil function and hepatocyte regeneration.

G-CSF was well tolerated in patients with cirrhosis and alcoholic steatohepatitis; 5 d of treatment was associated with an increase in circulating $CD34^+$ cells (a surrogate for haematopoietic stem cells), increased serum hepatocyte growth factor and proliferation of hepatic progenitor cells in day 7 liver biopsy specimens. However, there was no change in liver function compared to the control group[82]. A randomised open label trial of G-CSF treatment of Acute on Chronic Liver Failure (of which 57% had alcoholic hepatitis as the underlying aetiology) demonstrated increased hepatic $CD34^+$ cells after 28 d and significantly improved 60 d survival[83]. A recent open label RCT of 5 d of G-CSF *vs* standard care (including pentoxifylline) in the treatment of patients with AAH resulted in a greater number of serum $CD34^+$ cells and improved 3 mo survival compared to standard care[84].

These trials have demonstrated the potential benefit of G-CSF in the treatment of AAH but con-

firmation of its benefit in a large double-blind RCT is needed. Further study is required to elucidate the mechanisms of G-CSF action and evaluate its benefit in the context of steroid resistant disease.

CONCLUSION

Evidence drawn from studies on patients with AAH and chronic ALD as well as animal models has enhanced our understanding of the immune mechanisms that occur in AAH. In summary, chronic alcohol consumption leads to gut dysbiosis, disrupting the gut epithelial integrity and allowing the presentation of PAMPs to intrahepatic cells *via* the portal circulation. This in turn causes activation of a network of cells resulting in the alteration of surface molecular patterns and the release of a plethora of soluble mediators, which co-ordinates the influx of immune cells into the liver. In the context of AAH, these immune cell subsets cause direct damage to hepatocytes and stimulate HSCs to produce fibrogenic molecules leading to liver cell death and fibrosis. Additionally, there is evidence of immune paresis with poor innate cell responses and increased T cell exhaustion resulting in a reduced ability to prevent bacterial infection (Figure 1).

Many of the mechanisms of pathogenesis require confirmation and testing in patients with AAH but have the potential to yield new therapeutic targets in the future. Here, we have highlighted the gut microbiome, the expression of TLRs on different myeloid cell subsets, the chemokine pathway, the steroid responsiveness of Th17 cells, T cell exhaustion and G-CSF therapy as priority areas for further research.

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Protective Effect of Magnesium Isoglycyrrhizina on Acute Hepatic Injury in Mice

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OBJECTIVE: To investigate the protective effect of MgIG on acute hepatic injury in mice induced by D-GalN. METHODS: The acute hepatic injury model of mice was induced by D-GalN. The mice were divided into control group, patho-model group, MgIG high and low dose group,compound glycyrrhizin group,Essentiale N group and Tiopronin group. The changes of activity of ALT, AST in serum and MDA, SOD, XOD,GSH-PX,T-AOC, NO, NOS, iNOS content in liver tissue were measured. Then by HE staining the pathologic change of liver tissue was examined under light microscope. RESULTS: MgIG could decrease(P0.01) the activities of ALT, AST in serum and MDA, XOD,NO,NOS, iNOS content in liver tissue; and increase the SOD, GSH-PX, T-AOC content in liver tissue. It was observed that MgIG could significantly alleviate the necrotic pathologic change of liver tissue. CONCLUSION: MgIG has protective function against acute liver injury in mice induced by D-GalN.

【Key Words】: **Magnesium isoglycyrrhizinate D-GalN Mouse Acute hepatic injury Protective effect**

【CateGory Index】: R965

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Influence of antioxidants (mannitol and allopurinol) on oxygen free radical generation during and after cardiopulmonary bypass

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ABSTRACT Oxygen-derived free radicals (O_2^-, H_2O_2, OH·) are produced during oxidative metabolism, ischemia and reperfusion, and cardiopulmonary bypass (CPB). When oxygen free radical production exceeds scavenging capacity, peroxidation of structural lipids in cell membranes can occur with potentially injurious consequences. In this prospective study, 45 patients were evaluated to determine the effect of CPB on oxygen free radical generation. Twenty patients in group I were controls. Exogenous oxygen free radical antioxidants were administered before bypass to patients in group II ($n = 15$, mannitol) and group III ($n = 10$, allopurinol). In group I, plasma H_2O_2 increased during extracorporeal circulation from 65 ± 6.0 to 125 ± 12 μM/ml ($p < .001$). At similar sampling intervals, plasma H_2O_2 levels were significantly lower in group II ($p < .03$) and group III ($p < .05$) when compared with those in group I. Red blood cell H_2O_2 did not change in group I or group II. White blood cell H_2O_2 levels decreased in group I ($p < .04$) and group II during CPB. (Intracellular concentrations of H_2O_2 were not obtained in group III patients.) We conclude that cytotoxic oxygen radicals are generated during CPB and that pretreatment with free radical antioxidants, mannitol or allopurinol, may minimize the free radicals available for lipid peroxidation of biomembranes.
Circulation 74(suppl III), III-134, 1986.

ELECTRON REDUCTION of molecular oxygen by cytochrome oxidase produces oxygen free radicals (O_2^-, H_2O_2, and OH·) in metabolically active cells. These toxic intermediates are also formed during ischemia and reperfusion and after cardiopulmonary bypass (CPB). Catalase, superoxide dismutase, and glutathione peroxidase are intracellular enzymes that catalyze the disproportionation of these radicals preventing peroxidation of lipid membranes.[1-3] Specifically, free radical production is greatly increased in polymorphonuclear leukocytes (PMNs) via the flavoenzyme NADPH oxidase after leukocytes have been stimulated by complement C5a fragments.[4]

Chenoweth et al.[5] and Cavarocchi et al.[6] have demonstrated that C3a and C5a complement fragments and transpulmonary sequestration of leukocytes occur during CPB. In addition, studies suggest that the C5a fragment avidly binds to neutrophils and does not freely dissociate in plasma.[7] Weissmann et al.[8] and Del Maestro et al.[9] showed that stimulated PMNs release oxygen free radicals into the extracellular space. Sacks et al.[10] have demonstrated cytotoxic reactions due to oxygen radical production occurring when leukocytes are in close approximation to endothelial cells. Significant cell damage occurred only in the presence of C5a aggregated white blood cells. Thus the complement-activated leukocytes were a source of the oxygen free radicals. These workers and others[5, 9-17] have implicated superoxides and/or their byproducts as mediators of capillary endothelial damage. These theories provide a foundation for the hypothesis that the pathophysiologic mechanism of shock lung or adult respiratory distress syndrome involves oxygen free radicals.

The work of Tate and Repine[15] suggests that hydrogen peroxide or peroxide by-products are the main species responsible for the alterations in alveolar-capillary membrane permeability. The purpose of this study was to quantitate changes in oxygen free radical production during CPB as indicated by plasma hydrogen peroxide concentrations. We also investigated the effect of exogenous oxygen free radical antioxidants on plasma hydrogen peroxide. In addition, red cell and leukocyte peroxide concentrations were determined in selected patients.

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Materials and methods

This prospective trial involved 45 patients undergoing coronary artery bypass or cardiac valve replacement. Patients were placed into one of three groups: group I, 20 patients undergoing standard CPB; group II, 15 patients undergoing CPB with the addition of 12.5 g of mannitol to the pump prime; group III, 10 patients receiving 1200 mg of allopurinol preoperatively (600 mg po at 9 P.M. the night before surgery and 600 mg po at 5 A.M. on the day of surgery). Clinical characteristics of the patients are presented in table 1.

The protocol required withdrawal of five samples of 10 ml of arterial blood for assay of H_2O_2 at the following times: (1) after induction of general anesthesia but before sternotomy, (2) after 30 min of CPB, (3) immediately after CPB, (4) 10 min after infusion of protamine, and (5) after sternal closure. Plasma concentrations of H_2O_2 were corrected for hemodilution resulting from crystalloid pump prime as follows: $[H_2O_2]$ corrected = $[H_2O_2]$ measured × initial packed red cell volume/packed red cell volume during CPB.

CPB equipment consisted of a Sarnes roller pump, a Bentley BOS-10 bubble oxygenator, a Shiley CARDF, or a CARDF Plus cardiotomy reservoir and a Pall arterial filter. The extracorporeal circuit was primed with 2000 ml of crystalloid solution and 6000 U of bovine lung heparin, and whole blood was added to the prime for patients with preoperative anemia (hemoglobin < 10.0 g/dl). Before CPB, the patients were anticoagulated with bovine lung heparin administered intravenously at a dose of 350 U/kg body weight to achieve an activated clotting time in excess of 450 sec. Perfusion was maintained at a flow of 2.2 to 2.4 liters/min/m^2 and a temperature of 20° to 25° C. Hypothermic potassium cardioplegic solution (crystalloid or blood) was used during aortic cross-clamping for myocardial protection. After discontinuation of CPB, protamine (1.3 mg/100 U of heparin) was administered over 5 min to neutralize the heparin.

Plasma hydrogen peroxide assay. A hydrogen peroxide–specific assay was used in these experiments.[18] The specificity of this reaction depended on a peroxidase catalyzed oxidation reaction ($2H_2O_2$ + 4 aminoantipyrine + phenol in the presence of peroxidase form a chromogen + $4H_2$). The colored product is a quinoneimine dye with a maximum absorption at 505 nM that was measured spectrophotometrically.

Red blood cell intracellular H_2O_2 preparation. A red blood cell lysate was prepared according to a technique described by Maral et al.[19] The stable dye formed after the addition of color reagent to the red blood cell lysate was quantitated by standard colorimetric techniques.

Intracellular white blood cell H_2O_2 preparation. The leukocyte preparation was that of Percy and Brady[20] with the addition of two 45 sec "shock" treatments to eliminate residual red blood cells. In this procedure, 5% dextran and a sample of whole blood were mixed and allowed to settle at ambient temperature. The supernatant was aspirated, and the infranatant was centrifuged to obtain a button of white blood cells. These cells were then shocked by adding distilled H_2O and gently mixing for 45 sec. A solution of 1.8% NaCl was then added to stop hemolysis. After centrifugation, the supernatant was aspirated and the shock treatment was repeated. A 1:500 dilution of white cells and isotonic saline was made, and cell counts were performed with a Coulter counter. The leukocytes were then sonicated and added to the assay solution. This was allowed to react 5 min, and the product formed was measured spectrophotometrically.

Statistical analysis. A paired Students t test was used for intragroup comparisons. Analysis of variance was used to compare continuous variables between different patient groups.

TABLE 1
Characteristics of the patients

	Group I (Control)	Group II (Mannitol)	Group III (Allopurinol)
No. of patients	20	15	10
Age (yr, mean ± SD)	61 ± 11	56 ± 15	59 ± 9
Sex (male/female)	15/5	11/4	9/1
Procedure			
Coronary artery bypass	16	11	10
Valve replacement	4	4	0
CPB time (min, mean ± SD)	97 ± 26	100 ± 25	106 ± 37
Aortic cross-clamp time (min, mean ± SD)	56 ± 17	52 ± 16	61 ± 21

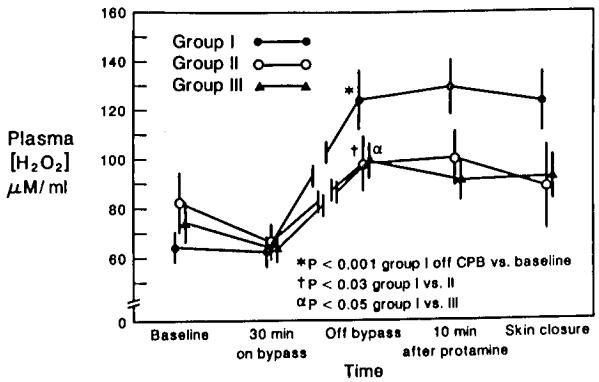

FIGURE 1. Plasma hydrogen peroxide levels during and after CPB.

Results

Plasma H_2O_2 data. The effects of CPB bypass on plasma hydrogen peroxide levels are illustrated in figure 1. Before CPB, plasma H_2O_2 levels were similar in all three groups. In control patients (group I), plasma H_2O_2 levels increased from 65 ± 6 μM/ml before CPB to 125 ± 12 μM/ml at the termination of bypass ($p < .001$). Patients in group II had a statistically nonsignificant increase in plasma H_2O_2 from 83 ± 12 μM/ml before CPB, to 99 ± 11 μM/ml at the end of CPB. In group III patients, plasma H_2O_2 was 75 ± 8 μM/ml before CPB and 100 ± 7 μM/ml after CPB ($p < .04$). The rise in H_2O_2 levels after CPB in group I was significantly greater than that in group II ($p < .03$) or group III ($p < .05$). Plasma H_2O_2 values in group III remained significantly lower than those in group I ($p < .01$) after protamine infusion and skin closure.

Leukocyte intracellular H_2O_2. In group I (control) the hydrogen peroxide concentration in leukocytes decreased from an initial level of 18 ± 1 μM/10^{10} cells to a postbypass value of 15 ± 1 μM/10^{10} cells ($p < .04$) (figure 2). There were no significant changes in H_2O_2 when comparing postprotamine (15 ± 1) and skin closure values (16 ± 1) with the post-CPB (15 ± 1)

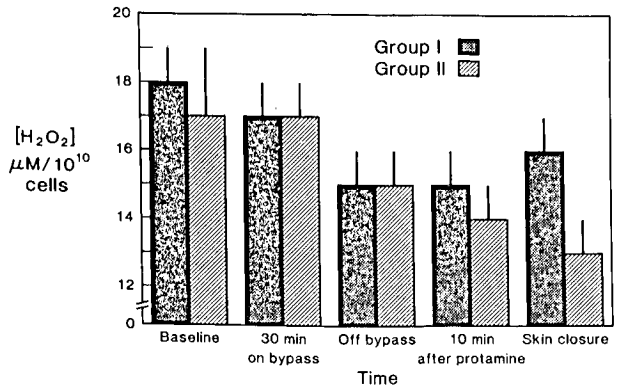

FIGURE 2. Leukocyte intracellular hydrogen peroxide levels during and after CPB.

levels. Leukocyte H_2O_2 in group II (mannitol) decreased from 17 ± 2 to 15 ± 1 $\mu M/10^{10}$ cells (p = NS). There were no significant differences when comparing postprotamine (14 ± 1) and post–sternal closure values (13 ± 1) with the immediately post-CPB value (15 ± 1). Leukocyte intracellular H_2O_2 was not measured in group III patients.

Red blood cell intracellular H_2O_2. In group I, intracellular H_2O_2 decreased from 123 ± 14 to 102 ± 7 μM/ml lysed packed red blood cells during CPB (figure 3). After extracorporeal circulation, red blood cell H_2O_2 did not change but remained significantly lower than values observed in group II ($p < .05$). The intracellular H_2O_2 concentrations were not ascertained in group III patients.

Discussion

Pulmonary gas transport mechanisms are often impaired after extracorporeal circulation, and there are several possible mechanisms. One possible mechanism that has been proposed is increased permeability of the pulmonary capillary membrane. Hydrogen peroxide, superoxide, and hydroxyl radicals have been implicated in this phenomenon.[11-16] The source of these toxic radicals appears to be PMNs and macrophages.[21-25] It is known that PMNs go through a hypermetabolic phase (oxygen burst) after being stimulated by C5a or bacteria,[4, 26] causing a transient increase in oxygen free radical production. Chenoweth et al.[5] and Cavarocchi et al.[6] have documented complement activation and transpulmonary leukosequestration during CPB.

In this study of patients undergoing extracorporeal circulation, we found significant elevations in plasma H_2O_2 after bypass. Hydrogen peroxide is a good marker for oxygen free radical production because of its stoichiometric relationship (2.1:1.0) to O_2^- and its comparative stability relative to superoxide.[27] The plasma elevations of H_2O_2 observed in our studies are greater than the steady-state levels reported by Chance et al.[1] Our data support the theory that increased oxygen free radical generation participates in the pathophysiology of extracorporeal circulation, but the study does not specifically correlate plasma H_2O_2 accumulation and pulmonary injury.

Our study demonstrates that the addition of mannitol to the pump priming solution retards the rise in plasma H_2O_2 levels observed at the conclusion of CPB. We attribute this effect to the following: first, H_2O_2 spontaneously forms hydroxyl radicals via the Haber-Weiss reaction[8, 28] ($O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH\cdot$), and second, mannitol scavenges $OH\cdot$ by forming a mannitol radical ($MH_2 + OH\cdot \rightarrow MH + H_2O$) that undergoes disproportionation or dimerizes. Theoretically, by mass action, H_2O_2 is decreased secondary to $OH\cdot$depletion. The finding that plasma levels of H_2O_2 were slightly increased (although not statistically significant) over baseline values may indicate incomplete scavenging at the mannitol dose given in this study.

Allopurinol also decreased plasma H_2O_2 levels when compared with control. The usefulness of this agent in preventing postischemic injury has been investigated by many workers.[29-33] It is generally believed that during ischemia ATP is broken down to inosine and then to hypoxanthine. Under anaerobic conditions, xanthine dehydrogenase is also converted to xanthine oxidase. During reoxygenation, hypoxanthine + O_2 in the presence of xanthine oxidase form xanthine, O_2^-, H_2O_2, and $OH\cdot$. Allopurinol is a xanthine oxidase inhibitor that blocks this reaction. The finding that plasma H_2O_2 was significantly decreased compared with control suggests that this pathway is a major source for toxic oxygen radical production during CPB. The demonstration that even with pretreatment with allopurinol H_2O_2 rose from 75 ± 8 to 100 ± 7 μM/ml ($p < .04$) suggests that the NADPH oxidase, glucose oxidase, aldehyde oxidase, and cytochrome oxygenase

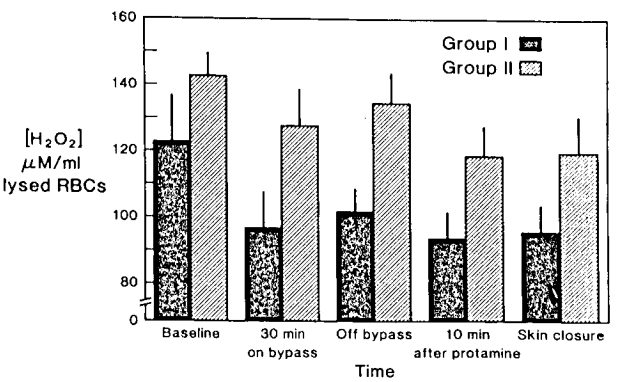

FIGURE 3. Red blood cell intracellular H_2O_2 during and after CPB.

systems contribute to the final plasma peroxide concentration.

PMN intracellular H_2O_2 levels decreased in control and in mannitol-treated patients. It is thought that toxic radicals can diffuse across cell membranes according to concentration gradients and high lipid permeability.[1] Our results reflect steady-state concentrations and not the transient oxygen burst phenomenon. The fact that both treated (group II) and nontreated (group I) groups had similar progressive declines in H_2O_2 supports the hypothesis that mannitol works on extracellular scavenging and does not affect production. The significance of this finding will require further investigation with more direct and sensitive assays for free radical production.

It is known that red blood cells contain substantial concentrations of catalase and superoxide dismutase and that these enzymes protect the cell from toxic oxidative processes. In mannitol-treated patients in whom plasma H_2O_2 rose only slightly during CPB, red blood cell H_2O_2 during and after CPB decreased only slightly. Additional study will be necessary to correlate changes in red blood cell intracellular H_2O_2 and plasma H_2O_2.

In summary, this study documents that CPB causes an increase in oxygen free radicals in plasma. The oxygen free radical antioxidants, mannitol and allopurinol, appear to reduce plasma hydrogen peroxide concentrations in patients after CPB and may be useful in reducing the risks of postperfusion sequelae such as increased capillary permeability by removing these toxic intermediates from plasma.

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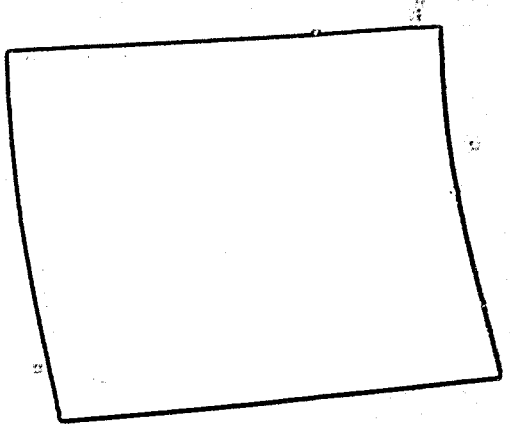

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Efficacy of a glycyrrhizin suppository for the treatment of chronic hepatitis C: a pilot study

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Abstract

Intravenous administration of glycyrrhizin has potential efficacy on decreasing serum aminotransferase levels in patients with chronic hepatitis. However, patients receiving this treatment are recommended to attend hospital regularly for several years. To improve the quality of life for these patients, we developed a glycyrrhizin suppository. In this pilot study, we examined the most effective and safe material contents of the suppository and revealed clinical efficacy for patients with biopsy-proven chronic hepatitis C comparing intravenous administration of glycyrrhizin. As content combinations of the suppository, a mixture of 300 mg of glycyrrhizinic ammonium salt and 60 μg of sodium capric acid, with pH neutralization, was confirmed to be most effective and safe condition, based on analysis of serum glycyrrhizin levels and the grade of rectal irritations in tested patients. The efficacy on decreasing serum alanine aminotransferase levels for 12-week administration of the suppository in 13 patients with chronic hepatitis C was similar to that in another 13 patients intravenously administered glycyrrhizin. Moreover, no serious side effects were observed. In conclusion, the usage of the newly developed suppository of glycyrrhizin can improve the quality of life for chronic hepatitis C patients, especially those who do not respond with viral clearance to interferon therapy. Using this suppository, larger and longer-term studies are needed.

Keywords: Glycyrrhizin suppository; Chronic hepatitis C; Quality of life

1. Introduction

Studies conducted in Western countries patients who acquired hepatitis C virus (HCV) by blood transfusion 15–25 years previously, suggested that 20–30% of them have progressed to liver cirrhosis, including 5–10% with end stage liver disease and 4–8% who died of liver-related causes [1,2]. In the Japanese population too, the incidence of HCV-positive patients with hepatocellular carcinoma has increased [3].

To prevent developing liver cirrhosis and hepatocellular carcinoma for patients with chronic hepatitis C, anti-viral therapies have recently been developed such as interferon and its combination with ribavirin [4–6]. However, totally sufficient effects have still not been obtained regardless of such anti-viral therapies. Therefore, different treatment strategies are required for those who do not respond. As one of these agents, an anti-inflammatory agent, intravenous administration of stronger neo-minophagen C™ (SNMC, Minophagen Pharmaceutical, Tokyo, Japan), which mainly consists of glycyrrhizin (GL), has potential efficacy for decreasing aminotransferase levels in patients with chronic hepatitis [7–12]. However, patients receiving SNMC are recommended to attend hospital regularly at least three times per week for several years. Most chronic hepatitis patients are asymptomatic. To improve the

Abbreviations: SNMC, stronger neo-minophagen C; GL, glycyrrhizin; HCV, hepatitis C virus; ALT, alanine aminotransferase; HPLC, high-performance liquid chromatography; CAH, chronic active hepatitis.

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quality of life for these patients, we developed a GL suppository.

In this pilot study, we developed the most effective and safe contents of the suppository, and estimated the clinical efficacy of the GL suppository comparing with intravenous administration of SNMC.

2. Materials and methods

2.1. Development of GL suppository

Glycyrrhizinic ammonium salt was used as a major substrate of the GL suppository. In addition, sodium capric acid was used as a promoting absorbent. Various dosage combinations of the two materials were prepared and the efficacy compared by analysis of the serum GL levels in three patients receiving each suppository. Considering drug-delivery systems, the effects of pH neutralizing treatment were evaluated. Rectal irritation for patients receiving the suppository was evaluated according to three degrees as follows: mild as a slight irritation, moderate as an intermediate irritation, and severe as an unbearable irritation.

2.2. Determination of the serum GL level

The serum GL level was determined by semi-micro high-performance liquid chromatography (HPLC) according to a previously described method [13]. Sera were collected 1 h after the administration after obtaining the patients' informed consent, and were stored at -70 °C until the assays were conducted.

2.3. Patients and therapeutic protocol

Twenty-six patients with biopsy-proven chronic hepatitis C in the Self-Defense Forces Central Hospital (Tokyo) were subjected in this study. All patients were positive for anti-HCV antibody and HCV RNA. These patients were randomly assigned into two groups by means of sealed, opaque, numbered envelopes: 13 patients received two pieces of GL suppository per day and the remaining other 13 patients were intravenously administered 40 ml SNMC (containing 80 mg of GL) per day, after obtaining the patients' informed consent. No significant differences in mean age, gender distribution, mean alanine aminotransferase (ALT) level, HCV RNA load, variations of HCV genotype, or pathological diagnosis adopted prior treatments were found between the two groups (Table 1). HCV RNA and HCV genotype were determined by conventional PCR and sequence specific priming-PCR (SSP-PCR). Pathological diagnosis was performed according to Ludwig's classification [14]. The therapeutic periods in both groups were set at 12 weeks. Subsequently, the frequencies of the major presenting side effects such as hypernatremia, hypokalemia, hypertension, and myopathy were compared.

All procedures in this study were performed with the approval of the local ethical committee and conducted under strict informed consents.

2.4. Statistic analysis

The findings in the two groups were compared using the χ^2-test and Welch's t-test (two-tail). P values < 0.05 were considered significant.

3. Results

3.1. Serum GL levels under various conditions

Considering the efficacy and drug-delivery system, we compared mean serum GL levels in tested each three patients under various concentrations of sodium capric acid and GL in the suppository. First, the concentrations of sodium capric acid in the suppository varied from 40 to 80 μg under the same GL concentration (200 mg). As shown in Fig. 1A, the mean serum GL level in patients treated with a suppository with 80 μg of sodium capric acid was the highest, however, it could not be clinically used because severe irritation to the rectal mucous membrane was observed. Therefore, we decided to set the concentration of sodium capric acid of 60 μg in the suppositories.

Secondly, the concentrations of GL ammonium salt in the suppositories varied between 200 and 400 mg under the same sodium capric acid concentration (60 μg). As shown in Fig. 1B, the mean serum GL levels in patients treated with suppositories including 300 and 400 mg of GL were the highest, however, suppositories including 400 mg GL could not be clinically used because irritation to the rectal mucous membrane was severe. Therefore, we decided to set the GL concentrations at 300 mg in the suppositories.

Finally, the effects on pH neutralization to reduce irritation to the rectal mucous membrane were evaluated. Mean serum GL levels in tested patients with the two suppositories were compared with and without the treatment of pH neutralization. As shown in Fig. 1C, the mean serum GL level in the patient receiving the suppository with the treatment of pH neutralization was higher than that without the treatment. Therefore, we decided to treat pH neutralization.

Taken together, we decided the new suppository should show the following conditions: 60 μg of sodium capric acid, 300 mg of GL, and with pH neutralization treatment.

Table 1
Profiles of the study patients with chronic hepatitis C

	Suppository	SNMC	*P*
No. of patients	13	13	
Male:female	6:7	7:6	ns
Mean age (years old)[a]	59.1±4.6	60.5±4.7	ns
Mean AST (IU/L)[a]	102.2±13.7	79.0±8.9	ns
Mean ALT (IU/L)[a]	138.8±20.3	109.5±15.0	ns
Mean HCV RNA load (kcopies/ml)	302±56	341±59	ns
HCV genotype (Ib:Iia)	11:2	10:3	ns
Pathological diagnosis[b] (moderate CAH: severe CAH)	8:5	5:8	ns

All data were adopted prior to treatment. ns, not significantly different. CAH, chronic active hepatitis.
[a] Values are expressed as mean±SEM.
[b] Pathological diagnosis was performed according to Ludwig's classification [14].

3.2. Clinical study

Using this GL suppository, we compared the decreases in the serum ALT levels with SNMC treated patients (suppository vs. SNMC). As shown in Fig. 2A, in both groups, ALT levels at 4, 8, and 12 weeks of administration in both groups were significantly decreased compared with each prior level. However, the ALT levels re-elevated 4 weeks after the end of the administrations. As shown in Fig. 2B, the decreasing ratios of the serum ALT levels during the administration periods were similar between the two groups. There were no patients showing the clearance of serum HCV RNA during the therapies in both groups.

The frequencies of the presenting side effects were compared between the two therapeutic groups. However, no significant differences were observed (Table 2).

4. Discussion

In Japan, SNMC has been empirically used for the treatment of chronic hepatitis since 1950s [7]. In 1983, Suzuki et al. first demonstrated the efficacy of SNMC on biochemical tests in chronic hepatitis using a double-blind control study [8]. Hino et al. also found an improvement in liver histology after treating chronic active hepatitis (CAH) with SNMC [15]. Recently, several studies on the efficacy of SNMC for patients with chronic hepatitis have also been reported from European and Asian countries [9–11]. As discussed above, regular intravenous administration for long periods limits the quality of life of patients. On the other hand, oral GL tablets have been used for these patients, although the biochemical improvements on the orally administered GL was lower than that of intrave-

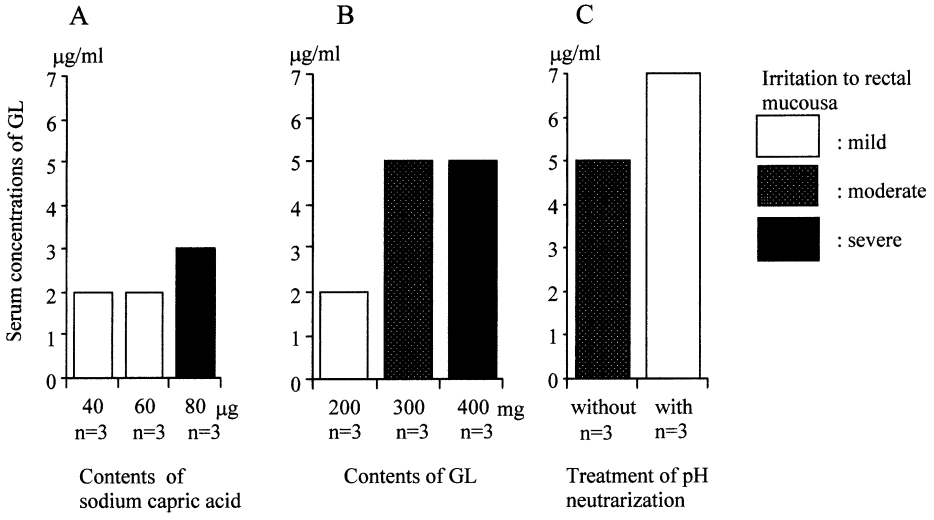

Fig. 1. Mean serum GL levels and the severity of rectal irritation in three patients receiving various doses and conditions of suppositories: (A) the concentration of sodium capric acid varied between 40 and 80 μg; (B) the concentration of GL varied between 200 and 400 mg; (C) with or without pH neutralization. The grades of rectal irritation were revealed as mild (opened bar), moderate (dotted bar), or severe (closed bar).

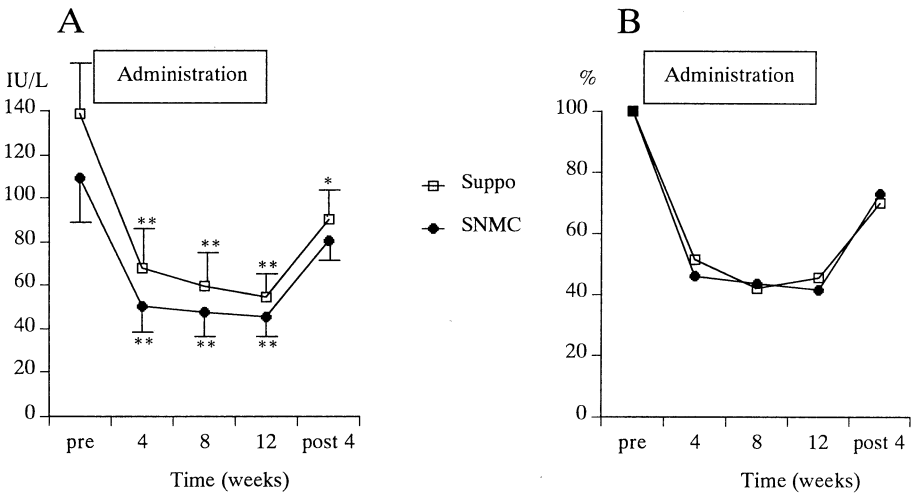

Fig. 2. Changes in the serum ALT levels in the two patient groups (suppository [opened square] and SNMC [closed circle]). (A) Changes of serum ALT levels (mean ± SEM) at each week of administration. (B) Decreasing ratios of the serum ALT levels (each prior level: 100%). $^{**}P < 0.01$, $^{*}P < 0.05$.

nously administered SNMC [16]. Moreover, pseudoaldosteronism such as hypokalemia and myopathy [17,18] in orally administered patients develops more frequently, because orally administered GL alternates 3-monoglucuronyl-glycyrrhetinic acid (GA), which was reported to be a major metabolite causing GL-induced pseudoaldosteronism [19]. Thus, oral administration of GL did not fully improve the quality of life for the patients with chronic hepatitis.

In the present study, we first succeeded in developing an effective and safe GL suppository. Considering the efficacy and the drug-delivery system, we set the conditions of the suppository materials at 60 μg of sodium capric acid, 300 mg of GL, and with pH neutralization. Using this suppository, we investigated the clinical efficacy and side effects in patients with chronic hepatitis C.

We previously reported that serial serum GL levels in patients receiving suppository (300 mg of GL) were not so high comparing those in patients receiving SNMC (40 ml). However, serial serum GA levels in patients receiving GL suppository were almost same as those in patients receiving SNMC [20]. Analysis of serum GA levels, in addition to serum GL, may implicate the dynamic kinetics in administered GL, including portal flow and intestinal re-absorption.

Table 2
Frequencies of the presenting side effects in the two patient groups

	Suppository ($n = 13$)	SNMC ($n = 13$)
Hypernatremia	0	0
Hypokalemia	0	0
Hypertension	0	0
Myopathy	0	0
Others	0	0

The present results suggested that the efficacy of the GL suppository was similar to that of 40 ml intravenously administered SNMC. Furthermore, no serious side effects were observed. Thus, it is suggested that these clinical results may greatly contribute to improve the quality of life of these patients because therapeutic choices can be widened.

In vitro GL has anti-viral activity against herpes simplex virus [21] and hepatitis A virus [22]. However, it is unlikely that GL induces viral clearance in chronic hepatitis C [9,10], because ALT rises directly after cessation of the treatment as shown in this study. It is important to define the changes in HCV RNA load in patients receiving GL for long-term period.

GL administered by suppository is considered to react directly with hepatic cells through portal circulation. On the other hand, intravenously administered GL reacts indirectly via systemic circulation [23,24]. Therefore, the reacting effect on hepatic cells of GL administered by suppository should be higher than that by intravenous administration. However, since it was reported that GL is metabolized to glycyrrhetinic acid by intestinal bacteria containing β-D-glucuronidase [25], further pharmacokinetic studies are needed using the GL suppository.

Recently, it was reported that long-term administration of SNMC for patients with chronic hepatitis C prevented developing liver cirrhosis and hepatocellular carcinoma [26,27]. Therefore, larger and longer-term studies are needed to evaluate the efficacy of the newly developed GL suppository for these patients, especially for those who do not respond with viral clearance to interferon therapy.

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Regular Paper

Radioprotection of DNA by Glycyrrhizic Acid Through Scavenging free Radicals

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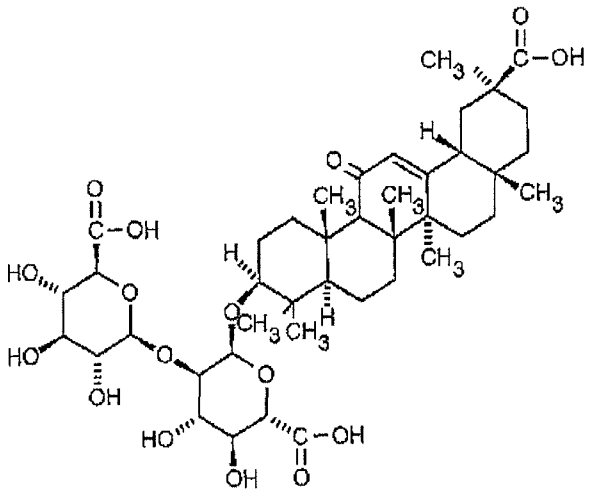
Glycyrrhizic acid/Pulse radiolysis/Radiation protection/Plasmid DNA/DNA strand breaks/Comet assay

Gamma-radiation induced strand breaks in plasmid pBR322 DNA. Glycyrrhizic acid (GZA) protected plasmid DNA from radiation-induced strand breaks, as the disappearance of super-coiled (ccc) form was prevented by the compound with a dose-reduction factor of 2.04 at 2.5 mM concentration. Studies of comet assay on human peripheral blood leukocytes exposed to gamma radiation in the presence and absence of glycyrrhizic acid ex vivo revealed that this compound protected the cellular DNA from radiation-induced strand breaks in a concentration-dependent manner. An intraperitoneal administration of the GZA to mice one hour before exposure to gamma radiation protected cellular DNA from radiation-induced strand breaks in peripheral blood leucocytes and bone marrow cells, as revealed by comet assay. Pulse radiolysis studies indicated that glycyrrhizic acid offered radioprotection by scavenging free radicals. The rate constants for the reaction of glycyrrhizic acid with $OH^{\cdot}$ and e_{aq}^{-} are $1.2 \times 10^{10}\ M^{-1}\ s^{-1}$ and $3.9 \times 10^{9}\ M^{-1}\ s^{-1}$, respectively.

INTRODUCTION

Root extracts of the plant *Glycyrrhiza glabra* L., known as *Yashtimadhu,* in Ayurveda have been used for curing various diseases because of its antiinflammatory, antibacterial, antiviral, and immune-modulating activities[1]. The extract, generally called liquorices, is widely used as a sweetener in food products and chewing tobacco. The active compounds of the extract have been reported to have immuno-modulating[2] antioxidant[3,4]. Our earlier studies have revealed the radio protective effect of the extract on gamma radiation induced DNA and membrane damages[5]. One major component of the extract is glycyrrhizic acid (Scheme I and its absorption spectra in Fig. 1). In biological systems, most of the damage induced by gamma rays is indirect and mediated through free radicals that interact with important macromolecules, including DNA and membranes[6,7]. Membrane damage occurs mainly because of the peroxidation of membrane lipids. In the present study we have investigated the mechanism of radiation protection of DNA by glycyrrhizic acid (GZA) *in vitro*, ex-vivo, and in vivo. The effect of GZA on the production of gamma radiation induced strand breaks in plasmid pBR322 DNA in vitro was monitored by agarose gel electrophoresis from the disappearance of supercoiled form (ccc) of DNA. The radiation-induced strand breaks in cellular DNA of human peripheral blood leukocytes was examined in ex vivo studies by the use of alkaline comet assay. Studies on in vivo radioprotection of DNA were undertaken in mice by an ip administration of GZA before whole-body irradiation and measuring strand breaks in the cellular DNA of peripheral blood leukocytes and bone mar-



Scheme 1

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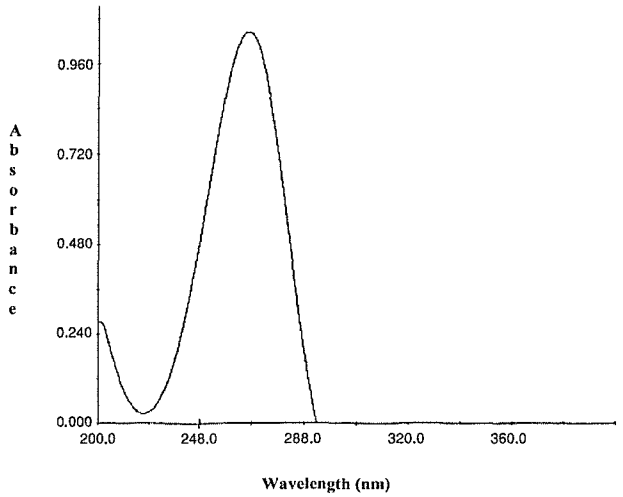

Fig. 1. Absorption spectra of GZA

row cells by alkaline comet assay. To understand the molecular mechanisms of radioprotection by GZA, the bimolecular rate constants for the reactions of primary radicals of water radiolysis with GZA were determined with the pulse radiolysis technique.

MATERIALS AND METHODS

Reagents

Plasmid pBR322 DNA was purchased from Bangalore Genei, Bangalore, India. Glycyrrhizic acid, propidium iodide, and ethylenediaminetetraacetic acid were obtained from Sigma Chemical Co., USA. Other reagents were of analytical grade obtained from local manufacturers.

Collection of human blood

Human blood samples were collected from three healthy nonsmoking volunteers, having a mean age of 25 ± 2 years, by the finger prick method and stored in heparinized eppendorf tubes at ice temperature.

Animals

Male Swiss mice, 8–10 weeks old and weighing 20–25 g each, were selected from an inbred group maintained under standard conditions of temperature (25 ± 2°C) and humidity, with a 12-hour light and 12-hour dark cycle. The animals were provided with food and water ad libitum. Usually four animals were housed in each sterile polypropylene cage containing a sterile paddy as bedding. All animal experiments were conducted with strict adherence to the ethical guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of the Government of India on the use of animals in scientific research.

Gamma irradiation

Plasmid pBR322 DNA (20–25 μg/ml) in 0.1M sodium phosphate buffer pH 7.0 was exposed to ^{60}Co-gamma rays in a Gamma Cell 220 (AECL, Canada) at a dose rate of 9.6 Gy per min in the presence and absence of GZA. For in vivo experiments, the animals after ip administration of GZA (4 mg / kg body weight in double distilled water) were kept in a well-ventilated, acrylic box and whole-body exposed to 4 Gy in a Junior Theratron unit (AECL, Canada) having a dose rate of approximately 0.5 Gy/min. Ex vivo irradiation of human peripheral leukocytes in the presence and absence of glycyrrhizic acid was also done in the Junior Theratron unit.

Effect of glycyrrhizic acid on radiation-induced DNA damage in blood leukocytes and bone marrow cells

The animals were divided into the following groups:
(1) DDW+ sham irradiation
(2) DDW+ 4 Gy irradiation
(3) Glycyrrhizic acid + sham irradiation
(4) Glycyrrhizic acid + 4 Gy irradiation

One hour after the administration of DDW or GZA, animals were whole-body exposed to 0 (sham-irradiation) or 4 Gy by use of the Junior Theratron unit in a well-ventilated acrylic box. After irradiation, all four groups of were kept back in a polycarbonate cage. All animals were sacrificed by cervical dislocation after two hours of irradiation. Blood was collected in heparinized eppendorf tubes by heart puncture, and bone marrow cells were collected from the femurs[8] in ice-chilled 10 mM potassium phosphate buffer, pH 7.4.

Analysis of DNA damage

Radiation-induced damage to plasmid DNA was determined by elctrophoresis in 1% agarose gel[9,10]. The ethidium bromide stained DNA bands were analyzed by the use of Syngene software.

Measurement of DNA damage by the use of Single-Cell Gel Electrophoresis (Comet Assay)

The DNA strand breaks in the bone marrow cells and blood leukocytes were measured by the use of single-cell gel electrophoresis (comet assay) based on the method of Singh *et al.* 2000[11], with some modification[8,12,13]. Fully frosted microscope slides (Gold Coin, Mumbai) were covered with 200 μl of 1% normal melting agarose (NMA) in PBS at 45°C, immediately cover –slipped and kept at 4±°C for 10 min to allow the agarose to solidify. The removal of the cover slip from the agarose layer was followed by an addition of a second layer of 200 μl of 0.5% low-melting agarose (LMA) containing approximately 10^5 cells at 37°C. Cover slips were placed immediately, and the slides were placed at 4°C. After a solidification of the LMA, the cover slips were removed and the slides were placed in the chilled lysing solution consisting of 2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris-HCl, pH 10, 1% DMSO, 1% Triton-X100, and 1%

sodium sarcosinate for 1 hour at 4°C. The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank filled with freshly prepared alkaline buffer (300 mM NaOH, 1 mM Na_2-EDTA, and 0.2% DMSO, pH ≥13.0). The slides were equilibrated in the same buffer for 20 min, and electrophoresis was carried out at 40 V, 350 mA for 15 min. After electrophoresis, the slides were washed gently with 0.4 M Tris-HCl buffer, pH 7.4, to remove the alkali. They were stained with propidium iodide and visualized with a fluorescent microscope (Carl Ziess Axioskop) with bright field phase contrast and an epifluorescence facility attached to a high-performance JVG TK 1280E camera. The integral frame grabber used in this system (Cvfbo1p) is a PC-based card, and it accepts color composite video output of the camera. Fifty cells/slide were captured. The quantification of the DNA strand breaks of the stored images was done with CASP software[14], by which a percent of DNA in the tail, tail migration, tail moment, and Olive tail moment could be obtained directly.

Pulse Radiolysis studies

Pulse radiolysis studies were carried out by irradiating solutions in rectangular quartz cells. The pulse radiolysis setup consists of an electron linear accelerator (Viritech Ltd., U.K.) capable of giving single shots of 50 or 500 ns or 2 μs of 7 MeV electron pulses. The pulse irradiates the sample contained in a 1 cm × 1 cm suprasil quartz cuvette kept about 12 cm from the electron beam window, where the beam diameter is approximately 1 cm. The transient changes in the absorbance of the solution caused by the electron pulse are monitored with the help of a collimated light beam from a 450 W xenon arc lamp. The output from the PMT is fed through a DC offset circuit to the Y input of an L & T storage scope that can transfer 400 mega- samples/sec on each input channel at a 250 ns/div time base range with a sensitivity of 2mV/div and having a bandwidth of 100 MHz. However, further details of the LINAC can be seen elsewhere[15,16]. An aerated 0.05 mol dm^{-3} KSCN solution was used for dosimetry, and the $(SCN)_2^{-\cdot}$ radical was monitored at 500 nm. We calculated the absorbed dose per pulse, assuming G.ε. for the $(SCN)_2^{-\cdot}$ radical to be 23889 dm^3 mol^{-1} cm^{-1} per 100 eV,[17], where G is the radiation chemical yield expressed as the number of molecules formed or destroyed per 100 eV of energy absorbed and ε is the molar absorptivity. The dose employed in the present study, unless otherwise stated, was a typical 16 Gy per pulse.

On irradiation of H_2O, the following primary radicals are produced[18]

$$H_2O \longrightarrow e_{aq}^-, H^\cdot, OH^\cdot \quad (1)$$

For studying the reaction of e_{aq}^- with glycyrrhizic acid, we used an N_2-bubbled aqueous solution containing 0.1 mol dm^{-3} 2-propanol. $OH^\cdot$ and $H^\cdot$ radicals get scavenged by 2-propanol. We measured the rate of reaction of e_{aq}^- with glycyrrhizic acid by monitoring the decay of e_{aq}^- at 700 nm.

To selectively produce $OH^\cdot$ radicals, we saturated solutions with N_2O before pulse irradiation. This resulted in a scavenging of e_{aq}^-, and under these conditions the yield of $OH^\cdot$ radicals is 90% of the total radical yield; $G(OH^\cdot) = 5.4$ mol/100 eV = 5.6×10^{-7} mol J^{-1}. The remaining 10% contribution is of $H^\cdot$ atoms $G(H^\cdot) = 0.6$ mol/100 eV = 0.62×10^{-7} mol J^{-1}.

$$e_{aq}^- + N_2O \xrightarrow{H_2O} OH^\cdot + OH^- + N_2 \quad (2)$$

We measured the rate of reaction of $OH^\cdot$ radicals with glycyrrhizic acid by monitoring the buildup kinetics at 320 nm.

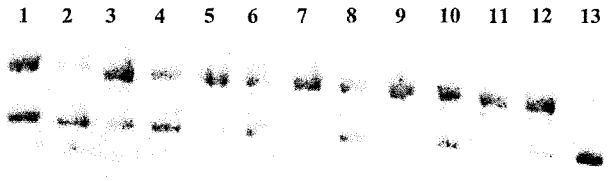

Fig. 2. Agarose gel electrophoresis pattern of pBR322 DNA exposed to various doses of γ-radiation in the presence and absence of 2.5 mM GZA (negative of the photograph). The upper and lower bands depict the open circular (oc) and the supercoiled (ccc) forms, respectively. Lanes 1, 3, 5, 7, 9, and 11 depict the results of pBR322 degradation to oc form at the radiation doses of 10, 20, 30, 40, 50, and 100 Gy, respectively, without the presence of the compound. Lanes 2, 4, 6, 8, 10, and 11 show the effect of the presence of GZA at the same radiation doses, respectively. Lane 13 is the control lane (without radiation).

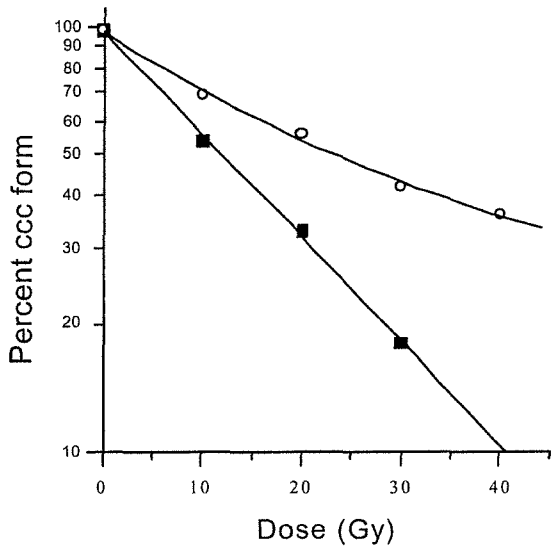

Fig. 3. Presents the quantified data from Fig. 1 analyzed by Syngene Software (Syngene Inc., USA). Each point is the average of the three independent experiments. The dose-reduction factor for glycyrrhizic acid (2.5 mM) for the protection of the DNA in vitro was determined to be 2.04.

The rates of reactions were determined by carrying out the experiments with at least three different concentrations of glycyrrhizic acid, varying by at least a factor of 4. Bimolecular rate constants were derived from plots of the first-order rates vs. concentration. The calculated bimolecular rates are within the limits of ± 15% experimental error.

Statistical analysis

The results are represented as mean ± standard error of mean (mean ± SEM). ANOVA was done by using origin 5.0. The results are significant at $p<0.01$ and $p<0.05$

RESULTS

The reverse of a photo negative of the agarose gel electrophoresis of pBR322 DNA exposed to various doses of γ-radiation in the presence and absence of 2.5 mM GZA is given in Fig. 2. As compared to control, the exposure of plasmid DNA to gamma radiation decreased the relative intensity of the ccc form of DNA. This decrease was dependent on the increase in the radiation dose, as could be seen in lanes 1, 3, 5, 7, 9, and 11. The presence of GZA along with DNA during irradiation prevented this decrease of the ccc form, as evident in lanes 2, 4, 6, 8, 10, and 12. This would suggest that GZA offered protection to DNA in vitro against gamma radiation induced strand breaks. The reduction in the quantity of the supercoiled (ccc) form of plasmid DNA is directly related to the radiation-induced damage, particularly strand breaks in DNA. There was a dose-dependent reduction of the ccc form of plasmid DNA when exposed to 0-100 Gy of gamma radiation, and the presence of 2.5mM GZA prevented this loss of ccc form, as can be seen in Fig. 3. The dose-reduction factor (DRF) for the DNA at 2.5mM GZA calculated from the data is 2.04.

An exposure to γ-radiation ex vivo induces damage to the DNA of human peripheral blood leukocytes, as can be

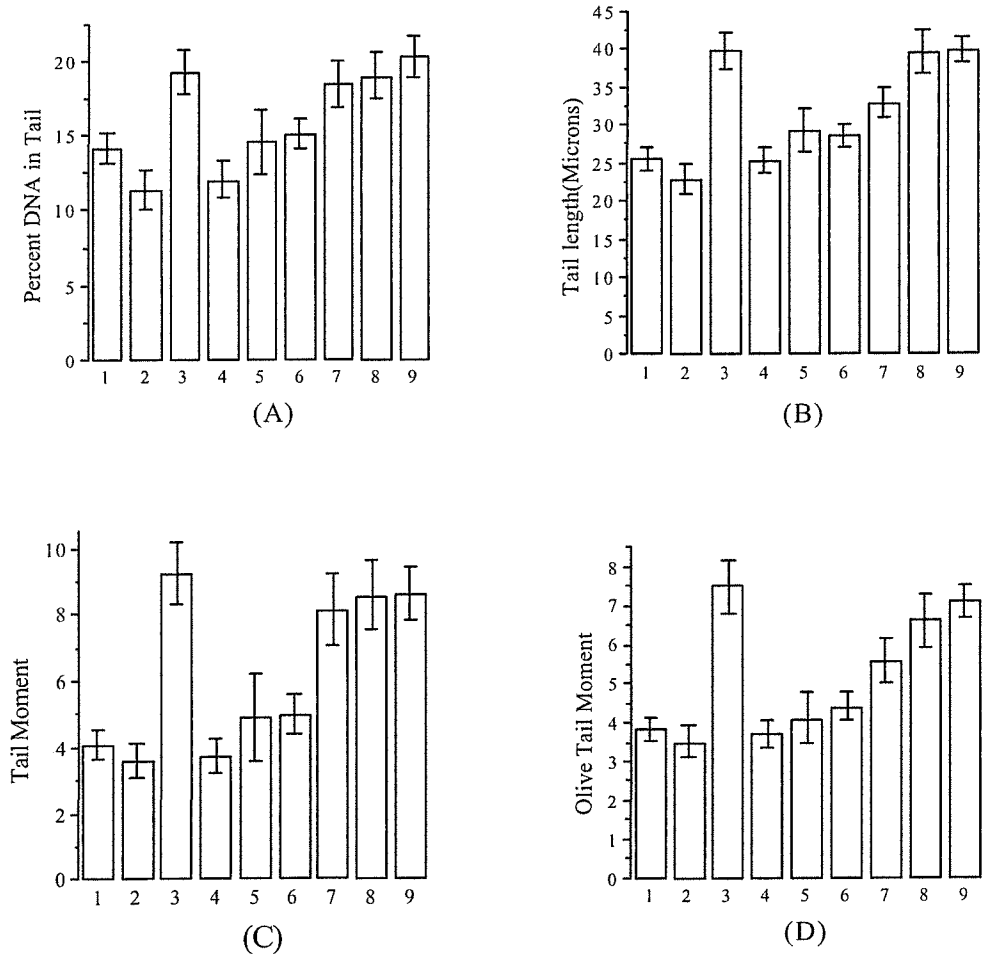

Fig. 4. Effect of Various concentrations on γ-radiation-induced DNA damage in human blood lymphocytes, estimated by comet assay in terms of the percent DNA in tail (A), tail length (B), tail moment (C), and Olive tail moment (D). 1: control; 2: GZA (1.0 mM) alone; 3: 2 Gy radiation alone; 4, 5, 6, 7, 8, and 9: 2 Gy radiation + 10, 7.5, 5, 2.5, 1.0, and 0.5 mM GZA, respectively.

inferred from the data on comet assay presented in Fig. 4. An exposure of human peripheral blood leucocytes to 2 Gy γ-radiation ex vivo resulted in increases of the comet parameters, such as % DNA in tail, tail length, tail moment, and Olive tail moment, and the presence of GZA during irradiation inhibited these increases in a concentration-dependent manner (Fig. 4, a–d). These results thus suggest a protection of cellular DNA by GZA from radiation damage.

Figures 5 and 6 depict the results of comet assay performed on blood leukocytes and bone marrow cells from whole-body irradiated mice treated with GZA. The whole-body exposure of animals to gamma radiation (4Gy) resulted in an increase in the comet parameters (such as % DNA in tail, tail length, tail moment, and Olive tail moment) of the cells of blood leucocytes and bone marrow as a result of damage to cellular DNA. When GZA was administered 1 hr befofre irradiation, there was a significant decrease in comet parameters in blood leucocytes and bone marrow cells of irradiated mice (Figs. 5 and 6). When the animals were exposed to gamma radiation (4 Gy), % DNA in the tail was increased from 3.117 ± 0.45 to 8.133 ± 0.54, tail length was increased from 8.631 ± 0.94 to 26.158 ± 1.49, tail moment was increased from 0.689 ± 0.21 to 3.475 ± 0.44, and Olive tail moment was increased from 0.869 ± 0.15 to 3.626 ± 0.32 in blood cells. But the administration of GZA 1 hour before irradiation brought these parameters down to levels of 6.496 ± 0.54, 22.016 ± 1.39, 2.728 ± 0.41, and 2.555 ± 0.25, respectively, in the irradiated animals (Fig. 5, A–D).

In the bone marrow cells of animals exposed to radiation (4 Gy), % DNA in tail, tail length, tail moment, and Olive

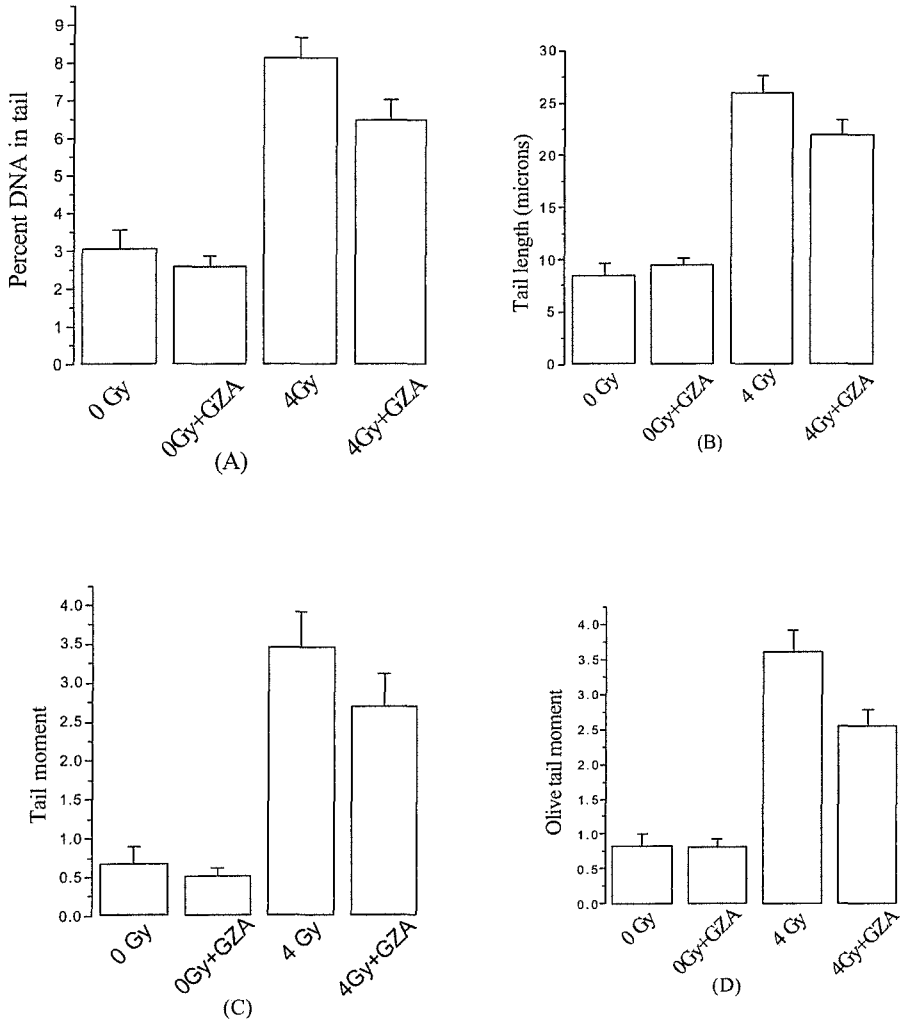

Fig. 5. Effect of Glycyrrhizic acid on DNA damage in murine blood leucocytes assayed by Comet assay: (a) % DNA in tail; (b) Tail length; (c) Tail moment; (d) Olive tail moment. (Why small letters here (a), (b), etc., and capital letters in Figs. 4 and 6 (A), (B), etc.?) (And why capitalize Tail length and Tail moment here and below when you didn't above in Fig. 5? Please be consistent.)

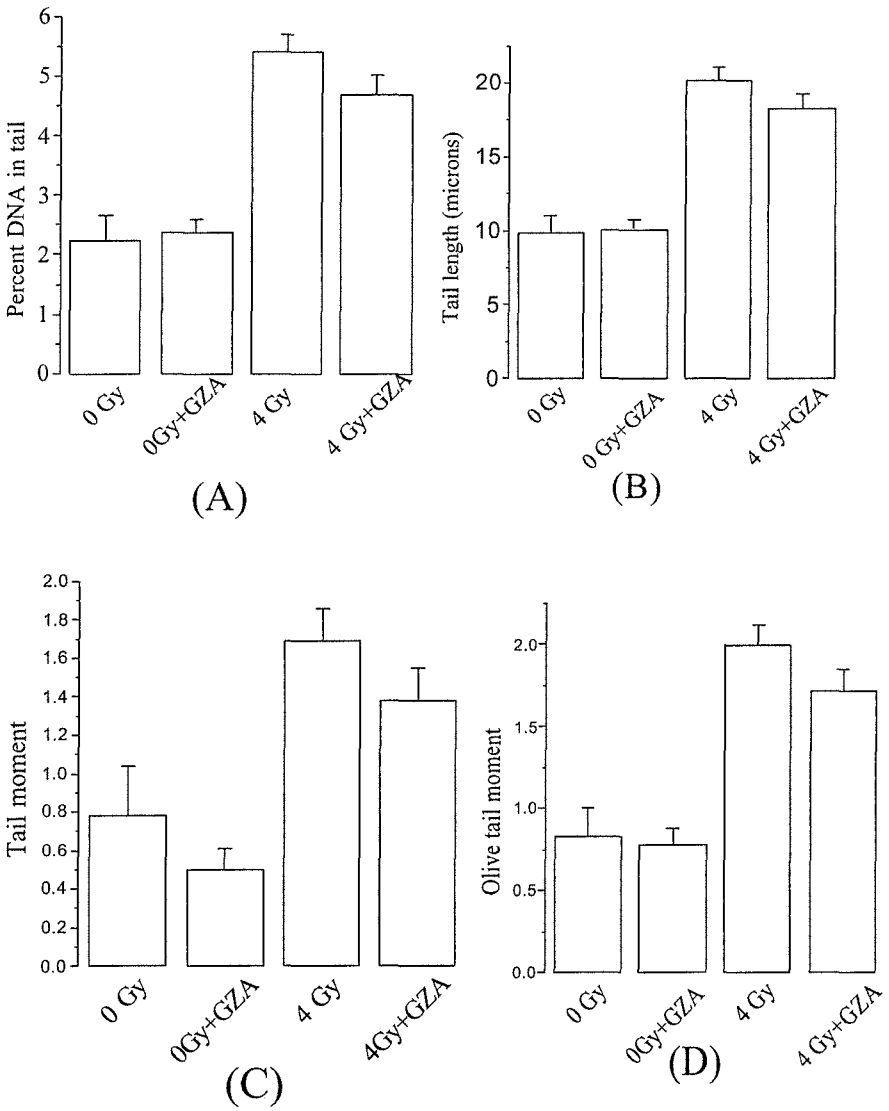

Fig. 6. Effect of Glycyrrhizic acid on DNA damage in murine bone marrow cells assayed by Comet assay, (A) % DNA in tail (B) Tail length (C) Tail moment and (D) Olive tail moment.

tail moment was increased from the respective control value 2.265 ± 0.4, 9.971 ± 1.12, 0.787 ± 0.27, and 0.832 ± 0.18 to 5.412 ± 0.29, 20.19 ± 0.95, 1.704 ± 0.16, and 2.001 ± 0.12. The administration of GZA 1 hour before irradiation brought these levels down to 4.702 ± 0.32, 18.376 ± 0.088, 1.392 ± 0.16, and 1.73 ± 0.13, respectively, in the irradiated animals (Fig. 6, A–D).

The mechanism of radioprotection by GZA was investigated by pulse radiolysis studies. The transient absorption spectra obtained on the reaction of glycyrrhizic acid with OH radicals is presented in Fig. 7. The bimolecular rate constants for the reactions of $OH^{\cdot}$ and e_{aq}^{-} with GZA were determined as explained in materials and methods and found to be 1.2×10^{10} M^{-1} s^{-1} and 3.9×10^{9} M^{-1} s^{-1}, respectively. The transient produced on reaction with OH radical shows an absorption maximum at 320 nm and did not decay up to 2 milliseconds, both in the presence and absence of air. However, we could observe no transient formation on the reaction of GZA with e_{aq}^{-} in the wavelength region of 280 nm to 700 nm under our experimental conditions.

To see the radioprotection of GZA, pulse radiolysis experiments were carried out. Experimental conditions were chosen in such a way that the electron and the OH radicals reacted selectively with Thymine in the presence of GZA. It was observed that e_{aq}^{-} and $OH^{\cdot}$ radical adducts of thymine were found to be nonreactive toward glycyrrhizic acid (our unpublished observations). The results thus indicate that glycyrrhizic acid offers radioprotection by a scavenging of free radicals.

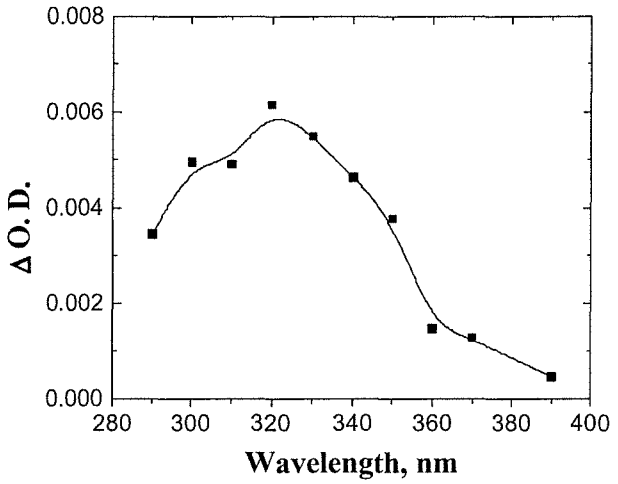

Fig. 7. Transient absorption spectrum obtained on pulse irradiation of N_2O saturated solution containing 1×10^{-4} mol dm^{-3} Glycyrrhizic acid at pH 7. Dose = 16 Gy.

DISCUSSION

DNA constitutes the primary vital target for cellular inactivation of living systems by ionizing radiation. The present study shows that DNA is protected from the deleterious effects of γ-radiation by GZA in the in vitro, ex vivo, and in vivo conditions of radiation exposure. Ionizing radiation-induced damages to cellular DNA are mainly strand breaks of the double- and single-strand types, base damages, elimination of bases, and sugar damage[19]. In the in vitro studies, it was found that when the plasmid pBR322 was exposed to γ-radiation, the ccc form of the molecule was converted to the oc form, with a difference in the mobility in the agarose gel because of the induction of strand breaks in the DNA. Several phytochemicals have been shown to be radioprotectors[20–24].

Nontoxic compounds that can protect DNA against ionzing radiation have considerable potential as radioprotectors and could be of use in preventing diseases like cancer and degenerative diseases arising from gene mutations. GZA does not induce DNA damage by itself, but it inhibits the induction of single- strand breaks in DNA by γ-radiation. The ability of GZA to protect DNA in vitro is determined, as a dose-reduction factor (DRF), from the dose response curve for undamaged ccc DNA remain after exposure to various doses of radiation. The DRF for the plasmid DNA at 2.5 mM GZA is 2.04.

The damages by ionizing radiation to DNA can cause the loss of viability of the cells exposed to radiation. The alkaline comet assay is an elegant and effective technique to monitor the extent of the DNA damage and its protection. When the human leucocytes are exposed to γ-radiation ex vivo, the cellular DNA undergoes damage, as reflected in the increase of the comet parameters (tail length, tail moment, % DNA in the tail, among others). GZA's presence during irradiation of the cells decreases the comet parameters in a concentration-dependent manner indicative of radiation protection.

Whole-body exposure of animals to gamma radiation (4 Gy) resulted in an increase in the comet parameters of cells of various tissues and tumors because of damage to cellular DNA[8,12,13]. The IP administration of GZA to mice before whole-body irradiation protected the mice because there was a decrease in the comet parameters in various tissues, such as bone marrow and blood cells. The results presented in our work clearly show that the GZA protects bone marrow cells from the radiation-induced damages. This would suggest that GZA could be a potential drug for the protection of the hemopoietic system from radiation-induced lesions.

To understand the mechanism of radioprotection by GZA at a molecular level, we have studied by pulse radiolysis technique the transient absorption spectra of glycyrrhizic acid on reaction with OH radicals. Glycyrrhizic acid reacted with $OH^{\cdot}$ and e_{aq}^{-} with rate constants 1.2×10^{10} M^{-1} s^{-1} and 3.9×10^{9} M^{-1} s^{-1}, respectively. The results thus indicate that glycyrrhizic acid offers radioprotection by the scavenging of free radicals.

GZA is the main active ingredient of liquorice, a natural sweet substance originating from the root of the genus *Glycyrrhizia*. This genus contains about 14 species with varying degrees of sweetness, such as *glabra* (Spanish), *glandulifera* (Russian and Persian), *echinata* (German), *uralensis* (Asian), and *lepidota* (American). Among them, the main source of medicinal liquorice is *Glycyrrhizia glabra*. Liquorice has been used since ancient times as an herbal remedy for coughs, peptic ulcers, constipation, stomachache, arthritis, insomnia, depression, fatigue, asthma, and liver problems. It is administered in such forms as liquid extract, powder, concentrate, infusion, tablet, capsules, tincture, candy, gum, tea, soft drinks, and alcoholic drinks. The undesirable side effects of liquorice include water and sodium retention, hypertension, hypokalemia, and alkalosis, and these are reversible if it is discontinued. Liquorice contains 0.2% GZA. An acceptable daily intake of GZA for human subjects was estimated to be 0.2 mg/kg body weight.[25]. Our findings reveal that GZA protects cellular DNA against radiation-induced damage. One deleterious consequence of DNA damage from exposure to ionizing radiation is the induction of cancer. Protecting cellular DNA from radiation damage might result in the prevention of the cancers induced by the radiation. Recently it has been reported that diagnostic X-rays -though they provide great benefits -do cause a risk of cancer[26]. GZA or liquorice administration to the patients undergoing medical X-ray exposures might reduce the incidence of these cancers.

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Protective effect of mannitol, glucose-fructose-sucrose-maltose mixture, and natural honey hyperosmolar solutions against ethanol-induced gastric mucosal damage in rats

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Key words: Acid secretion; cytoprotection; ethanol; experimental gastric ulcer; gastric emptying; honey; mannitol; osmolality.

Summary

Background: We have previously shown that natural honey is able to protect the rat stomach against acute ethanol- and indomethacin-induced lesions. The present investigations were undertaken to examine the role of intraluminal osmolality in this protective effect. **Methods:** Mannitol, glucose-fructose-sucrose-maltose mixture (GFSM) and natural honey (300, 600, 1800 mOsmol/kg water) were given orally to rats 30 min before administration of 70% ethanol for a further 15-min period. Lesions area of the excised stomachs were evaluated. Pylorus-ligated stomachs were filled with mannitol, GFSM mixture and honey (1800 mOsmol/kg water) to test the effect of the hyperosmolar solutions on gastric fluid content and acid secretion. The rate of gastric emptying of the three test solutions (1800 mOsmol/kg) was measured by the phenol red method. **Results:** Intragastric administration of mannitol, GFSM mixture or honey prevented the formation of mucosal lesions in an osmolality-dependent manner. Using the pylorus-ligated stomach model, the test solutions led to a net increase of luminal fluid volume without affecting acid content. Hyperosmolar solutions presented a delayed gastric emptying if compared to a nonnutrient solution made of carboxymethyl cellulose. **Conclusions:** The observed results suggest that hyperosmolar solutions can prevent the formation of hemorrhagic lesions by luminal dilution of the necrotising agent and acid, an effect which may be potentiated by a lowered gastric emptying rate.

Honey is elaborated by honey bees from sugars present in the nectar of various plants. Besides carbohydrates which are the major constituents (70–80%), honey contains, in low amounts, various substances such as organic acids, proteins, amino acids, vitamins, enzymes, minerals and different other molecules (pigments, flavonoids, antibacterial factors, etc.) (1). Since ancient times, people know the curative properties of honey. The ancient Greeks, Romans, Chinese and Egyptians used honey to heal wounds and to cure gut diseases (2). Several reports have shown the effectiveness of natural honey in the treatment of wound ulcers and skin burns (3–8). Furthermore, it has been recently shown that the growth of *Helicobacter pylori*, a causative agent in duodenal ulcer, can be prevented by manuka honey (9). Few reports have shown the effectiveness of honey in gastric ulcers and gastroenteritis (10, 11). It has been reported that natural honey is able to protect rat stomach against acute ethanol- and indomethacin-induced lesions and to accelerate the healing of rat antral ulcers induced by indomethacin (12–14). Ali et al. have pointed out that protection of the stomach against necrotising agents by honey is dose- and time-dependent (15). Enhancement of gastric fluid volume subsequent to intragastric instillation of hyperosmolar solutions may be an important mechanism by which honey or a combination of sugars provide mucosal protection. The present study was undertaken to examine the role of luminal osmolality in the protective effect of honey against ethanol-induced

gastric lesions in rats. Gastric protection induced by honey was compared with that of mannitol and a glucose-fructose-sucrose-maltose (GFSM) mixture formulated to match the content of sugars in honeys. In a second experiment, hyperosmolar and efficiently protective solutions were used to test their effect on water and acid secretion by the pylorus-ligated stomach and to measure their gastric emptying rate.

Material and methods

Animal treatment: Male Wistar rats weighing between 200–250 g were deprived of food for 48 h, but they were allowed free access to tap water until 1 h before the experiment. During the fasting period, the animals were placed individually in cages with wide-mesh wire bottoms to prevent coprophagy.

Ethanol-induced gastric lesions: Treated animals received 5 ml/kg of 300 mOsmol/kg water NaCl (controls) or the same dose of test solution by orogastric intubation. Thirty minutes after their pretreatment, the animals were gavaged with 70% ethanol (5 ml/kg). They were killed 15 min later by cervical dislocation and their stomach rapidly removed after ligating both oesophageal and pyloric ends. Each stomach was opened along the greater curvature, its content was drained and completely recovered by washing with 10 ml isotonic saline. The recovered solution was weighed and centrifuged (3500 rpm for 10 min) to remove contaminating debris. Samples with more than 0.3 ml of sediment were discarded. Supernatants were used for acid determination.

Each stomach was pinned flat on a corkboard and fixed with 10% formalin (10–15 min). Using a colour CCD-camera (DIC-D, World Precision Instr., Sarasota, Florida), the stomach images were coded and stored in a personal computer for subsequent lesions analysis by an observer unaware of the treatments (S. K.). Lesions analysis was performed using the UTHSCA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas, and available from the Internet by anonymous FTP from maxrad6.uthsca.edu). Lesions were delimited manually and the resulting pixels area converted in mm^2 by the ImageTool program using an internal conversion factor. The total area of lesions was determined for each stomach and the treatment mean of the group used for comparison.

Gastric secretion: In order to test the effect of the gastroprotective test solutions on water and acid secretion, the pylorus ligated stomach model was used. Laparotomy and pylorus ligation were performed under light ether anaesthesia. Just after ligation of the pylorus, animals received 5 ml/kg of mannitol, GFSM mixture or honey solution (1800 mOsmol/kg water). Animals regain righting reflex 5–15 min after operation. Conscious animals were killed 4 h after pylorus ligation by cervical dislocation. The stomachs were removed immediately and processed as previously described for fluid recovery.

Water and acid determinations: Aliquots of the test solutions were used to measure free acidity. Free acidity (mEq/l) was determined by titration of the sample to pH 7 using 0.1 N NaOH and an automatic titrator (Orion EA960 Titrator, Boston, USA). Concentration and free acidity of the test solutions are shown in table 1.

Table 1. Composition and total acidity of the test solutions.

Test solution (mOsmol/kg water)	Composition (g/100 g water)	Total acidity (mEq/l)
Mannitol		
300	5.088	0.15
600	9.460	0.15
1800	23.072	0.15
GFSM mixture		
300	6.262	0.88
600	12.768	0.88
1800	35.758	0.88
Honey		
300	6.264	2.43
600	12.040	4.05
1800	29.400	7.90

Stomach fluid content was calculated by subtracting the volume of the washing solution from that of the recovered solution. Acid content was determined in the same manner as in the test solutions. Water and acid contents were expressed in ml and µEq per 200 g body weight, respectively.

Measurement of gastric emptying: Gastric emptying was measured according to the method described by Barquist et al. (1992) (16). A solution containing honey, GFSM mixture or mannitol (1800 mOsmol/kg water) and phenol red (50 mg/100 ml) was given intragastrically (1.5 ml) through a stainless steel tube in conscious rats. After a period of 20 min, animals were killed by cervical dislocation. Control animals received a nonnutrient solution containing 1.5% carboxymethyl cellulose and phenol red. Animals serving as standards (reference stomach, 0% emptying rate) were killed immediately after the intubation. Stomachs were ligated at the pylorus and cardia, removed and homogenized in 100 ml 0.1 N NaOH. After allowing the suspension so settle at room temperature for 60 min, 5 ml of the supernatant was added to 0.5 ml of 20% TCA and centrifuged (3000 rpm for 30 min at 4 °C). The supernatant was mixed with 4 ml 0.5 N NaOH and the absorbance was read at 560 nm. Gastric emptying was calculated according to the following formula:

$$\frac{(A_{560}\ \text{ref} - A_{560}\ \text{sample}) \times 100}{A_{560}\ \text{ref}}$$

Substances and test solutions: An unprocessed specimen of natural honey were gathered directly from a local bee-keeper. GFSM mixture was prepared by mixing the sugars together in the same proportions as they are found in honeys (17). To 17.5 ml of distilled water were added 38.2 g fructose, 31.3 g glucose, 1.5 g sucrose and 7.3 g maltose. The solution was heated briefly to 50 °C to accelerate the dissolving of sugars.

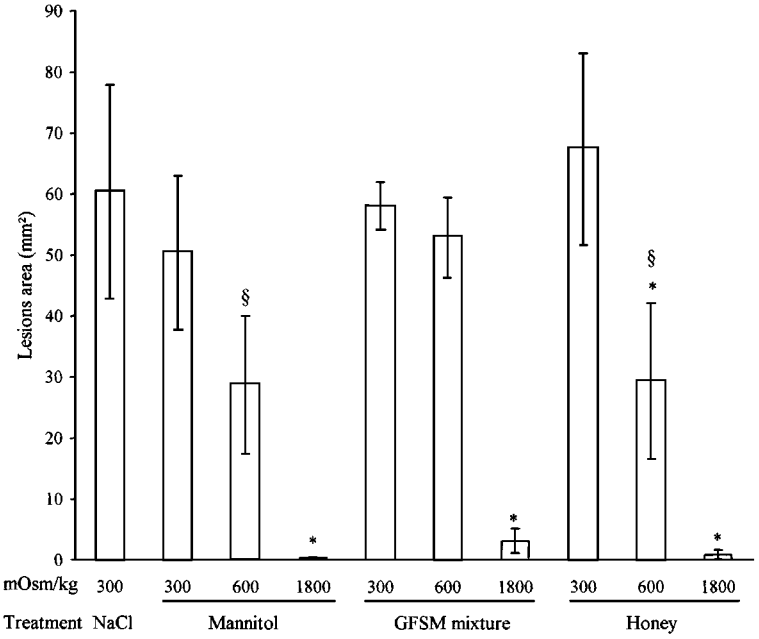


Fig. 1. Effect of intragastric instillation of mannitol, GFSM mixture and honey on ethanol-induced gastric lesions. Test solutions were administered 30 min prior to 70% ethanol instillation. Values are means ± SE (n = 9–10). *: comparisons with respect to control (NaCl), $P < 0.05$; §: comparisons with respect to 600 mOsmol/kg GFSM mixture, $P < 0.05$.

Table 2. Effect of intragastric instillation of mannitol, GFSM mixture and honey and subsequent treatment with ethanol on luminal fluid content.

Treatment	Luminal fluid volume (ml/200 g)	Acid content (µEq/200 g)	Acid concentration (µEq/ml)
Control	1.21 ± 0.11	14.11 ± 4.23	12.42 ± 3.86
Mannitol (mOsmol/kg water)			
300	1.27 ± 0.19	3.94 ± 1.49*	3.15 ± 1.69*
600	1.50 ± 0.20	13.25 ± 4.09	6.34 ± 1.53*
1800	1.64 ± 0.16	16.63 ± 2.67	9.97± 1.29
GFSM mixture (mOsmol/kg water)			
300	1.31 ± 0.17	1.90 ± 3.21*	0.10 ± 3.04*
600	1.52 ± 0.10	9.30 ± 2.72*	6.64 ± 2.07*
1800	1.57 ± 0.12	11.68 ± 2.26	7.33 ± 3.56*
Honey (mOsmol/kg water)			
300	1.61 ± 0.14	1.04 ± 2.32*	1.31 ± 1.52*
600	1.25 ± 0.17	11.97 ± 1.57	9.72 ± 1.39*
1800	1.28 ± 0.10	14.22 ± 2.78	11.29 ± 2.27

Test solutions were administered 30 min prior to 70% ethanol instillation. Values are means ± SE (n = 9–10). Comparisons are made with respect to control (isotonic NaCl). * $P < 0.05$.

The test solutions of mannitol, GFSM mixture and honey were prepared by dissolving the appropriate amount of matter in distilled water to obtain an osmolality equivalent to 300, 600 and 1800 mOsmol/kg water. Osmolality was determined using a cryoscopic osmometer (Osmomat 030, Gonotech, Germany) calibrated with NaCl solution (2500 mOsmol/kg water). All chemicals were of analytical grade.

Statistical analysis: Data are expressed as means ± SE. Statistical analyses were performed using analysis of variance followed by Dunnett's test for multiple comparisons with respect to control and Student test for comparisons between treatment groups. Values of $P < 0.05$ were regarded as indicating significant differences.

Results

Gastric damage: Gastric instillation of 70% ethanol resulted in mucosal damage. The area covered by hemorrhagic lesions was 60.5 ± 22.6 mm^2. Pretreatment of animals with 300 mOsmol/kg water mannitol, GFSM mixture or honey solutions was without effect on mucosal lesions area (mean area: 58.7 mm^2). Increasing luminal osmolality from 300 to 600 and 1800 mOsmol/kg led to a significant reduction of the lesions ($P < 0.05$), except for 600 mOsmol/kg GFSM mixture which induced only 12.4% protection. The protection of the stomach was almost total when using the highest osmolality (fig. 1).

Water and acid content of ethanol-treated stomachs: Fluid content of the stomach did not vary significantly when animals were given mannitol, GFSM mixture or honey solutions prior to ethanol intubation (table 2). Oral administration of each test solution showed an increase of acid content with increasing osmolality of the solution. If compared to controls, acid content was significantly reduced when using 300 mOsmol/kg mannitol, GFSM mixture or honey ($P < 0.05$; table 2).

Water and acid secretion by pylorus-ligated stomach: In control animals (stomach filled with

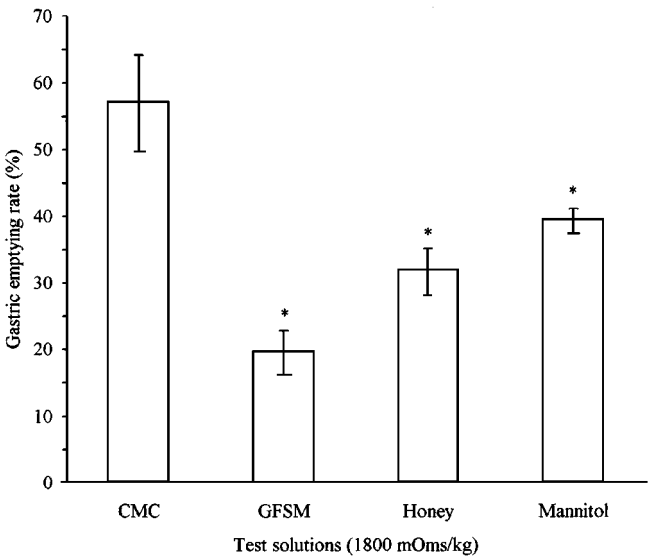
Table 3. Effects of mannitol, GFSM mixture and honey (1800 mOsmol/kg water) on fluid and acid secretion by the pylorus ligated stomach.

Treatment	Luminal fluid volume (ml/200 g)	Acid content (µEq/200 g)	Acid concentration (µEq/ml)
Control	2.91 ± 0.44	282.4 ± 40.8	91.2 ± 20.4
Test solutions (1800 mOsmol/kg water)			
Mannitol	4.57 ± 0.40*	329.2 ± 61.6	62.5 ± 8.9*
GFSM mixture	4.68 ± 0.20*	276.2 ± 67.4	43.6 ± 7.6*
Honey	5.18 ± 0.40*	340.8 ± 56.1	57.2 ± 5.9*

The stomachs were filled with test solution for 4 h. Values are means ± SE (n = 7–8). Comparisons are made with respect to control (isotonic NaCl). * $P < 0.05$

300 mOsmol/kg water NaCl), the volume of luminal fluid reached 2.91 ± 0.44 ml/200 g 4 h after ligation of the pylorus. When the stomach was filled with 1800 mOsmol/kg mannitol, GFSM mixture or honey solution, fluid content was increased by 57%, 61% and 78%, respectively (table 3). Parallel to a significant decrease of acid concentration in the lumen, ranging from 30.5 to 53.7%, acid content was not significantly changed (table 3).

Gastric emptying: A CMC solution (1.5%) was used as a control solution for two main reasons: (1) it possesses a viscosity similar to the test solutions and is considered as non-nutritive, (2) it is devoid of molecules which can directly interfere with osmoreceptors. In animals given the nonnutrient solution, the rate of gastric emptying was 57.1 ± 7.1%. Honey, GFSM mixture and mannitol solutions (1800 mOsmol/kg) presented a significantly lower rate than that of CMC solution ($P < 0.05$; fig. 2). The observed rates were varying according to the nature of the test solution (mannitol > honey > GFSM mixture).



Discussion

The present results show that gastric instillation of isotonic mannitol, GFSM mixture or honey did not prevent mucosal lesions formation induced by 70% ethanol. Whereas animal pretreatment with hyperosmolar solutions (1800 mOsmol/kg) reduced lesions area to almost zero. The effects of 600 mOsmol/kg solutions was variable according to the nature of the solution used. Both 600 mOsmol mannitol and honey solutions led to approximately 52% protection whereas GFSM mixture had no effect at the same osmolality. Parallel to an increase of the percent protection, acid content of the stomach tended to increase with increasing the osmolality of the test solution. These results are in agreement with those reported earlier (12–15, 18). Furthermore, it has been shown that hyperosmolar solutions of mannitol (5–30%) present a concentration-dependent protective effect against absolute ethanol-induced mucosal damage in rats (19). The absence of protective effect of 600 mOsmol/kg GFSM mixture solution suggests that both sugars and non sugar components of natural honey are involved in gastroprotection. At higher osmolality, the role of carbohydrates becomes indiscernible from that of non sugar components. It has been reported that honey can induce a partial protection if given at a dose of 5 g/kg body weight (1 g mixed with 1 ml 100% ethanol) and that a GFSM mixture at the same dose had no effect (12). Gastric protection by mannitol may be attributed to both its osmotic and antioxidant properties.

Gastric administration of the test solutions prior to ethanol led to an increase of acidity in the stomach parallel to a reduction of lesions area; this change was not correlated to initial acidity of the test solution. It has long been recognised that gastric damage is followed by a disappearance of acid from the gastric lumen and an increase of gastric blood flow (20, 21). Acid disappearance has been attributed to back-diffusion of acid through the disrupted gastric barrier (22) and neutralisation of the influxing acid by an increased efflux of bicarbonate (23).

Mannitol, GFSM mixture and honey (1800 mOsmol/kg) led to a significant increase of fluid in the ligated stomach. The net decrease of acid concentration in the gastric lumen is due to the increase of fluid volume without parallel change in acid content. These results suggest that these test solutions act on fluid secretion by an osmotic effect. A slight increase of fluid was also observed in ethanol-induced lesions model but without reaching a statistically significant level. Experiments undertaken to measure gastric emptying showed that

Fig. 2. Gastric emptying rate of hyperosmolar solutions of mannitol, GFSM mixture and honey. Measurements were made 20 min after intragastric administration of the test solutions. Values are means ± SE (n = 7–8). Comparisons are made with respect to control (CMC, 1.5% carboxymethyl cellulose). * $P < 0.05$.

hyperosmolar solutions of mannitol, GFSM mixture and honey present a lower rate of gastric emptying. Acutely induced hyperglycaemia in man and hyperosmolar glucose solution were shown to slow the rate of gastric emptying (24–26). In pylorus-ligated stomach model, both luminal hypertonicity and stimulation of fluid secretion may be involved in the rise of fluid volume. The slowing of gastric emptying by hyperosmolar solutions has been postulated to result from the triggering of duodenal osmoreceptor feedback on the stomach (26, 27). The delay of gastric emptying induced by hyperosmolar mannitol, GFSM mixture or honey solution may lead to a dilution of ethanol given 30 min later. Under these conditions, the luminal concentration of ethanol, given as a necrotising agent, would be insufficient to provoke mucosal damage. Ethanol is a potent gastric barrier disrupting agent if administered at a concentration greater than 50% (28). Despite the fact that hyperosmolar solutions can slow gastric emptying and induce fluid secretion, an increased fluid volume in the ethanol-induced lesions model was not observed. This may be due to an insufficient time that allows accumulation of secreted fluid in the lumen, and to the contractions of the stomach that follow ethanol administration (29). It has been shown that some mild irritants (HCl, NaCl) can induce volume increase that by itself is enough to afford protection through luminal dilution (28).

In conclusion, it appears that mannitol, GFSM mixture and natural honey solutions prevented gastric lesions in an osmolality-dependent manner. Total protection of the stomach against 70% ethanol can be obtained with hyperosmolar sugars solutions (osmolality greater than 600 mOsmol/kg water). The gastroprotective effect of these substances may be attributed to luminal osmolality and delayed gastric emptying which act to lower the concentration of the necrotizing agent and secreted acid.

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CONTENTS

FOREWORD

I am pleased to present the World Health Organization's Global status report on alcohol and health 2014. WHO has published several reports in the past on this topic with the last one being published in 2011, but this report of 2014 has some unique features. First, it describes some progress made in alcohol policy development in WHO Member States after endorsement of the Global strategy to reduce the harmful use of alcohol in 2010. Second, this report provides a wealth of information on alcohol-related indicators for the comprehensive global monitoring framework for the prevention and control of non-communicable diseases (NCDs) adopted by the 66th World Health Assembly. The global monitoring framework was developed to fulfil the mandate given by the Political Declaration of the High-level Meeting of the General Assembly on the Prevention and Control of Non-communicable Diseases (NCDs) and includes the voluntary target of a 10% relative reduction in harmful use of alcohol by 2025 measured against a 2010 baseline. Thirdly, this report presents an overview of some of the mechanisms and pathways which underlie the impact of the harmful use of alcohol on public health.

The report highlights some progress achieved in WHO Member States in the development and implementation of alcohol policies according to the ten areas of action at the national level recommended by the Global strategy. This progress is uneven and there is no room for complacency given the enormous public health burden attributable to alcohol consumption. Globally, harmful use of alcohol causes approximately 3.3 million deaths every year (or 5.9% of all deaths), and 5.1% of the global burden of disease is attributable to alcohol consumption. We now have an extended knowledge of the causal relationship between alcohol consumption and more than 200 health conditions, including the new data on causal relationships between the harmful use of alcohol and the incidence and clinical outcomes of infectious diseases such as tuberculosis, HIV/AIDS and pneumonia. Considering that beyond health consequences, the harmful use of alcohol inflicts significant social and economic losses on individuals and society at large, the harmful use of alcohol continues to be a factor that has to be addressed to ensure sustained social and economic development throughout the world. In the light of a growing population worldwide and the predicted increase in alcohol consumption in the world, the alcohol-attributable disease burden as well as the social and economic burden may increase further unless effective prevention policies and measures based on the best available evidence are implemented worldwide. And, importantly, we know that in countries with lower economic wealth the morbidity and mortality risks are higher per litre of pure alcohol consumed than in the higher income countries.

Following the endorsement of the Global strategy to reduce the harmful use of alcohol WHO has strengthened its actions and activities to prevent and reduce alcohol-related harm at all levels. Several regions have developed and adopted regional strategies focusing on the target areas recommended in the global strategy. At the global level the WHO Secretariat has facilitated establishment of a global network of WHO national counterparts as well as a coordinating council to ensure effective collaboration with and between Member States. At the same time all the efforts and resources available at all levels are clearly not adequate to confront the enormous public health burden caused by the harmful use of alcohol, and further progress is needed at all levels and by all relevant actors to

achieve the objectives of the Global alcohol strategy and the voluntary global target of at least a 10% relative reduction in the harmful use of alcohol by 2025. WHO is prepared and committed to continue to monitor, report and disseminate the best available knowledge on alcohol consumption, alcohol-related harm and policy responses at all levels, which is key to monitoring progress in implementing the Global strategy and regional action plans. Accurate and up-to-date information is vital for alcohol policy development, and I hope that you will find this report, which is largely based on the information submitted from Member States, useful in contributing to the public health objectives articulated in the Global strategy to reduce the harmful use of alcohol.

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ABBREVIATIONS

15+	population of those aged 15 years and older
AAF	alcohol-attributable fraction
AD	alcohol dependence
AFR	WHO African Region
AIDS	acquired immunodeficiency syndrome
AMR	WHO Region of the Americas
APC	alcohol per capita consumption
ASDR	age-standardized death rate
AUD	alcohol use disorder
BAC	blood alcohol concentration
CI	confidence interval
CVD	cardiovascular disease
DALY	disability-adjusted life year
EMR	WHO Eastern Mediterranean Region
EUR	WHO European Region
ESPAD	European School Survey Project on Alcohol and Other Drugs
FAOSTAT	Food and Agriculture Organization of the United Nations (FAO) statistical database
FAS	fetal alcohol syndrome
GDP	gross domestic product
GENACIS	Gender, alcohol, and culture: an international study
GISAH	WHO Global Information System on Alcohol and Health
GSHS	Global School-based Student Health Surveys
HED	heavy episodic drinking
HIV	human immunodeficiency virus
HU	harmful use of alcohol
ICD	International Classification of Diseases
MA	moving average
mhGAP	WHO Mental Health Gap Action Programme
MLPA	minimum legal purchase age

NCD	noncommunicable disease
NICE	National Institute for Health and Care Excellence
NIS	Newly Independent States
OIV	Organisation Internationale de la Vigne et du Vin
PPP	purchasing power parity
RBS	responsible beverage service
SBIRT	screening, brief intervention and referral to treatment
SEAR	WHO South-East Asia Region
SES	Socioeconomic status
STEPS	STEPwise approach to surveillance
UN	United Nations
WHA	World Health Assembly
WHO	World Health Organization
WPR	WHO Western Pacific Region
YLL	years of life lost

EXECUTIVE SUMMARY

This report provides a global overview of alcohol consumption in relation to public health (Chapter 1) as well as information on: the consumption of alcohol in populations (Chapter 2); the health consequences of alcohol consumption (Chapter 3); and policy responses at national level (Chapter 4). The main messages of these chapters can be summarized as follows:

CHAPTER 1: ALCOHOL AND PUBLIC HEALTH

Alcohol is a psychoactive substance with dependence-producing properties that has been widely used in many cultures for centuries. The harmful use of alcohol causes a large disease, social and economic burden in societies.

- Environmental factors such as economic development, culture, availability of alcohol and the level and effectiveness of alcohol policies are relevant factors in explaining differences and historical trends in alcohol consumption and related harm.
- Alcohol-related harm is determined by the volume of alcohol consumed, the pattern of drinking, and, on rare occasions, the quality of alcohol consumed.
- The harmful use of alcohol is a component cause of more than 200 disease and injury conditions in individuals, most notably alcohol dependence, liver cirrhosis, cancers and injuries.
- The latest causal relationships suggested by research are those between harmful use of alcohol and infectious diseases such as tuberculosis and HIV/AIDS.
- A wide range of global, regional and national policies and actions are in place to reduce the harmful use of alcohol.

CHAPTER 2: ALCOHOL CONSUMPTION

- Worldwide consumption in 2010 was equal to 6.2 litres of pure alcohol consumed per person aged 15 years or older, which translates into 13.5 grams of pure alcohol per day.
- A quarter of this consumption (24.8%) was unrecorded, i.e., homemade alcohol, illegally produced or sold outside normal government controls. Of total recorded alcohol consumed worldwide, 50.1% was consumed in the form of spirits.
- Worldwide 61.7% of the population aged 15 years or older (15+) had not drunk alcohol in the past 12 months. In all WHO regions, females are more often lifetime abstainers than males. There is a considerable variation in prevalence of abstention across WHO regions.

- Worldwide about 16.0% of drinkers aged 15 years or older engage in heavy episodic drinking.
- In general, the greater the economic wealth of a country, the more alcohol is consumed and the smaller the number of abstainers. As a rule, high-income countries have the highest alcohol per capita consumption (APC) and the highest prevalence of heavy episodic drinking among drinkers.

CHAPTER 3: HEALTH CONSEQUENCES

- In 2012, about 3.3 million deaths, or 5.9% of all global deaths, were attributable to alcohol consumption.
- There are significant sex differences in the proportion of global deaths attributable to alcohol, for example, in 2012 7.6% of deaths among males and 4.0% of deaths among females were attributable to alcohol.
- In 2012 139 million DALYs (disability-adjusted life years), or 5.1% of the global burden of disease and injury, were attributable to alcohol consumption.
- There is also wide geographical variation in the proportion of alcohol-attributable deaths and DALYs, with the highest alcohol-attributable fractions reported in the WHO European Region.

CHAPTER 4: ALCOHOL POLICY AND INTERVENTIONS

- Alcohol policies are developed with the aim of reducing harmful use of alcohol and the alcohol-attributable health and social burden in a population and in society. Such policies can be formulated at the global, regional, multinational, national and subnational level.
- Many WHO Member States have demonstrated increased leadership and commitment to reducing harmful use of alcohol in recent years. A higher percentage of the reporting countries indicated having written national alcohol policies and imposing stricter blood alcohol concentration limits in 2012 than in 2008.

The report also contains country profiles for all 194 WHO Member States as well as data tables to support information provided in chapters 2–4 (Appendices I–III) and a section explaining data sources and methods used in this report (Appendix IV).

global status report on alcohol and health

1. Alcohol and public health

1. ALCOHOL AND PUBLIC HEALTH

The protection of the health of populations by preventing and reducing the harmful use of alcohol is a public health priority, and one of the objectives of the World Health Organization (WHO) is to reduce the health and social burden caused by the harmful use of alcohol. The Global strategy to reduce the harmful use of alcohol defines "harmful use" as drinking that causes detrimental health and social consequences for the drinker, the people around the drinker and society at large, as well as the patterns of drinking that are associated with increased risk of adverse health outcomes.[1] The vision of this strategy is to improve the health and social outcomes of individuals, families and communities, considerably reducing morbidity and mortality due to harmful use of alcohol and their ensuing social consequences (WHO, 2010a).

Alcohol is a psychoactive substance with dependence-producing properties. As described in this report, consumption of alcohol and problems related to alcohol vary widely around the world, but the burden of disease and death remains significant in most countries. The harmful use of alcohol ranks among the top five risk factors for disease, disability and death throughout the world (WHO, 2011a; Lim et al., 2012). It is a causal factor in more than 200 disease and injury conditions (as described in Statistical Classification of Diseases and Related Health Problems (ICD) 10th revision, WHO, 1992). Drinking alcohol is associated with a risk of developing such health problems as alcohol dependence, liver cirrhosis, cancers and injuries (WHO, 2004a; Baan et al., 2007; Shield, Parry & Rehm, 2013). The latest causal relationships suggested by research findings are those between alcohol consumption and incidence of infectious diseases such as tuberculosis and HIV/AIDS (Lönnroth et al., 2008; Rehm et al., 2009b; Baliunas et al., 2010) as well as between the harmful use of alcohol and the course of HIV/AIDS (Hendershot et al., 2009; Azar et al., 2010). As described in chapter 3 of this report, the net effect of harmful use of alcohol is approximately 3.3 million deaths each year, even when the beneficial impact of low-risk patterns of alcohol use on some diseases is taken into account. Thus, harmful use of alcohol accounts for 5.9% of all deaths worldwide.

As described in section 1.6 of this chapter, harmful use of alcohol can also have serious social and economic consequences for individuals other than the drinker and for society at large (e.g. Anderson et al., 2006; Sacks et al., 2013).

Despite the large health, social and economic burden associated with harmful use of alcohol, it has remained a relatively low priority in public policy, including in public health policy (see section 1.7). However, recent international policy frameworks and action plans, such as the WHO Global strategy to reduce the harmful use of alcohol and the WHO Global action plan for the prevention and control of noncommunicable diseases (NCDs) 2013–2020 (see section 1.7.2) are expected to shift the political compass towards

1 The word "harmful" in the strategy refers only to public-health effects of alcohol consumption, without prejudice to religious beliefs and cultural norms in any way. The concept of "harmful use of alcohol" in this context is different from "harmful use of alcohol" as a diagnostic category in the ICD-10 Classification of Mental and Behavioural Disorders (WHO, 1992)

an increased focus on the harmful use of alcohol. In fact, since 2008 WHO Member States have already made improvements in several areas of action recommended by the Global strategy to reduce the harmful use of alcohol. In particular, an increased number of Member States reported having written national alcohol policies and taking action to reduce the prevalence of drink–driving, to limit availability of alcohol and to implement restrictions on alcohol marketing. This positive trend is expected to continue as society's ability and willingness to tackle NCDs and their risk factors, including the harmful use of alcohol, is "a precondition for, an outcome of and an indicator of all three dimensions of sustainable development: economic development, environmental sustainability, and social inclusion" (Global NCD Action Plan 2013–2020; WHO, 2013a).

1.1 ALCOHOL CONSUMPTION IN ITS HISTORICAL CONTEXT

The use of alcoholic beverages has been an integral part of many cultures for thousands of years (McGovern, 2009). Prior to the modern era, fermented alcoholic beverages were known in all tribal and village societies except in Australia, Oceania and North America. In societies where there was no aboriginal alcohol consumption, the encounter with alcoholic beverages was often abrupt and highly problematic. Where alcohol was traditionally consumed, production of alcoholic beverages commonly occurred on a small scale as a household or artisanal activity, particularly when and where agricultural surpluses were available. Drinking alcohol was thus often an occasional and communal activity, associated with particular communal festivals (Gumede, 1995; Parry & Bennets, 1998; Room et al., 2002). There are many places in the world today where versions of these traditional patterns originating from tribal and village societies persist (Obot, 2000; Room et al., 2002; Willis, 2006).

Superimposed upon, and often replacing the aforementioned traditional patterns of drinking, are patterns of production and consumption which developed in European empires and during early modern industrialization. These involved new beverages, new modes of production, distribution and promotion, and new drinking customs and institutions (Jernigan, 2000). As distilled spirits became available and transportation improved, alcoholic beverages became a market commodity which was available in all seasons of the year, and at any time during the week. This increased supply and availability often proved disastrous for indigenous economies (Colson & Scudder, 1988) and public health (e.g. Coffey, 1966). The consequences were also often catastrophic elsewhere in the world (Room et al., 2002). By the nineteenth century, leaders of industry were viewing alcohol as a major impediment to industrial livelihoods, which demanded a sober and attentive workforce. Eventually, and with great difficulty, industrializing societies in Europe and elsewhere came to see the flood of alcohol as a substantial social and health problem. In a number of countries, popular social movements to limit drinking and even to prohibit it gained broad membership and eventually political strength. In most of these countries, after a century or more of popular movements and political activity, a new and fairly stable alcohol control structure was put in place (Aaron & Musto, 1981; Room et al., 2002; WHO, 2011a).

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Original Article

Glycyrrhizin attenuates rat ischemic spinal cord injury by suppressing inflammatory cytokines and HMGB1

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Aim: To investigate the neuroprotective effect of glycyrrhizin (Gly) against the ischemic injury of rat spinal cord and the possible role of the nuclear protein high-mobility group box 1 (HMGB1) in the process.
Methods: Male Sprague-Dawley rats were subjected to 45 min aortic occlusion to induce transient lumbar spinal cord ischemia. The motor functions of the animals were assessed according to the modified Tarlov scale. The animals were sacrificed 72 h after reperfusion and the lumbar spinal cord segment (L2–L4) was taken out for histopathological examination and Western blotting analysis. Serum inflammatory cytokine and HMGB1 levels were analyzed using ELISA.
Results: Gly (6 mg/kg) administered intravenously 30 min before inducing the transient lumbar spinal cord ischemia significantly improved the hind-limb motor function scores, and reduced the number of apoptotic neurons, which was accompanied by reduced levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) in the plasma and injured spinal cord. Moreover, the serum HMGB1 level correlated well with the serum TNF-α, IL-1β and IL-6 levels during the time period of reperfusion.
Conclusion: The results suggest that Gly can attenuate the transient spinal cord ischemic injury in rats via reducing inflammatory cytokines and inhibiting the release of HMGB1.

Keywords: glycyrrhizin; ischemic spinal cord injury; high-mobility group box 1; tumor necrosis factor-α; interleukin-1β; interleukin-6

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Introduction

Ischemic spinal cord injury (ISCI) is a serious complication that can result from thoracoabdominal aortic surgery and can cause paraplegia in 2% to 18% of patients[1, 2]. In a recent report, only 5% of 127 patients who underwent clamp/sew surgery developed paraplegia[3]; paraplegia caused by ISCI remains a problem that should be solved. Multiple studies have suggested that calcium overload, inflammatory processes, free radical production, platelet aggregation, neutrophil accumulation and adhesion following ischemia might contribute to the neuronal damage that was observed in patients with ISCI[4, 5]. However, the cellular and molecular mechanisms of ischemic spinal cord injury are not fully understood. Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) are key proinflammatory cytokines that play important functions in the central nervous system during inflammatory injury. Besides causing direct damage to cell membranes, free oxygen radicals activate the accumulation of neutrophils and stimulate various types of cells to produce TNF-α and IL-1β[6]. These cytokines further contribute to the production of other cytokines and to the expression of endothelial leukocyte adhesion factor-1, ultimately leading to endothelial cell damage and spinal cord ischemia[7, 8]. Recent studies have shown that the high-mobility group box 1 (HMGB1) protein, an abundant nuclear protein that acts as an architectural chromatin binding factor, can be passively released by necrotic or damaged cells and serves as a signaling molecule that is involved in acute and chronic inflammation[9, 10]. A wealth of evidence indicates that HMGB1 is massively released during the excitotoxicity-induced, acute damaging process in the post-ischemic brain, where it triggers inflammatory processes, and suggests that HMGB1 acts as a novel mediator that links excitotoxicity-induced acute damage and subsequent inflammatory processes in the post-ischemic brain[11–13].

Along these lines, we have recently recognized glycyrrhizin (Gly), a natural triterpene glycoconjugate that is derived from the root of licorice (*Glycyrrhiza glabra*), as an additional HMGB1 inhibitor. Gly binds directly to both HMG boxes in

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HMGB1, thereby inhibiting its chemoattractant functions in fibroblasts and smooth muscle cells[14, 15]. Of note, Gly is a natural compound that is commonly used in Japan to treat patients with chronic hepatitis[16]; however, no study has been designed to examine its use in preventing ISCI.

The current study was designed to investigate the protective efficacy and dose-response relationship of Gly against the neurologic and histopathological outcomes of spinal cord ischemia and reperfusion injury that are related to aortic occlusion in rats, and to determine whether HMGB1 plays a pathogenetic role in ischemic spinal cord injury. First, we injected ischemic rats with either glycyrrhizin or a placebo. Second, we monitored the concentration of HMGB1, TNF-α, IL-1β, and IL-6 in the plasma of the rats. Third, we detected the expression of HMGB1 and cell death within the ischemic spinal cords of those rats.

Materials and methods

Animals and groups

Male Sprague-Dawley rats weighing 300–350 g were obtained from the Experimental Animal Center of Sichuan University (Chengdu, China) and were allowed free access to laboratory chow and tap water in day-night regulated quarters at 25 °C.

Rats were randomized into the following three experimental groups, each consisting of 15 animals: (i) ISCI rats that were pretreated with saline (NS group); (ii) ISCI rats that were pretreated with Gly (Minophagen Pharmaceutical Co, Tokyo, Japan) at a dose of 6 mg/kg (Gly group); and (iii) healthy, control, sham-operated rats (Sham group). In the pretreatment groups, glycyrrhizin or saline was administered intravenously via the tail vein 30 min before the induction of ischemia/reperfusion (I/R) ISCI.

Experimental I/R spinal cord injury

The detailed surgical method for transient lumbar spinal cord ischemia has been described previously[17]. Briefly, rats were initially anesthetized with intramuscular ketamine (50 mg/kg) and then by a half dose of ketamine, as required for the procedure. During the surgery, body temperature was monitored using a rectal probe and was maintained at 35.5–37.5 °C with a heat lamp. During the procedure, an intravenous catheter was placed into the tail vein, and 0.9% NaCl was infused. Cefazolin was injected intravenously at a single dose of 10 mg/kg immediately before the surgery to prevent infection. To monitor proximal and distal aortal blood pressures, catheters were surgically placed into the left common carotid artery and the left femoral artery, respectively. The abdominal aorta was accessed through a midline laparotomy, and animals in the sham group were subjected to laparotomy without aortic occlusion. For the other groups, animals were subjected to 45 min of cross clamping, where vascular clamps were placed under the left renal vein and above the bifurcation in the aorta. Each rat received 150 IU/kg of heparin before aortic occlusion, the aortic clamps were removed after 45 min, and the abdomen was closed appropriately. Animals were allowed to recover in a plastic box at 28 °C for 3 h and were subsequently placed in their cages with free access to food and water. The Crede maneuver was used twice daily to empty the urinary bladders of paraplegic animals. Animals that never recovered completely from the surgery and died within 24 h after reperfusion were excluded from the analyses.

Serum detection

Blood samples (0.4 mL) were collected from the femoral vein at 0 h, 0.5 h, 2 h, 6 h, 12 h, 24 h, 48 h, and 72 h after reperfusion. Serum was isolated from the blood after centrifugation at 1500 r/min for 15 min and was frozen at -80 °C until enzyme-linked immunosorbent assay (ELISA) analyses were performed. HMGB1 concentrations and the levels of inflammatory mediators (TNF-α, IL-1β, and IL-6) in the serum samples were quantified using specific ELISA kits for rats according to the manufacturers' instructions (Biosource International Inc, Camarillo, CA, USA).

Neurological assessment

The motor functions of the rats were assessed at 24 h, 48 h, and 72 h after the procedure using the following modified Tarlov scale[6, 17, 18]: 0, no voluntary movement (complete paraplegia); 1, perceptible movement at the joint; 2, good joint mobility but unable to stand; 3, ability to stand but unable to walk; 4, weak walking; 5, complete recovery.

Spinal cord HMGB1 contents

The lumbar enlargements of the spinal cords of rats that were killed at the completion of behavioral testing were removed. Five samples from every group were stored at -80 °C until Western blotting analyses were performed. Briefly, frozen samples were mechanically lysed in a homogenization buffer on ice. The lysates were centrifuged at 12000 r/min for 20 min at 4 °C, and the protein concentrations were estimated using a BCA protein assay kit (Jiancheng Bioengineering Institute, Nanjing, China). Each sample was adjusted to a final total protein concentration of 5 μg/μL in 4×sample buffer, heated at 95 °C for 10 min, and then stored at -20 °C. Protein samples (50 μg per lane) were loaded into a 12% SDS-PAGE gel and run at 100 V for 120 min in running buffer. Proteins were then transferred from the gel to a PVDF membrane at 250 mA for 90 min using transfer buffer. The membrane was blocked with 5% skimmed milk for 2 h at room temperature and incubated overnight at 4 °C with primary antibody directed against HMGB1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:500. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (diluted in 1:6000, Sigma-Aldrich Inc, St Louis, MO, USA) was used as a loading control. After 6×10 min rinses with PBS/Tween, the membrane was incubated in the appropriate HRP-conjugated secondary antibody (diluted 1:1000 in PBST) for 2 h. The blotted protein bands were visualized using enhanced chemiluminescence (ECL) Western blotting detection reagents (Millipore, Billerica, MA, USA), and the blots were exposed to X-ray film. Developed films were digitized using an Epson Perfection 2480 scanner (Seiko, Nagano, Japan), and optical densities were obtained

using Glyko Bandscan software (Glyko, Novato, CA, USA). All experiments were repeated at least three times.

Histological examination

The remnant samples (n=10 for each group) were fixed in 10% formalin, embedded in paraffin and cut to a thickness of 6 μm, with a routine follow-up procedure. An observer who was uninformed of the experimental conditions of the animals recorded the data.

Coronal sections were stained with hematoxylin and eosin (HE) for light microscopic examination. Changes in rat motor neurons caused by ischemia were identified to be shrunken cellular bodies, a disappearance of Nissl granules, an intensely eosinophilic cytoplasm and triangular and pyknotic nuclei. The remaining normal neurons in the ischemic ventral spinal cord of each animal, as judged by their morphological appearance, were counted in three sections that were selected randomly from the rostral, middle, and caudal levels of the L4 segment and then averaged. The numbers of normal neurons per section of the anterior spinal cords of the rats (anterior to an imaginary line drawn through the central canal, which was perpendicular to the vertical axis) were compared between three groups.

TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) reactions were applied to identify cells with fragmented DNA according to the instruction manual of a commercial TUNEL kit (Roche, Basel, Switzerland). Cell viability was assessed by visual inspection of damaged cells that had been stained with TUNEL, and data are presented as the number of TUNEL-positive cells from three sections of the same animal.

Statistical analysis

All data, except neurologic scoring, were presented as the mean±SEM (standard error of mean), which was calculated using SPSS (Statistical Package for the Social Sciences) 12.0 software (SPSS Inc, Chicago, IL, USA). The Mann-Whitney U-test was used to compare the behavior and activity score among groups, and the concentrations of serum HMGB1 and inflammatory mediators were analyzed using two-way repeated-measures (time and group) analysis of variance followed by the *post hoc* Student-Newman-Keuls test. Correlations between HMGB1 levels and concentrations of inflammatory mediators were analyzed using Spearman's rank correlation test, and the number of normal neurons and TUNEL-positive motor neurons in the anterior spinal cord were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U-test with the Bonferroni correction. The P<0.05 level of probability was used as the criteria for significance.

Results

Serum HMGB1 concentrations

As shown in Figure 1, the HMGB1 serum concentrations in the sham animals were unchanged during the period of the experimental procedure. However, the concentrations of HMGB1 in the serum of the NS and Gly groups significantly increased

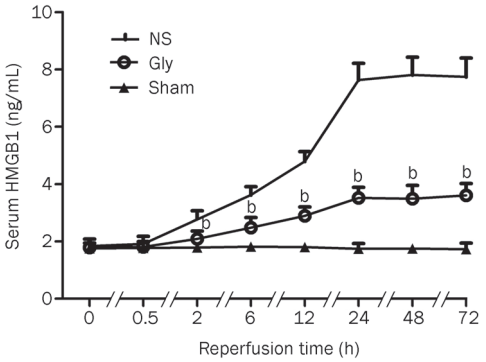


Figure 1. Time course of serum HMGB1 concentrations. The serum HMGB1 levels were significantly increased after spinal cord ischemia-reperfusion compared with that of preischemia (P<0.05), whereas they were significantly decreased in the animals treated by Gly compared with that of animals in NS group (P<0.05). Data are means±SEM. n=15 for each group. ^bP<0.05 *vs* NS group.

2 h after reperfusion, when compared to pre-ischemia levels, and the concentrations remained at higher levels thereafter (P<0.05). Furthermore, HMGB1 serum concentrations in the animals that were treated with Gly were significantly lower than in those of the NS group from 2 h to 72 h after reperfusion (P<0.05).

Concentrations of inflammatory cytokines in the serum

The concentrations of TNF-α, IL-1β, and IL-6 were low in the serum of the rat sham group (Figure 2); however, serum levels of these inflammatory cytokines were greatly induced from 6 h to 72 h after reperfusion in the experimental groups (P<0.05). As shown in Figure 2, Gly administration before I/R resulted in significantly decreased IL-1β, TNF-α, and IL-6 concentrations compared to the NS group (P<0.05). Moreover, the serum HMGB1 contents correlated well with the levels of TNF-α (r=0.947), IL-1β (r=0.906), and IL-6 (r=0.935) at 2 h, 6 h, 12 h, 24 h, 48 h, and 72 h after reperfusion (Figure 3).

Neurologic outcomes

All animals survived until the final neurologic behavior assessments at 24 h, 48 h, and 72 h after reperfusion. The hind-limb motor function scores of the 3 groups at 24 h, 48 h, and 72 h after reperfusion are shown in Table 1. In the NS group, most of the animals developed complete paraplegia of the hind-limbs (grade 1) at 72 h after reperfusion. Importantly, the neurologic statuses of members of the Gly group were signifi-

Table 1. Tarlov scores in each group (mean). ^bP<0.05 compared with sham group. ^eP<0.05 compared with NS+SCI group.

Group	24 h	48 h	72 h
Sham	4.64^e	5^e	5^e
NS+SCI	2.18^b	1.27^b	1^b
Gly+SCI	3.25^{be}	2.33^{be}	2.17^{be}

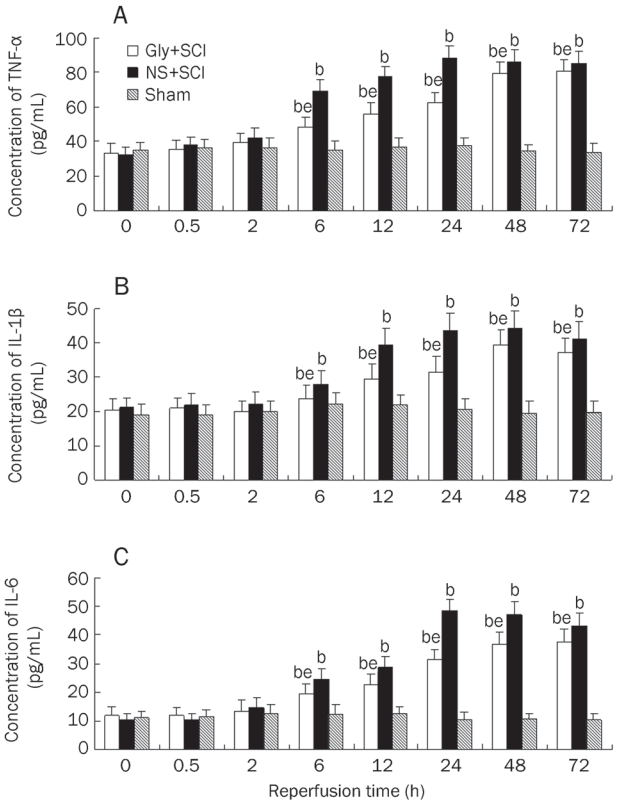


Figure 2. Serum concentrations of inflammatory cytokines. (A–C) respectively show the concentrations of TNF-α, IL-1β, and IL-6 at different time after I/R. Means±SEM. *n*=15. [b]*P*<0.05 *vs* Sham group. [e]*P*<0.05 *vs* NS group.

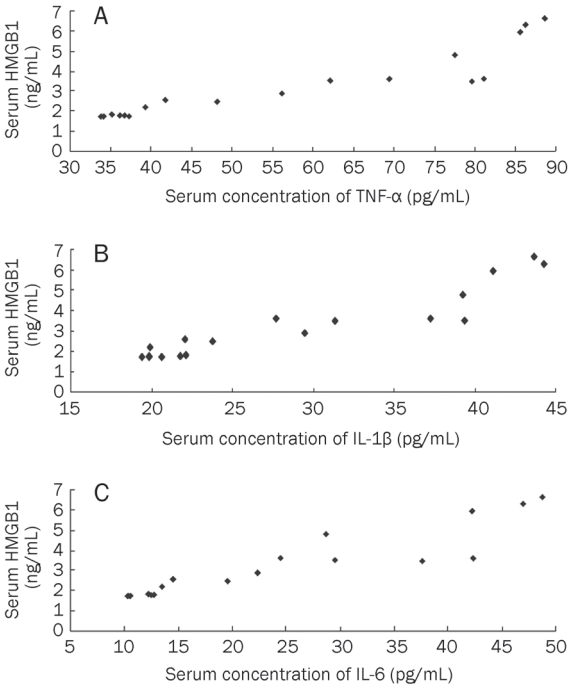


Figure 3. The serum HMGB1 contents correlated well with the levels of TNF-α (r=0.947, *P*=0.004), IL-1β (*r*=0.906, *P*=0.000), and IL-6 (*r*=0.935, *P*=0.000) at 2 h, 6 h, 12 h, 24 h, 48 h, and 72 h after reperfusion. *n*=17 pairs.

cantly improved, compared to those of the NS group, at 24 h, 48 h, and 72 h after reperfusion (*P*=0.029, 0.001, 0.004, respectively).

Western blotting

The protein levels of HMGB1 were detected by Western blot analysis (Figure 4). The protein was expressed at low levels in the spinal cords of the sham group members; however, the levels of HMGB1 significantly increased in the spinal cords of members of the experimental groups as compared with HMGB1 levels of the sham groups (*P*=0.006). Furthermore, the protein expression of HMGB1 in the spinal cords of members of the Gly group was significantly lower than that of the NS group (*P*=0.035).

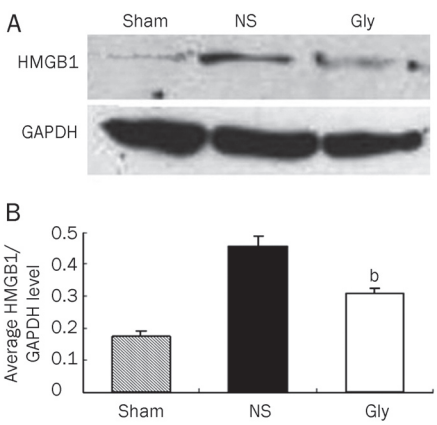
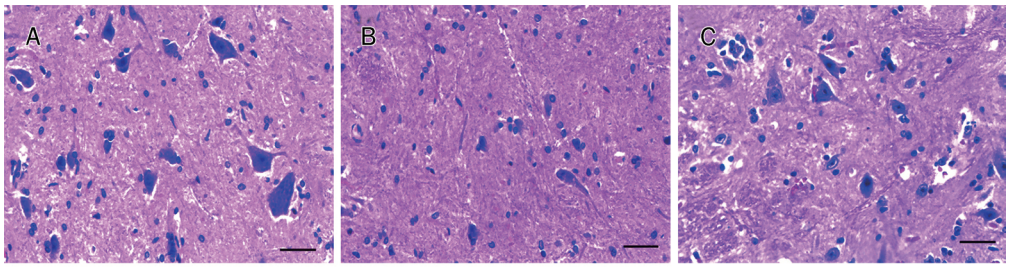


Figure 4. The spinal cord HMGB1 contents in sham, NS or Gly group animals at 72 h after I/R. (A) Result of Western blotting. (B) The bar graph showing the quantitative analysis of the protein levels of HMGB1 in the injured spinal cord in 3 groups. Means±SEM. *n*=5. [b]*P*<0.05 *vs* NS group.

Histological examination

The representative micrographs of HE staining of the ventral horn of the L4 spinal cord segment 72 h after reperfusion are shown in Figure 5A–5C. The number of normal cells in the Gly group was more than that in the NS group (Figure 5D, *P*=0.019), and TUNEL staining identified a few dead cells in the cord sections of the sham-operated animals (Figure 6A). In the spinal cords of members of the NS group, numerous cells were strongly positive for TUNEL staining (Figure 6B). However, in samples from the Gly group, only a few cells were positive for TUNEL staining (Figure 6C). For quantitative measurement, the number of cells that were positive or negative for TUNEL was recorded for each specimen in a blind fashion. Administration of Gly 30 min before ischemia significantly reduced the total number of dead cells, compared to that of the NS group (Figure 6D, *P*=0.016). Moreover, the



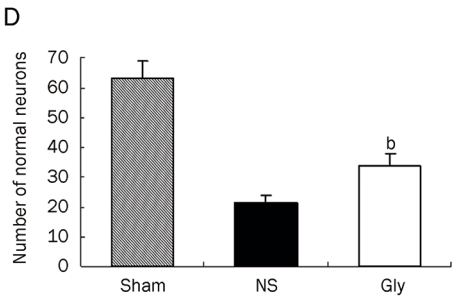
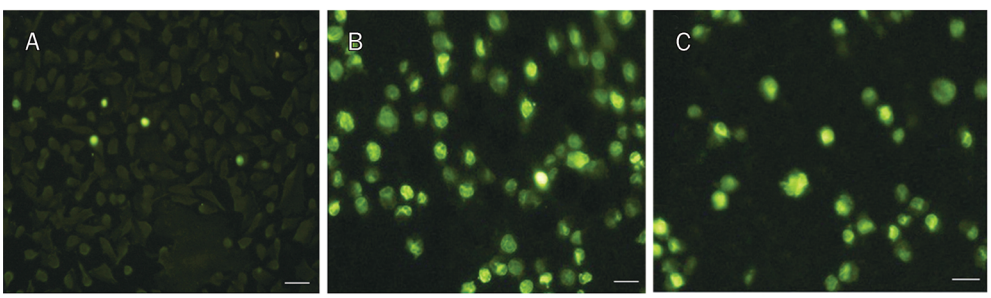
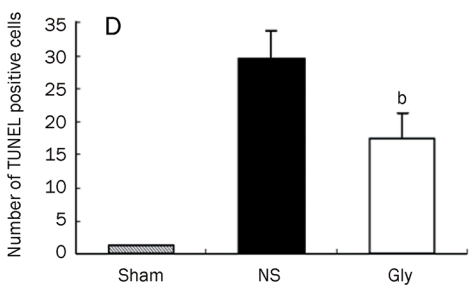


Figure 5. Coronary section of the lumbar spinal cords and quantification of normal motor neurons. (A–C) Representative micrographs of H&E staining in the ventral horn of spinal cord of L4 segments in the Sham, NS and Gly groups at 72 h after reperfusion, respectively (×200). (D) The bar graph showing the quantitative analysis of the number of normal motor neurons in the anterior horn of spinal cord of L4 segments in 3 groups. Data are means±SEM. n=10 for each group. ^bP<0.05 *vs* NS group. Scale bars=80 μm.





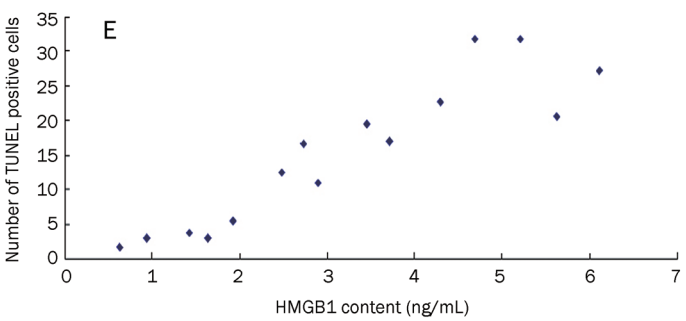


Figure 6. Representative fluorescence micrographs of TUNEL staining and quantification of apoptotic motor neurons. (A–C) Representative fluorescence micrographs of TUNEL staining in the ventral horn of spinal cord of L4 segments from animals in the sham, NS and Gly groups at 72 h after reperfusion, respectively (×200). (D) Quantitative analysis of the number of TUNEL-positive cells in the anterior horn of spinal cord of L4 segments in three groups. Data are means±SEM. n=10 for each group. ^bP<0.05 *vs* NS group. Scale bars=80 μm. (E) The numbers of dead cells correlated well with the HMGB1 levels of spinal cord tissue in the spinal cord at 72 h after reperfusion. n=15 pairs, r=0.929, P=0.005.

number of dead cells correlated well with the HMGB1 levels in spinal cord tissue at 72 h after reperfusion (Figure 6E, n=15 pairs, r=0.929, P=0.005).

Discussion

There is a wealth of evidence to suggest that the systemic inflammatory response that is associated with I/R injury contributes to the morbidity and mortality that is associated with the repair of thoracoabdominal aortic aneurysms[19]. The principal mechanisms of pharmacological therapy, such as the administration of high doses of the glucocorticoid steroid methylprednisolone that are used in humans, are likely to inhibit posttraumatic lipid peroxidation and inflammatory responses. In this study, we show that Gly significantly atten-

uated spinal cord I/R injury when administered 30 min before ischemia, and this protection was accompanied by a reduction in serum inflammatory factors and the protein HMGB1.

HMGB1 is a non-histone, nuclear protein with dual functions. Inside cells, HMGB1 binds DNA and plays a role in transcriptional regulation. Outside cells, HMGB1 serves as a late cytokine-like mediator of systemic inflammation[20]. HMGB1 can activate inflammatory pathways when released from ischemic cells, and studies indicate that HMGB1 acts as an early mediator of inflammation and organ damage in hepatic I/R injury. HMGB1 levels were increased during liver I/R as early as 1 h after reperfusion and then further increased, in a time-dependent manner, up to 24 h. Inhibition of HMGB1 activity with a neutralizing antibody significantly decreased liver damage after I/R, whereas administration of recombinant HMGB1 worsened I/R injury[21, 22]. Moreover, HMGB1 is massively released extracellularly and plays a cytokine-like function in the postischemic brain[11, 12]. HMGB1, as a mediator of postischemic brain damage, plays a critical role in the development of brain infarction through the amplification of plural inflammatory responses in the ischemic region and could be an outstandingly suitable target for treatment for this damage[23]. Intravenous injection of a neutralizing, anti-HMGB1 monoclonal antibody provides a novel therapeutic strategy for ischemic stroke[24]. In addition, serum HMGB1 levels were significantly elevated in patients with myocardial ischemia and cerebral ischemia, suggesting that systemic HMGB1 levels are elevated in human ischemic disease[25]. In this study, serum HMGB1 concentrations and levels of IL-β, TNF-α, and IL-6 increased during spinal cord I/R as early as 2 h after reperfusion and in a time-dependent manner up to 72 h. These results indicate that HMGB1 is involved in the proinflammatory stress response to I/R injuries of the spinal cord in a time-dependent manner after spinal cord I/R in rats.

Obviously, inhibition of HMGB1 secretion or release represents a novel and promising strategy for the therapy of I/R injuries[23]. A growing amount of information implicates a possible responsibility of inflammatory mediators in the pathogenesis of spinal cord injury. In a rat model of traumatic SCI, the tissue level of TNF-α in the spinal cord significantly increased 24 h after injury[26]. Similarly, in a mouse model of traumatic SCI, TNF-α, and IL-1β were produced almost immediately following injury, and this production was followed by the expression of IL-6[27]. Clinical research has also revealed increased immunoreactivity of TNF-α, IL-1β, and IL-6 in neurons at both early and late phases of trauma in human spinal cord tissues after injury[28]. In the present study, we demonstrated that serum proinflammatory cytokine levels (TNF-α, IL-1β, and IL-6) significantly increased after spinal cord I/R in rats. These increases were accompanied by elevated HMGB1 concentrations, and by analyzing histopathological specimens, tissue damage to the spinal cord was evident. In the NS group, all three proinflammatory cytokine levels reached significantly higher levels when compared to the sham-operated group, and these elevated levels were relieved by treatment with Gly. Treatment with Gly attenuated serum HMGB1 levels after spinal cord I/R injury when the drug was administered 30 min before ischemia. Moreover, the HMGB1 contents of spinal cord tissue in animals that had been treated with Gly 72 h after reperfusion were found to be significantly lower than those of the controls. To the best of our knowledge, this is the first study to demonstrate a protective effect of Gly that is related to its inhibitory effect on HMGB1 release in spinal cord I/R injuries. We found that I/R upregulated the expression of HMGB1 in injured tissue and the levels of IL-β, TNF-α, and IL-6 in the peripheral blood, which was inhibited by Gly administration. These results suggest that I/R could activate HMGB1, which might play a central role in the inflammatory response that leads to secondary insults after ischemia. Therefore, the therapeutic benefit of pre-I/R Gly administration might be due to its salutary effect on modulating HMGB1.

However, apoptosis has been demonstrated to be an important mechanism of neuron death in the ischemic spinal cord, and to play an important role in delayed paraplegia in the animal model of aortic occlusion[29]. It is important to note that the TUNEL assay does not distinguish between cell death mechanisms (necrosis or apoptosis); however, this method is useful for detecting damaged cells using light or fluorescence microscopy[30]. Furthermore, histopathological examinations of the spinal cords in our study revealed that there was significant neuronal loss in both 72-h I/R groups, when compared to the sham-operated groups. In this study, dead cells were detected based on positive TUNEL staining because the fluorescent nucleus developed a granular pattern. We used this method because of its high sensitivity and specific means of identifying DNA fragmentation. As noted, numerous dead cells were observed in the spinal cords of the control animals, and the total number of TUNEL-positive cells was reduced significantly after Gly treatment. The results showed that Gly alleviated cell apoptosis that was induced by spinal cord I/R. In line with this, the animals that were treated with Gly had better neurologic outcomes than those of the NS group. Moreover, at 72 h after reperfusion, the HMGB1 levels in spinal cord tissue from animals that had been treated with Gly were significantly lower than those of the NS group, and these levels correlated well with the numbers of dead cells in the spinal cord 72 h after reperfusion. Together, these results indicate that inhibiting the release of HMGB1 with Gly results in less tissue damage and better functional recovery of neurons. In accordance with our experimental results, the relationship between apoptosis and HMGB1 release in macrophages and other cells was investigated in an *in vitro* study, and those results indicated that the release of HMGB1 from macrophages correlated with the occurrence of apoptosis, and suggested that these processes reflected common mechanisms and could occur concomitantly[31]. However, other studies have shown that HMGB1 production occurred downstream of apoptosis in the final common pathway to organ damage in severe sepsis[32]. Thus, the crosstalk between HMGB1 and apoptosis must be further explored.

Because there are two contradictory pathways for inflammation and apoptosis, it is interesting that Gly influences the two

pathways simultaneously. Here, two mechanisms could be considered to cause this result. First, Gly could inhibit inflammation by suppressing HMGB1 expression. HMGB1 has been thought to take part in anti-inflammation because it activates inflammatory responses through multiple pathways, including activating the MAPK pathway and then NF-κB translocation, which triggers inflammatory responses[33]. These pathways lead to a cascade of inflammatory responses that can cause tissue damage and the release of inflammatory mediators. Secondly, the TUNEL assay is only useful for detecting dead cells, and this method does not distinguish between cell death mechanisms (necrosis or apoptosis)[30]. Because inflammatory responses can cause tissue damage and even death, Gly could reduce the number of TUNEL-positive cells by suppressing HMGB1 expression in this study. A limitation to our present study is that we did not assay the mechanism of Gly in attenuating cell damage.

In conclusion, our results confirmed that HMGB1 release plays an important role in spinal cord I/R damage[34], and we showed the Gly affords strong protection against transient spinal cord I/R injury by reducing inflammatory factors and cell apoptosis. Moreover, this protective effect by Gly is related to the inhibition of HMGB1 release that is induced by spinal cord I/R. These data suggest a new therapeutic possibility for treating ISCI with Gly. Future research should be directed toward developing a better understanding of the crosstalk between HMGB1 and apoptosis, as this ultimately might lead to therapeutic strategies for humans.

Author contribution

Prof Le-shun ZHOU designed the research and revised the manuscript; Gu GONG conducted the research, analyzed the data and wrote the paper; Li-bang YUAN, Ling HU, Wei WU, Liang YIN, and Jing-li HOU helped with portions of the research, and Ying-hai LIU helped write the manuscript.

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Protective Effect of Glycyrrhizin, a Direct HMGB1 Inhibitor, on Focal Cerebral Ischemia/Reperfusion-Induced Inflammation, Oxidative Stress, and Apoptosis in Rats

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Abstract

Aim: Glycyrrhizin (GL) has been reported to protect against ischemia and reperfusion (I/R)-induced injury by inhibiting the cytokine activity of high mobility group box 1 (HMGB1). In the present study, the protective effects of GL against I/R injury, as well as the related molecular mechanisms, were investigated in rat brains.

Methods: Focal cerebral I/R injury was induced by intraluminal filamentous occlusion of the middle cerebral artery (MCA) in Male Sprague-Dawley rats. GL alone or GL and rHMGB1 were administered intravenously at the time of reperfusion. Serum levels of HMGB1 and inflammatory mediators were quantified via enzyme-linked immunosorbent assay (ELISA). Histopathological examination, immunofluorescence, RT-PCR and western blotting analyses were performed to investigate the protective and anti-apoptotic effects and related molecular mechanisms of GL against I/R injury in rat brains.

Results: Pre-treatment with GL significantly reduced infarct volume and improved the accompanying neurological deficits in locomotor function. The release of HMGB1 from the cerebral cortex into the serum was inhibited by GL administration. Moreover, pre-treatment with GL alleviated apoptotic injury resulting from cerebral I/R through the inhibition of cytochrome C release and caspase 3 activity. The expression levels of inflammation- and oxidative stress-related molecules including TNF-α, iNOS, IL-1β, and IL-6, which were over-expressed in I/R, were decreased by GL. P38 and P-JNK signalling were involved in this process. All of the protective effects of GL could be reversed by rHMGB1 administration.

Conclusions: GL has a protective effect on ischemia-reperfusion injury in rat brains through the inhibition of inflammation, oxidative stress and apoptotic injury by antagonising the cytokine activity of HMGB1.

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Introduction

Ischemic stroke remains one of the leading cause of death and disability worldwide. Recent insight into the basic mechanism involved in ischemic stroke indicates that endothelial dysfunctions along with oxidative stress and neuroinflammation represent key elements in the occurrence and development of ischemic brain damage that results in cell damage and death [1,2]. Within hours of the ischemic insult, infiltrating leukocytes, as well as resident brain cells including neurons and glia, may release pro-inflammatory mediators such as cytokines, chemokines, and oxygen/nitrogen free radicals that contribute to the evolution of tissue damage [3]. Furthermore, the cerebral ischemia that occurs in brain cells affected by a stroke triggers a complex array of molecular and cellular alterations including the activation of signalling pathways that may either contribute to neuronal damage or protect neurons. Mitogen-activated protein kinases (MAPKs) have crucial roles in signal transduction from the cell surface to the nucleus and regulate cell death and survival processes. Among the MAPK pathways known to be activated in neurons in response to ischemia are the JNK, ERK, and p38 MAPK pathways [4,5].

High mobility group box 1 (HMGB1), a ubiquitous and abundant nuclear protein, can either be passively released into the extracellular milieu in response to necrotic signals or actively secreted in response to inflammatory signals [6–8]. Recently, HMGB1 has been reported to be a potent pro-inflammatory cytokine-like factor that contributes to the pathogenesis of vasculature and connects excitotoxicity-induced acute damage

processes with delayed inflammatory processes in the post-ischemic brain [9,10]. The receptor for advanced glycation end products (RAGE), one of the most important receptors for HMGB1, functions as a sensor of necrotic cell death, and the HMGB1–RAGE signalling axis contributes to inflammation and ischemic brain damage [11]. Intravenous injection of neutralising anti-HMGB1 mAb or intranasal delivery of HMGB1 siRNA conferred robust neuroprotection in the post-ischemic brain by antagonising the pro-inflammatory function of HMGB1 [12,13].

Glycyrrhizin (GL) is a major active constituent of *Glycyrrhiza glabra* root and is composed of a molecule of glycyrrhizic acid and two molecules of glucuronic acid. This compound has been associated with numerous pharmacological effects, including anti-inflammatory, anti-viral, anti-tumour, and hepatoprotective activities, and is commonly used in Asia to treat patients with chronic hepatitis [14–17]. It was reported by Sitia et al [18] that, as an HMGB1 inhibitor, GL binds directly to HMGB1 (Kd ~150 μM), interacting with two shallow concave surfaces formed by the two arms of both HMG boxes. GL has been reported to protect from I/R-induced injury in many organs, including the liver [19], spinal cord [20] and heart [21], by inhibiting the chemoattractant and mitogenic functions of HMGB1. Recently, a robust neuroprotective effect of Stronger Neo-Minophagen C (SNMC), a GL-containing preparation, has been reported in the post-ischemic brain, and this neuroprotective effect is due, at least in part, to an anti-inflammatory effect [22]. However, it is not known if the neuroprotective effect of GL occurs through the antagonism of HMGB1 and the ensuing molecular signalling events. Therefore, the aim of this study was to investigate the potential protective effect of GL, as well as the related mechanisms, against I/R injury in the rat brain, mainly in relation to the following aspects: (1) the neuroprotective effects of GL on focal cerebral ischemia; (2) the release of HMGB1 in rat serum and brain; (3) the effect of GL on the alleviation of apoptosis caused by I/R injury; (4) the expression of HMGB1-dependent inflammation- and oxidative stress-related molecules; (5) the involvement of certain MAPK pathways that are modulated by GL.

Materials and Methods

Animals and groups

All experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US NIH (National Institutes of Health Publication No. 85-23, revised 1996) and were approved by the Committee on Animal Experiments of the Sichuan Neurosurgical Institute. Male Sprague-Dawley rats weighing 325±25 g were obtained from the Experimental Animal Centre of the Sichuan Neurosurgical Institute (China) and were allowed free access to laboratory chow and tap water in day-night quarters at 25°C. Rats were randomly divided into the following four experimental groups of 8 animals each: (1) healthy control, sham-operated rats (the sham group); (2) I/R rats pre-treated with saline (the NS group); (3,4,5) I/R rats pre-treated with GL separately at doses of 2 mg/kg, 4 mg/kg and 10 mg/kg (the 2 mg/kg, 4 mg/kg and 10 mg/kg GL groups); (6) I/R rats pre-treated with GL and recombinant HMGB1 (100 μg per rat) (the GL+rHMGB1 group). In the pre-treatment groups, after 30 min ischemia and before reperfusion, NS, GL or GL plus rHMGB1 was administered intravenously in the tail vein in a volume of 0.5 ml followed by a 48 h reperfusion.

Focal cerebral I/R

Focal cerebral ischemia was induced by performing intraluminal filamentous occlusion of the middle cerebral artery (MCA) for 60 min, according to methods that have been described previously [23]. After 60 min of MCA occlusion, reperfusion of the MCA was initiated by removing the MCA-occlusive filament. The right femoral artery was cannulated to monitor mean arterial blood pressure, arterial blood gases, and pH. Regional cerebral blood flow was monitored using a laser Doppler flowmeter (Periflux System 5000; Perimed, Jarfalla, Sweden). A thermoregulated heating pad and an overhead heating lamp were used to maintain a rectal temperature of 37±0.5°C. In sham-operated rats, an incision was made over the MCA, but the artery was not occluded. The cerebral I/R injury model was developed with 60 min of MCA occlusion followed by 48 h reperfusion.

Triphenyltetrazolium chloride (TTC) staining and infarct volume assessments

Coronal brain sections (2-mm thickness) were incubated with 2% TTC at 37°C for 30 min with gentle shaking and then fixed with 10% formalin in PBS. The stained slices were photographed, and the size of the infarct was quantified using NIH image software.

TTC staining was used to measure infarction volumes two days after I/R (n = 8 per group) and whole brains were dissected coronally into 2-mm brain slices. Two investigators blinded to the study protocol measured the infarct sizes with a computerised image analyser. To account for cerebral oedema and differential shrinkage resulting from tissue processing, the areas of ischemic lesions were determined by subtracting the areas in ipsilateral hemispheres from those of contralateral hemispheres. Infarct volumes were calculated (in mm^3) by multiplying the summed section infarct areas by the section thickness.

Evaluation of neurological deficits

Neurological deficits were evaluated by two methods. In the rota-rod test, rats were conditioned for 3 days before MCA occlusion on an accelerating rota-rod cylinder at 5 to 15 rpm. Rats that could stay on the rotating rod at 15 rpm for 180 s were subjected to MCA occlusion. After MCA occlusion, each rat was subjected to trials conducted at 3 different speeds (5, 10 and 15 rpm), and the mean duration of three trials at each speed on the rota-rod was recorded. The other test was a neurological scoring method, which was performed essentially as described by Bederson et al [24]. The scores were categorised according to four grades (0, normal; 1, moderate; 2, considerable; 3, severe). The modified Neurological Severity Scores system consists of motor, sensory, balance, and reflex tests, all of which are graded using a scale of 0–18 (normal: 0, maximal deficit: 18). The neurological evaluation was performed by an investigator who was blind to the treatment condition.

Serum ELISA detection

Blood samples (0.5 ml) were collected from the femoral vein at 6, 12, 24 and 48 h after reperfusion. Serum was isolated from the blood after centrifugation at 14 000 rpm for 20 min at 4°C. After centrifugation, serum was frozen at −80°C until enzyme-linked immunosorbent assay (ELISA) analyses were performed. HMGB1 concentrations and the levels of inflammatory mediators (TNF-α, iNOS,IL-1β, COX-2 and IL-6) in the serum samples were quantified using specific ELISA kits for rats according to the manufacturer's instructions (Biosource International Inc., Camarillo, CA, USA).

Western blot

Total protein extracts or detached subcellular fractions from rat cerebral tissue were prepared as described previously [25]. The antibodies and the dilutions were as follows: p-JNK No. 9255 (1:2000), t-JNK No. 9252 (1:1000), p-ERK [1/2] No. 9101 (1:1000), t-ERK [1/2] No. 4695 (1:1000), p-p38 No. 9211(1:1000), t-p38 No. 9212 (1:1000) (Cell Signaling Technology, Danvers, Mass), Cytochrome c (1 μg/ml, No. ab90529, Abcam Plc., Cambridge, UK) and HMGB1 (1 μg/ml, No. ab18256, Abcam Plc., Cambridge, UK). Horseradish peroxidase–coupled rabbit and mouse IgG (1:2000) were used as secondary antibodies. Proteins were separated on 10–20% SDS polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes, which were then incubated overnight at 4°C with the primary antibody diluted in blocking solution. After washing, membranes were treated with horseradish peroxidase-conjugated secondary antibodies and then with enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). The film was scanned with a GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA), and the results were quantified with Multi-Analyst software (Bio-Rad Laboratories).

RNA preparation and reverse transcription-PCR

Total RNA was prepared using Trizol reagent (Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions, and 1 μg RNA samples were used for cDNA synthesis. First-strand cDNA synthesis was primed with random hexamers and conducted according to the manufacturer's specifications (RT-PCR kit; Roche, Mannheim, Germany). cDNA equivalent to 200 ng of total RNA was subjected to PCR using the manufacturer's protocol (PCR core kit; Roche). The sense and antisense primers used for the analysis of rat HMGB1, TNF-α, IL-1β, iNOS, COX-2, IL-6 and GAPDH expression were as follows. HMGB1: 5′-CTGATGCAGCTTA TACGAAG-3′ and 5′-TCAGGTAAGGAGCAGAACAT- 3′ (460 bp). TNF-α: 5′-CCC CTTTATCGTCTACTCCTC-3′ and 5′-GCTGGTAGTTTAGCTCCGTTT-3′ (553 bp). IL-1β: 5′-TCATTGTGGCTGTGGAGAAG-3′ and 5′-CTATGTCCCGACCATTGCTG-3′ (579 bp). iNOS: 5′-GCATCCCAAGTACGAGTGGT-3′ and 5′-GAAGGCGTAG CTGAACAAGG-3′ (700 bp). COX-2: 5′-GTGGGATGACGAGCGACTGT-3′ and 5′ -TTTCAGGGAGAAGCGTTTGC-3′ (454 bp). IL-6: 5′-GGATACCACCCACAACAGAC-3′ and 5′-TTGCCGAGTAGACCTCATAG-3′ (520 bp). GAPDH: 5′-CCATCAC TGCCACTCAGAAGA-3′ and 5′-CATGAGGTCCACCACCCTGT- 3′ (446 bp). The annealing temperature was 55°C for all primer pairs.

Immunofluorescence and TUNEL staining

Brains were isolated and fixed with 4% paraformaldehyde by transcardial perfusion and post-fixed in the same solution overnight at 4°C. Coronal brain sections (20 μm) from the ischemic core region were prepared using a vibratome (Leica, Solms, Germany), and immunological staining was performed using a previously described floating method [26]. Rabbit anti-HMGB1 (ab18956; Abcam, Cambridge, UK) antibody was used at a 1:500 dilution. FITC-labelled anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary antibody for anti-HMGB1.

The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assay was carried out with a commercial TUNEL kit (Roche, Switzerland) according to the manufacturer's instructions. Apoptotic cells in the rat heart after I/R in each group were counted separately using Image J software.

Analysis of superoxide and peroxynitrite formation

The oxidative fluorescent dye dihydroethidium (DHE; Sigma-Aldrich) was used to evaluate in situ superoxide production [27]. Frozen, enzymatically intact brains from the ischemic core region were cut into 30-μm thick sections and placed on glass slides. The sections were simultaneously incubated with DHE (10 μmol/L) in phosphate-buffered saline for 30 min at 37°C, in a humidified chamber that was shielded from light. We also monitored peroxynitrite formation by detecting nitrosylated tyrosine residues on proteins. We performed immunostaining with an anti-3-nitrotyrosine (3-NT) antibody (1:1000; Upstate Biotechnology). Coronal sections cut through the striatum (3 sections per brain, 1 mm in width) were imaged in parallel. Superoxide or 3-NT–positive cells were manually counted in the eight regions around the striatum under ×100 magnification using a laser scanning confocal microscope equipped with a Bio-Rad MRC 1024 (argon and krypton).

Measurement of lipid peroxidation

Malondialdehyde (MDA) was estimated as an indicator of lipid peroxidation (n = 8 per group). Brain tissues were homogenised with sodium phosphate buffer (pH 7.4). The reagents (1.5 ml acetic acid, 1.5 ml thiobarbituric acid, and 0.2 ml sodium dodecyl sulphate) were added to 0.1 ml of processed tissue sample. The mixture was then heated at 100°C for 60 min. The mixture was cooled with tap water and 5 ml of n-butanol:pyridine (15:1), and 1 ml of distilled water was added. After centrifugation at 4000 rpm for 10 min, the organic layer was withdrawn and the absorbance was measured at 532 nm using a spectrophotometer.

Statistical analysis

All data were expressed as the means±SEM (standard error of the mean). SPSS 17.0 was used for statistical analysis of the data. The concentrations of serum HMGB1 and inflammatory mediators were analysed using two-way repeated measures (time and group) analysis of variance followed by the post hoc Student-Newman-Keuls test. The significance level was set at $p<0.05$.

Results

Neuroprotective effects of GL on focal cerebral ischemia

No damage was observed upon TTC staining in the cerebrums of sham-group rats, whereas MCA occlusion for 60 min in rats produced massive infarction 48 h after reperfusion. Pre-treatment with different concentrations of GL significantly reduced the infarct volumes in a dose-dependent manner in focal cerebral I/R rats. There were no significant differences between the infarct volumes of the 4 mg/kg and 10 mg/kg GL-pre-treatment groups. Hence, a dose of 4 mg/kg of GL was selected for subsequent experiments, considering the potential for drug toxicity (Figure 1A).

High Neurological Severity Scores in the NS rats persisted for up to 48 h. Consistent with the reduced infarct volumes, when 4 mg/kg GL was administered at 30 min after ischemia and before reperfusion, the mean modified Neurological Severity Scores were significantly lower than those of rats in the PBS-treated NS group from 6 h to 48 h. From 6 h to 24 h, the Neurological Severity Scores declined slowly and reached the minimum value at 48 h after reperfusion in the GL group (Figure 1B). Motor activities were assessed using the rota-rod test at 5, 10 or 15 rpm speed loads. Pre-treatment with 4 mg/kg GL markedly improved the neurological deficits observed on the rota-rod test from 6 h to 48 h, regardless of whether the 5, 10 or 15 rpm speed load was used (Figure 1C). These results showed that pre-treatment with GL had neuroprotective effects on the

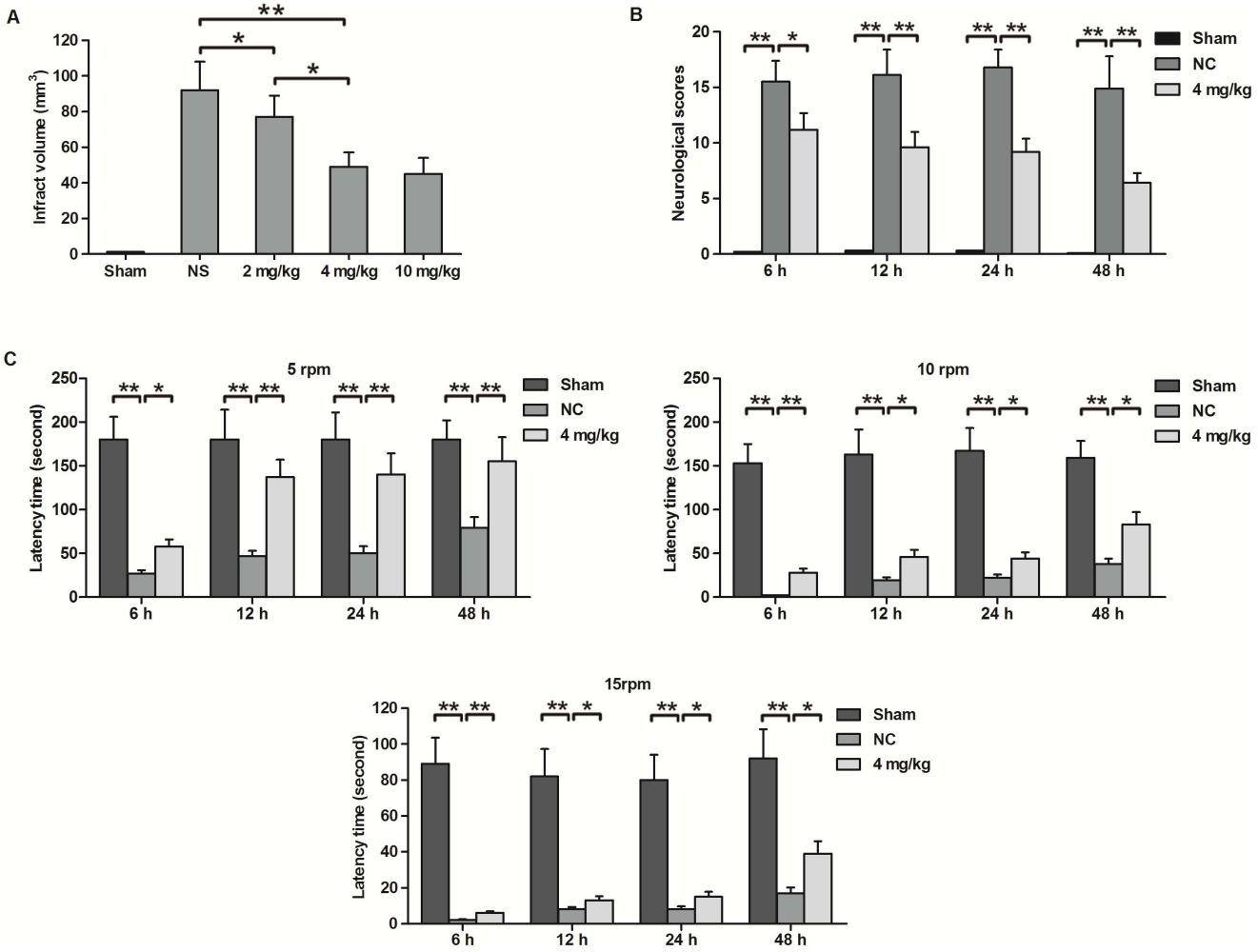

Figure 1. The neuroprotective effect of GL against cerebral I/R injury in rats. A) Cerebral infarction induced by MCA occlusion was evaluated 48 h after reperfusion by TTC staining of brain slices from rats treated with different doses of GL or NS. Sham-operated rats showed no infarction or TTC staining. The infarct volumes were quantified using computerised image analysis. B) Neurological scoring was carried out according to the categories described in the Materials and Methods. C) Neurological deficits in rats after MCA occlusion were examined using the rota-rod test. In the rota-rod test, trials were performed at 3 different speeds, and the time intervals running on the rod were determined for each rat after reperfusion. Values are means±SEM, n = 8 for each group. *P<0.05, **P<0.01 (t test).
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post-ischemic brain, which were manifested as improvements in motor impairments and neurological deficits scores.

Inhibition of HMGB1 release by GL in rats that underwent MCAO

We observed that plasma HMGB1 levels rapidly increased starting 6 h after MCAO for 1 h, peaked at 24 h after reperfusion, and then declined slowly (Figure 2A–B). When rats were pre-treated with 4 mg/kg GL, the plasma HMGB1 level was significantly decreased compared with that observed in the NS group at any time during the experimental procedure (Figure 2A–B). At 12 h after MCA occlusion (MCAO)/reperfusion, brain HMGB1 levels in the infarct area declined significantly to below the basal level and reached a minimum value at 24 h after MCAO/reperfusion. Moreover, at each time point from 12 h to 48 h after MCAO/reperfusion, brain HMGB1 levels after treatment with GL were significantly higher than those observed in the NS group (Figure 2C).

Immunofluorescence staining of nuclear HMGB1 was observed in the cerebral cortex in rats from the sham group (Figure 2D). However, the staining almost disappeared in the core of the infarct in the NS group 48 h after reperfusion, and markedly increased HMGB1 staining was observed in the extracellular space. The number of nuclear HMGB1-positive cells significantly increased upon pre-treatment with 4 mg/kg GL, and this was accompanied by a decrease in extracellular HMGB1 staining. These data suggested that increased serum levels of HMGB1 are probably attributable to spill-over from necrotic neural cells during cerebral ischemia and that GL may have a protective effect against MCAO/reperfusion-mediated cell death in neural cells, thereby decreasing the amount of HMGB1 released from the cerebral cortex into the serum.

GL inhibits HMGB1-dependent apoptotic injury

To further demonstrate that the protective effect of GL against brain I/R injury occurs via antagonism of HMGB1 function, we determined the infarct volumes of rat brains under conditions in

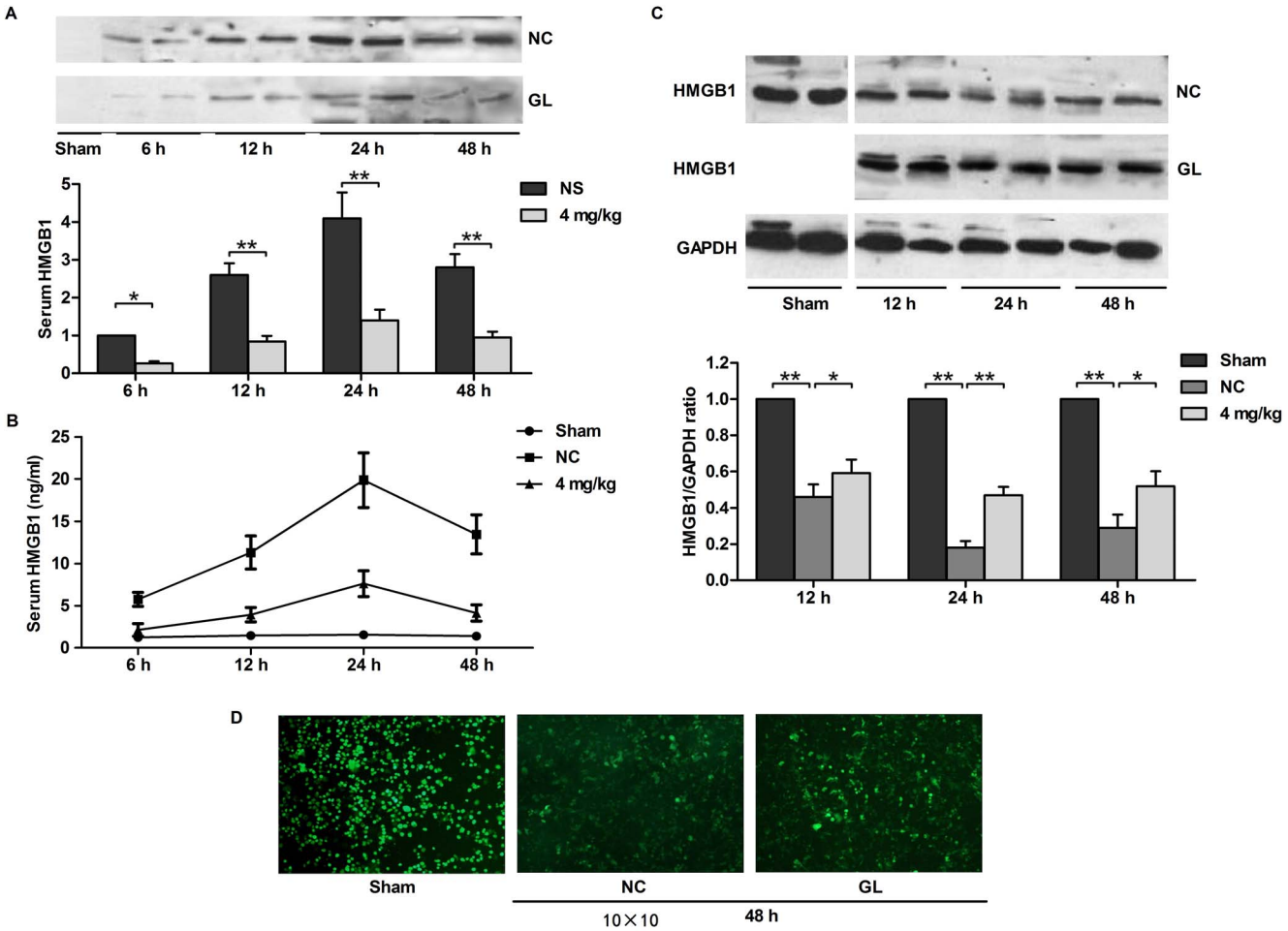

Figure 2. Inhibition of HMGB1 release by GL in the I/R brain. A) The serum HMGB1 concentrations of 4 mg/kg GL-pre-treated rats were determined by immunoblot at the indicated times after 1 h of MCAO. B) Serum HMGB1 concentrations were also determined by ELISA. Values are means±SEM, n = 8. **P<0.01 (t test). C) HMGB1 levels in I/R hemispheres were determined by immunoblot at various times after 1 h of MCAO. GAPDH was used as a loading control. D) Immunofluorescence staining showing different extracellular and intracellular distributions of HMGB1 in the I/R hemispheres after 48 h reperfusion. Values are means±SEM, n = 8 for each group. *P<0.05, **P<0.01 (t test).
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which rHMGB1 was included. As shown in Figure 3A, although the infarct volume was significantly decreased in the 4 mg/kg GL group compared with the NS group, co-treatment with 4 mg/kg GL and 100 μg rHMGB1 30 min before ischemia significantly alleviated these changes, resulting in infarct volumes similar those observed in the NS group.

Next, we investigated whether the anti-inflammatory effect of GL can alleviate I/R-induced, HMGB1-dependent apoptosis and whether cytochrome C and caspase 3, the two most important players, are involved in this process. As depicted in Figure 3B, a large number of TUNEL-positive cells was observed in the right cortex of rats subjected to I/R injury, whereas TUNEL positive cells were not detected in the right cortex of sham-operated rats. Upon pre-treatment with 4 mg/kg GL, the number of TUNEL-positive cells was significantly reduced in the right cortex compared with the NS group. Moreover, co-treatment with rHMGB1 increased the number of TUNEL-positive cells, as expected.

In the sham group, under basal conditions, cytochrome C was predominantly expressed in the mitochondrial fraction (Figure 3C). The NS group exhibited a significant decrease in cytochrome C expression in the mitochondrial fraction when compared with the sham group, whereas an increase was observed in the cytosolic fraction. Pre-treatment with GL resulted in a significant increase in cytochrome C expression in the mitochondrial fraction and a decrease in the cytosolic fraction compared with the NS group. However, expression still did not reach the level observed in the sham group. Moreover, co-treatment with rHMGB1 can partly abolish the effect of GL on the subcellular distribution of cytochrome C proteins between the cytosolic and mitochondrial fractions. There was no significant difference in the total expression of cytochrome C protein between each group (Figure 3C). As an executioner caspase, the change in caspase 3 activity was similar to that observed with cytochrome C expression in the cytosolic fraction (Figure 3D). These results demonstrate that the administration of GL significantly alleviates the cerebral cell apoptotic injury caused by I/R through the antagonism of HMGB1 function and that the mechanism involved regulation of cytochrome C release and caspase 3 activation.

GL inhibits HMGB1-dependent inflammatory molecule expression and oxidative stress

The expression of mRNA encoding inflammation- and oxidative stress-related molecules was determined by RT-PCR. As

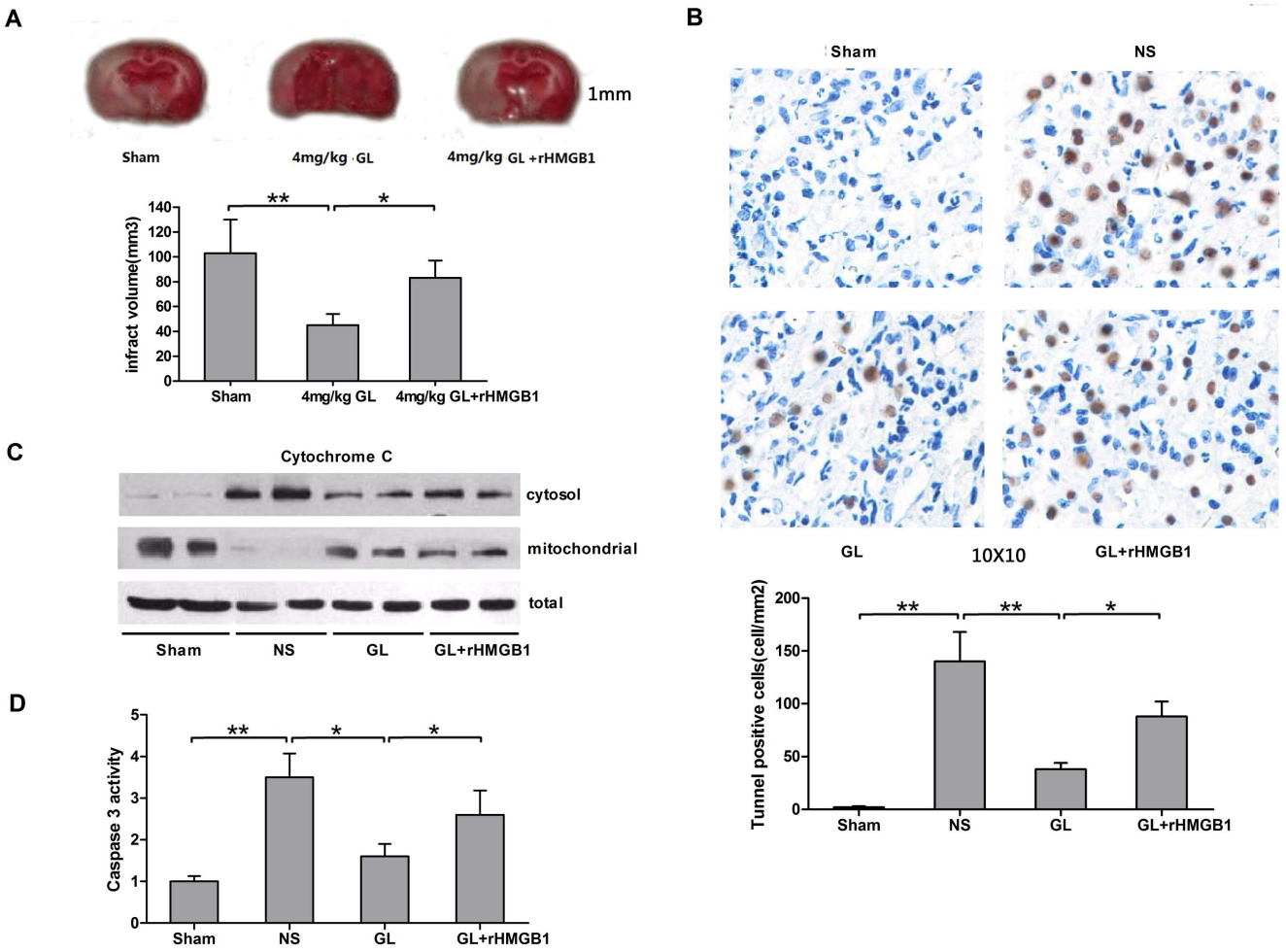

Figure 3. The protective effect of GL on I/R-induced infarct volume and apoptosis injury is HMGB1 dependent. A) Brain infarction induced by right MCA occlusion was evaluated by TTC staining from rats pre-treated with 4 mg/kg GL with or without 100 μg recombinant HMGB1, and the infarct volumes 48 h after reperfusion were quantified using NIH image software. B) Representative photomicrographs show TUNEL staining for apoptotic cells in rat brains at 48 h after reperfusion in the sham, NS, GL and GL+rHMGB1 groups. Effects on the severity of cerebral apoptosis are shown in an average quantitative analysis of the number of TUNEL-positive cells. C) Representative blots showing the effects of GL with or without rHMGB1 treatment on Cytochrome c translocation between the mitochondrial and cytosolic fractions. D) Effects of GL with or without rHMGB1 treatment on caspase-3 activity in areas at the risk zone of cerebral tissues at 48 h after reperfusion. Values are means±SEM, n = 8 for each group. *P<0.05, **P<0.01 (t test).
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showed in Figure 4A, MCA occlusion/reperfusion significantly up-regulated the expression of TNF-α, iNOS, IL-1β, COX-2 and IL-6 in the cerebral cortex compared with the non-ischemic side in the sham group. Treatment with GL significantly inhibited the expression of TNF-α, iNOS, IL-1β and IL-6 but had no apparent effect on the expression of COX-2. In contrast, co-treatment with rHMGB1 and GL again enhanced the expression of TNF-α, iNOS, IL-1β and IL-6 compared with the GL-pre-treatment alone group ($P<0.01$). The serum concentrations of TNF-α, iNOS, IL-1β and IL-6 in each group showed a similar trend to that of the mRNA expression levels, as determined by ELISA (Figure 4B). These results indicated that GL can inhibit the I/R-induced expression of HMGB1-dependent inflammation- and oxidative stress-related molecules in rat brains.

In the sham group, little DHE-induced EtBr fluorescence or 3-NT immunoreactivity was observed. The relative fluorescence intensities of superoxide-generating cells and 3-NT–positive cells were significantly increased in the NS-treated I/R hemisphere (Figure 5A–B). When rats were pre-treated with 4 mg/kg GL, the fluorescence intensity caused by superoxide and 3-NT production was significantly reduced compared with the NS group. In contrast, co-treatment with 4 mg/kg GL and 100 μg rHMGB1 enhanced the fluorescence intensity of superoxide and 3-NT production compared with the GL group, though the intensity was still lower than that in the NS group (Figure 5A–B). These histological results were further supported by a biochemical assay for MDA, a lipid peroxidation product. Pre-treatment with 4 mg/kg GL significantly decreased the level of MDA, compared with the NS group, while co-treatment with 100 μg rHMGB1 again increased the level of MDA, though this was still lower than that in the NS group (Figure 5C).

GL modulates P38 and P-JNK but not p-ERK signalling

The mitochondrial-dependent apoptosis pathway is tightly regulated by the mitogen_activated protein (MAP) kinase family, and among these, JNK, ERK1/2, and p38 have been demonstrated to be activated during I/R injury [28,29]. To study whether GL can modulate MAP kinase activity, we analysed these

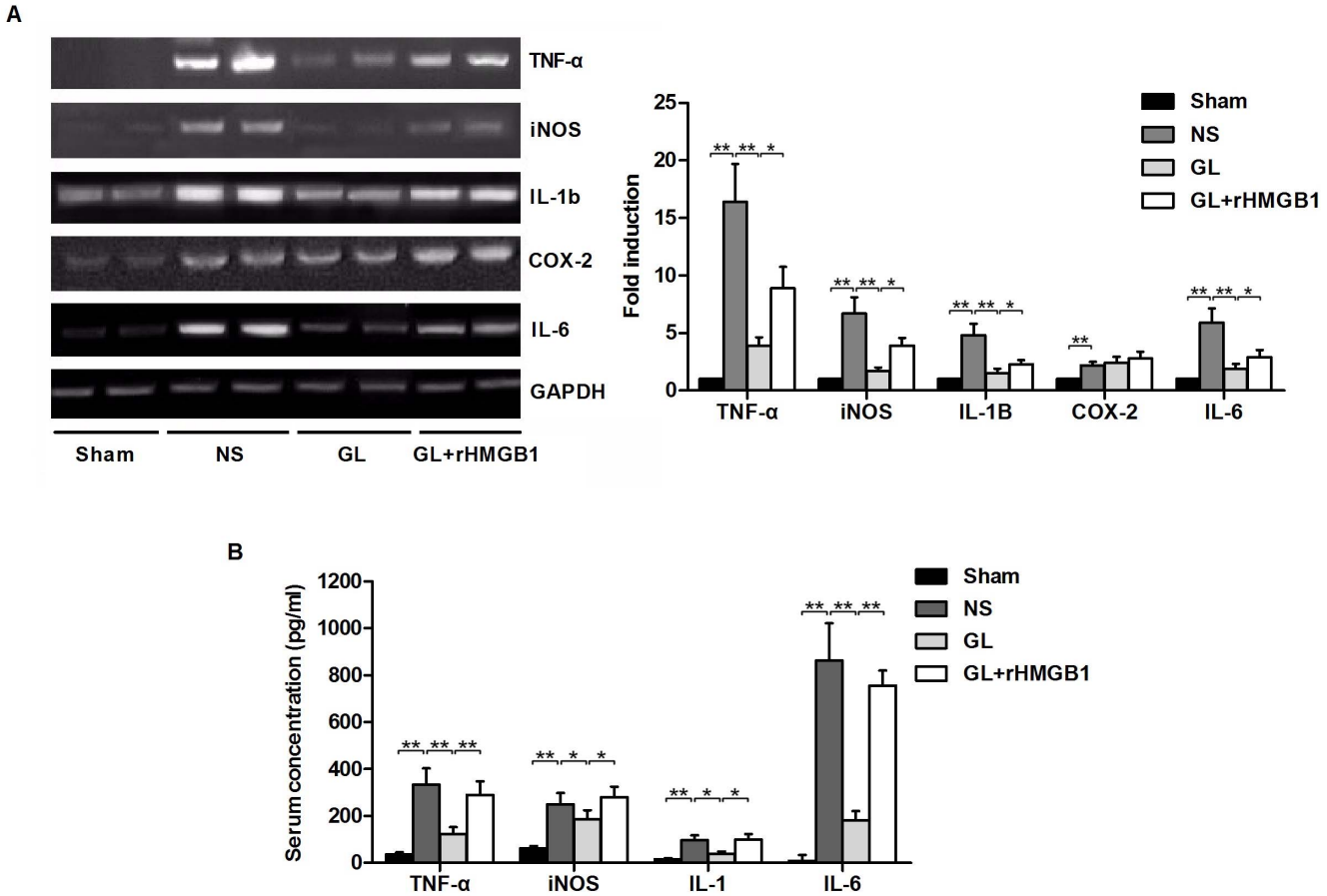

Figure 4. Expression of inflammation- and oxidative stress-related molecules in the brain and serum of MCA-occluded rats at 48 h after reperfusion. A) Representative blots showing the effects of GL with or without rHMGB1 treatment on mRNA expression levels of pro-inflammatory and oxidative stress markers: TNF-α, iNOS, IL-1β, COX-2 and IL-6. GAPDH was used as a loading control. The bar graph showing semi-quantitative densitometric analysis summarises the fold change of TNF-α, iNOS, IL-1β, COX-2 and IL-6 expression in each group. B) Serum concentrations of TNF-α, iNOS, IL-1β, COX-2 and IL-6 at 48 h after I/R in each groups are determined. Values are means±SEM, n = 8 for each group. *P<0.05, **P<0.01 (t test).
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MAP kinases by immunoblotting. As shown in Figure 6, I/R induced phosphorylation of JNK, ERK, and p38 in isolated rat brain cerebral cortex. In GL-pre-treated rats, the phosphorylation levels of p38 and p-JNK were decreased compared with the NS-treated group, whereas p-ERK1/2 was not affected. Co-treatment with rHMGB1 enhanced the phosphorylation of p38 and JNK compared with the GL group, but the levels were still lower than those observed in the NS group. Pre-treatment with GL alone or with rHMGB1 did not affect the total levels of the JNK, ERK1/2, and p38 proteins. These results demonstrated that GL can modulate the p38 and JNK signalling pathways, but not the ERK signalling pathway, in the brains of MCA-occluded rats and that this effect is HMGB1 dependent.

Discussion

Traditional Chinese medicine has become increasingly important in the treatment of cardiovascular ischemia/reperfusion injury. GL is a natural anti-inflammatory compound that is commonly used in Japan to treat patients with chronic hepatitis [14–17]. In the present study, we demonstrated that intravenous pre-treatment with a wide range of concentrations of GL, from 2 mg/kg to 10 mg/kg, reduced cerebral infarct volumes and ameliorated the neurological deficits of rat cerebral I/R injury. The use of a medium concentration of 4 mg/kg yielded considerable improvements, similar to those found at the high concentration of 10 mg/kg GL. Furthermore, this neuroprotective effect was accompanied by a reduction in the release of HMGB1 into the extracellular space and in the related inflammatory factors TNF-α, iNOS, IL-1β, and IL-6, both in the brain and serum of rats (Figure 2 and 4). Using this dose, no significant toxicities were observed in the effects investigated, including changes in the baseline hemodynamic values, cardiac electrophysiology, and in histopathological analysis of vital organs including liver, spleen, lung, kidney, and brain (data not shown).

HMGB1 is a nonhistone DNA-binding protein that possesses two HMG boxes that are DNA binding domains [30]. As a chromosomal protein, it has been implicated in diverse intracellular functions including stabilising nucleosomal structure and facilitating of gene transcription [31]. Moreover, HMGB1 is massively released extracellularly and plays a cytokine-like function in the post-ischemic brain [26,32]. HMGB1 is actively secreted by macrophages and monocytes or released by necrotic cells into the extracellular milieu, where it might be involved in triggering inflammation [33–36]. The present study demonstrated that the predominantly nuclear HMGB1 staining of neurons in rats from the sham group weakened and disappeared during

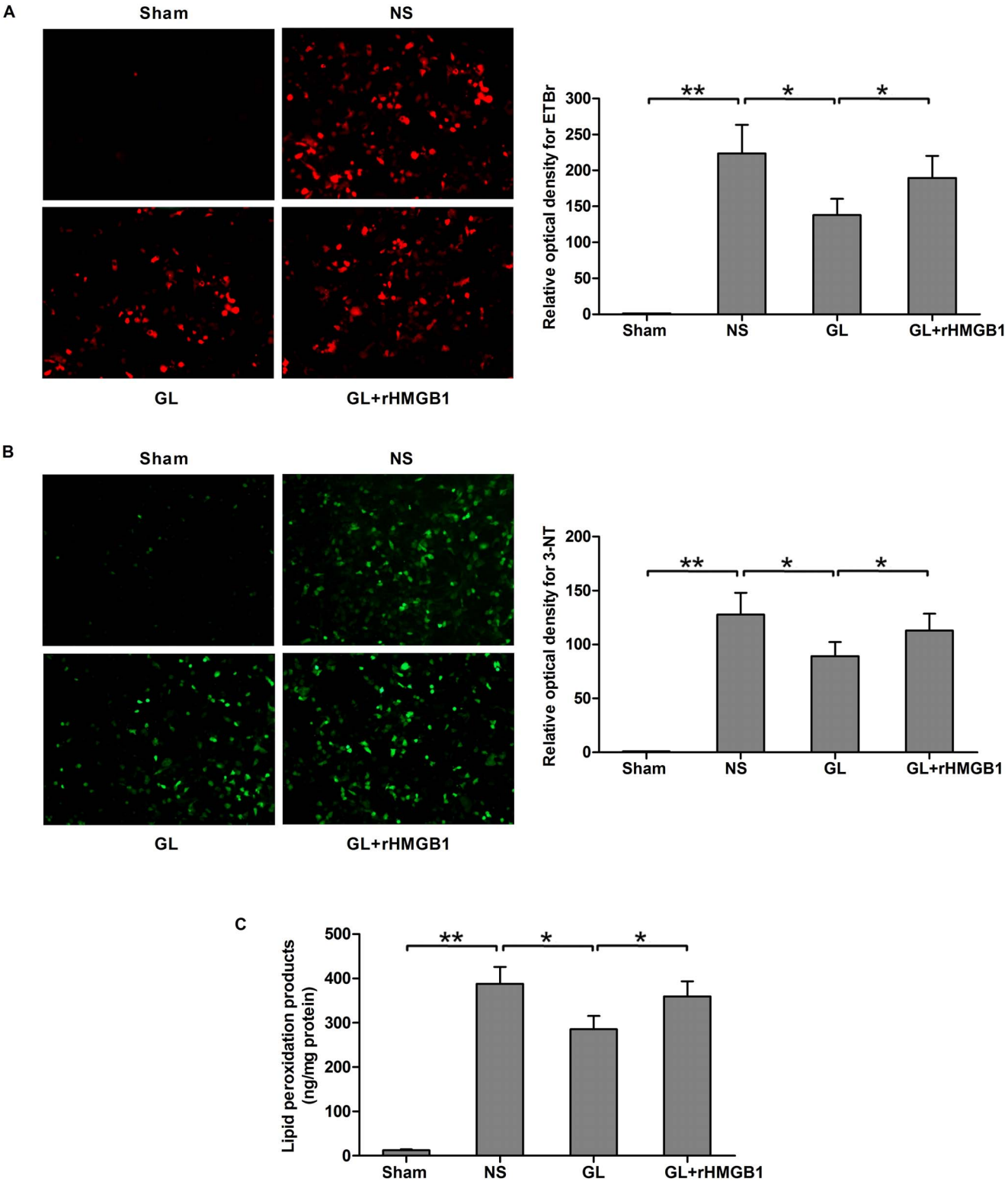

Figure 5. The antioxidant effects of GL are HMGB1 dependent. A) Fluorescent images after incubation with DHE showing superoxide levels in the I/R brain (red). Quantitative measurements of the fluorescence intensity indicated a significant change in the 4 mg/kg GL-treated group with or without rHMGB1, compared with the saline-treated I/R (NS) group. B) Representative photomicrographs showing 3-NT formation in the ischemic brain (green). Data from image analysis also indicated a significant change in the 4 mg/kg GL-treated group with or without rHMGB1, compared with the saline-treated I/R (NS) group. Bar = 50 μm. C) The effects of 4 mg/kg GL with or without rHMGB1 on MDA content in rat brains after I/R. Values are means±SEM, n = 8 for each group. *P<0.05, **P<0.01 (t test).
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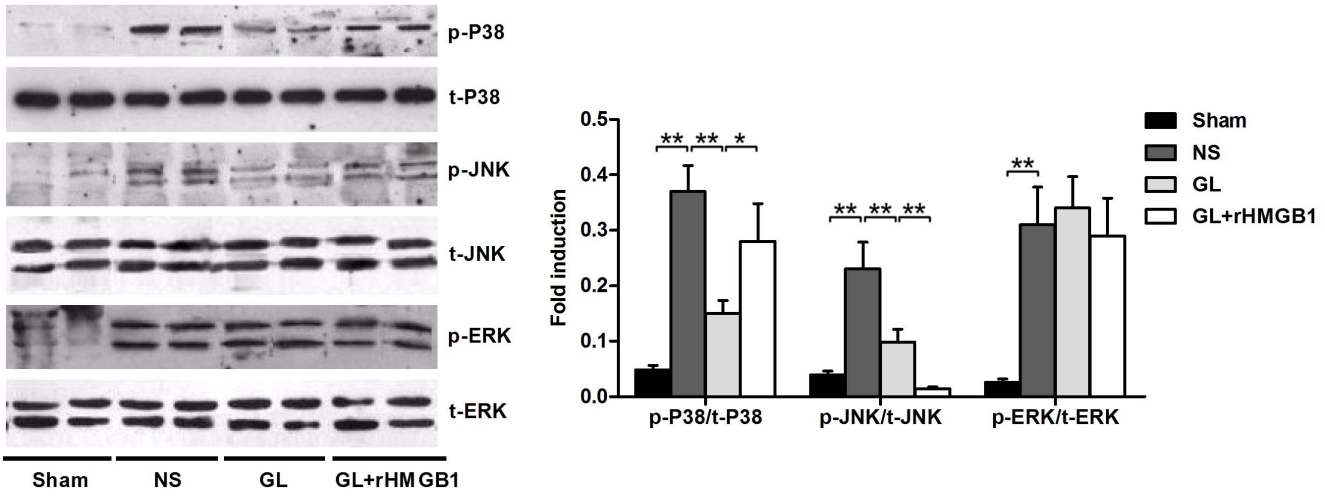

Figure 6. Representative blots showing the effects of GL with or without rHMGB1 treatment on phosphorylated (p) and total (t) p38, JNK, and ERK expression. Each blot shown is representative of 3 experiments with similar results. The bar graph showing semi-quantitative densitometric analysis summarises the fold change in phosphorylated to total p38, JNK and ERK in each group. Values are means±SEM, n = 8 for each group. *P<0.05, **P<0.01 (t test).
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cerebral ischemia (Figure 2D). Thus, release from necrotic neural cells is the most likely source of elevated HMGB1 concentrations in the serum, which were further determined by immunoblot analysis in rats subjected to focal cerebral ischemia. A recent report of elevated serum concentrations of HMGB1 in stroke patients confirmed our findings [37]. Antagonising the pro-inflammatory function or blocking the expression of HMGB1 by a neutralising antibody [11,12], HMGB1 box A [11], or a short hairpin RNA [10,13] ameliorated brain damage. In particular, the expression of TNF-α, iNOS and IL-1β, which were all up-regulated in the post-ischemic brain, was reduced when HMGB1 was inhibited [10,11], consistent with our results. HMGB1 has been reported to stimulate the production of IL-1, TNF-α, IL-6, and IL-8 and to induce iNOS expression [38,39]. In the present study, we demonstrated that the ischemia-induced up-regulation of iNOS, IL-1β, IL-6 and TNF-α was inhibited by treatment with GL. Co-treatment with rHMGB1 can partially reverse this effect (Figure 4). The induction of iNOS and TNF-α following an ischemic insult was reported to occur mainly in microglia. Thus, it is likely that HMGB1 activates microglia in the brain, leading to the up-regulation of iNOS and TNF-α expression. In fact, the induction of iNOS and TNF-α has been reported to be involved in the inflammatory response and the disruption of the blood-brain barrier, leading to the aggravation of brain infarction [40,41]. The regulation of any one of these factors has been postulated to reduce ischemic injury. Therefore, it is reasonable that GL, which has the ability to substantially reduce the expression of pro-inflammatory factors, exerted profound therapeutic effects on brain infarction.

In this study, pre-treatment with GL alleviated apoptosis injury resulting from cerebral I/R, and this was at least partly due to the inhibition of cytochrome C release and caspase 3 activity. Similarly, GL exhibited an anti-apoptotic effect by preventing HMGB1-induced cytochrome C release and caspase 3 activation in vitro in Huh-BAT cells [42]. Though the anti-apoptotic effect of GL has been linked with HMGB1 inhibition and caspase-dependent cytochrome c release, the question remains how GL regulates caspase-dependent cytochrome c release through the inhibition of HMGB1. The exact answer is not known at the present time, but we speculate that GL may modulate the activity of a particular kinase that contributes to cytochrome c translocation and is involved in I/R-induced HMGB1-dependent apoptosis.

Apoptosis is tightly regulated by the mitogen-activated protein (MAP) kinase family, and the JNK, ERK1/2, and p38 members of this family have been demonstrated to be activated in I/R injury. MAPKs play important roles in transducing signals by phosphorylating intracellular enzymes, transcription factors and cytosolic proteins involved in apoptosis and inflammatory cytokine production. Sustained MAPK activation has been shown to be associated with neuronal cell death/apoptosis following ischemic stroke, and the inhibition of this pathway is neuroprotective. In a Huh-BAT cell model, GL prevented HMGB1_induced cytochrome c release and p38 activation but had no effect on phospho_JNK and ERK1/2 [42]. In I/R-induced myocardial injury of rat heart, treatment with HMGB1 box A significantly reduced ERK1/2 and JNK phosphorylation, but did not affect the level of phospho-p38 [43]. However, in another similar study, treatment with GL significantly decreased JNK phosphorylation, but did not affect the level of phospho-p38 and ERK1/2[21]. Interestingly, in our studies, GL could modulate the p38 and JNK signalling pathways, but did not affect the ERK signalling pathway in the brains of MCA-occluded rats, and rHMGB1 could reverse this effect (Figure 6). This ambiguity regarding which single class of MAPK can be modulated by GL appears to primarily depend on the cell and tissue types used and differences in MAPK levels in vivo and in vitro.

In conclusion, our results demonstrated that pre-treatment with GL blocked and inhibited the extracellular cytokine activity of HMGB1 and explored the protective effect on I/R-induced apoptosis through the blockage of the JNK and p38-mediated pathways in rats in vivo. These data suggest a new therapeutic possibility for the treatment of ischemic stroke with GL. Considering the biological differences between species that may influence drug adsorption, metabolism, distribution, and toxicity in rats and humans, it is not clear whether GL has similar protective effects in humans. Hence, future research should be performed to evaluate the beneficial protective effect of GL in

clinical settings with humans, as this might ultimately lead to a new therapeutic strategy for ischemic stroke.

Author Contributions

Conceived and designed the experiments: GG LX HD. Performed the experiments: LY LH WW LC LY. Analyzed the data: GG LX HD. Contributed reagents/materials/analysis tools: LY LH WW LC LY. Wrote the paper: GG LX HD.

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Original articles

Effect of glycyrrhizin on traumatic brain injury in rats and its mechanism

*Gu Xiangjin, Xu Jin, Ma Banyou, Chen Gong, Gu Peiyuan, Wei Dong, Hu Weixing**

【**Abstract**】**Objective:** To investigate the neuroprotective effects of glycyrrhizin (Gly) as well as its effect on expression of high-mobility group box 1 (HMGB1) in rats after traumatic brain injury (TBI).

Methods: Male Sprague-Dawley rats were randomly divided into three groups: sham group, TBI group, and TBI+Gly group (*n*=36 per group). Rat TBI model was made by using the modified Feeney's method. In TBI+Gly group, Gly was administered intravenously at a dosage of 10 mg/kg 30 min after TBI. At 24 h after TBI, motor function and brain water content were evaluated. Meanwhile, HMGB1/HMGB1 receptors including toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE)/nuclear factor-κB(NF-κB) signaling pathway and inflammatory cytokines in the injured brain tissues were detected using quantitative real-time polymerase chain reaction, western blot, electrophoretic mobility shift assay and enzyme-linked immunosorbent assay. Furthermore, HMGB1, RAGE and TLR4 immunohistochemistry and apoptosis were analyzed.

Results: Beam walking performance impairment and brain edema were significantly reduced in TBI+Gly group compared with TBI group; meanwhile, the over-expressions of HMGB1/HMGB1 receptors (TLR4 and RAGE)/NF-κB DNA-binding activity and inflammatory cytokines were inhibited. The percentages of HMGB1, RAGE and TLR4-positive cells and apoptotic cells were respectively 58.37%±5.06%, 54.15%±4.65%, 65.50%±4.83%, 52.02%±4.63% in TBI group and 39.99%±4.99%, 34.87%±5.02%, 43.33%±4.54%, 37.84%±5.16% in TBI+Gly group (all $P<0.01$ compared with TBI group).

Conclusion: Gly can reduce secondary brain injury and improve outcomes in rat following TBI by down-regulation of HMGB1/HMGB1 receptors (TLR4 and RAGE)/NF-κB-mediated inflammatory responses in the injured rat brain.

Key words: ***Glycyrrhizic acid; HMGB1 protein; Brain injuries; Neuroprotective agents***

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Traumatic brain injury (TBI) is one of the major causes of mortality and neurological disability. TBI has been classified into primary injury and secondary injury, and the latter plays a crucial role in the clinical outcome of patients with TBI.[1] Posttraumatic inflammation can aggravate secondary brain injury and result in neurological deterioration.[2] Therefore, reduce of inflammation after TBI contributes to better prognosis of TBI patients.

High-mobility group box 1 (HMGB1) is a kind of non-histone protein that widely exists in the eukaryotic cells. After injury, it is released outside cells and becomes an important mediator of inflammation.[3] The extracellular HMGB1 can bind to cell surface receptors like receptor for advanced glycation end products (RAGE) and toll-like receptor 4 (TLR4).[4,5] These combinations activate nuclear factor-κB (NF-κB) signaling way[5,6] and produce many inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6),[6,7] resulting in inflammatory responses. It has been reported that the levels of HMGB1 in cerebrospinal fluid and plasma are increased in patients after TBI, and the increased levels are associated with poor outcomes of patients.[8,9] HMGB1 may implicate a critical role in promoting inflammation and aggravating damage after TBI.

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Glycyrrhizin (Gly) is a natural anti-inflammatory triterpene that largely exists in the roots and rhizomes of licorice. Recently, Gly has been found to be able to bind to HMGB1 and inhibit cytokine-like activities of HMGB1.[10] Here we investigate the neuroprotective effects of Gly against TBI in a rat model and its preliminary mechanism.

METHODS

Drugs and reagents

The main drugs and reagents included Gly (Minophagen Pharmaceutical Co, Japan), Trizol reagent (15596-026, Invitrogen Life Technologies, USA), the first chain of cDNA synthesis kit (K1622, Thermo Fisher Scientific Inc, USA), Taq DNA polymerase and EMSA Kit (EP0405 and 20148, both Thermo Fisher Scientific Inc, USA), oligonucleotide probe (5-agttgaggggactttcccaggc-3) (GS056B, Beyotime Institute of Biotechnology, China), anti-HMGB1 antibody (ab18256, Abcam, Cambridge, USA), anti-RAGE antibody and anti-TLR4 antibody (bs-0177R and bs-1021R, both Biosynthesis Biotechnology, China), rat IL-1β ELISA Kit (ERC007, Neobioscience Technology Company, China), as well as EliVision plus Kit and DAB Kit (Kit-9902 and DAB-1031, Maixin-Bio, China). Other main reagents were from Nanjing KeyGen Biotech, China.

Subjects and groups

Altogether 108 male Sprague-Dawley rats weighting 250-300 g were provided by the experimental animal center of Nanjing Medical University, China. All the programs followed the guides for care and use of experimental animals. Rats were placed on a 12-hour light-dark cycle with free access to food and water. Rats were randomly divided into three groups: sham group, TBI group and TBI+Gly group (n=36 for each). Rat model of TBI was established according to the modified Feeney's free weight-drop method.[11] Sham rats received right parietal craniotomy alone without head trauma. Rats in the other two groups suffered TBI; 30 min later TBI+Gly group were intravenously administered Gly at the dose of 10 mg/kg, while sham and TBI groups received equal volume of 0.9% saline solution.

At 24 h after operative procedure, randomly 6 rats in each group received beam walking test.[12] Then all the 108 rats were sacrificed and each group was further divided into 6 subgroups (n=6 for each) for tissue assays including brain water content test, quantitative real-time polymerase chain reaction (RT-PCR), western blot (WB), electrophoretic mobility shift assay (EMSA), enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry and apoptosis detection.

For RT-PCR, WB, EMSA and ELISA, the tissue specimens were harvested within 3 mm area to the injured cortex and immediately stored in liquid nitrogen until detection. For immunohistochemistry and apoptosis detection, the rats were transcardially perfused with 4°C 0.1 mol/L phosphate buffered saline (PBS), followed by 4% buffered formaldehyde combined with PBS. Subsequently brains were removed, soaked with 4% formaldehyde and then embedded in paraffin.

Beam walking test for evaluation of motor function

Under the distraction of bright light and loud white noise, rats escaped along a narrow wooden beam (2.5 cm wide and 120 cm long) to enter a darkened goal box. A score of 7 was given when animals traversed the beam with 2 or less foot slips; 6 was given with less than 50% foot slips; 5 was given for more than 50% but less than 100% foot slips; 4 was given for 100% foot slips; 3 was given for traversal with the affected limb extended and not reaching the surface of the beam; 2 was given when the animal was able to balance on the beam but not traverse it; and 1 was given when the animal could not balance on the beam.[12]

Measurement of water content in brain tissue

After the rats were killed, brains were rapidly harvested and cut through midline. Both cerebral hemispheres (injured and uninjured) were immediately weighed to gain the wet weight, and then placed in an oven at 100°C for 72 h to obtain dry weight. The brain water content was calculated as: (wet weight-dry weight)/wet weight×100%.[13]

RT-PCR detection of HMGB1, RAGE and TLR4 mRNA expression

Total cellular RNA was isolated from brain samples using Trizol reagents. RNA was transcribed to cDNA using the first chain of cDNA synthesis kit and oligo dT primers (Nanjing Genscript Biotechnology, China). The forward and reverse primers were as follows: 5'-ATGGGCAAAGGAGATCCTA-3' and 5'-ATTCATCATCATCATCTTCT-3' for HMGB1 (646 bp), 5'-AGTCCAACTACCGAGTCCGA-3' and 5'-

CAACCAACAGCTGAATGCCC-3' for RAGE (396 bp), 5'-TTGCCTTCATTACAGGGACTT-3' and 5'-CAGAGCGGCTACTCAGAAACT-3' for TLR4 (179 bp), as well as 5'- GCCATGTACGTAGCCATCCA-3' and 5'-GAACCGCTCATTGCCGATAG-3' for β-actin (375 bp). The reaction system was 50 μl. PCR products were detected by 1.5% agarose gel electrophoresis and visualized by the gel imaging system (SynGene G: BOX Chemi XR5, UK). The intensity of every band was quantified using Bandscan 4.3 software, and the ratio of every gene product to β-actin product was considered as the expression of every gene.

WB detection of protein expression of HMGB1, TLR4 and RAGE

Total protein was extracted from brain tissue samples and protein concentration was assayed with Bradford method. Samples (50 μg per lane) were subjected to electrophoresis on 10% SDS-polyacrylamide gels and then transferred onto nitrocellulose. The membranes were blocked with 5% skimmed milk for 1.5 h at 37°C then incubated for over night at 4°C in the presence of anti-HMGB1 polyclonal antibody (diluted 1: 500), anti-RAGE polyclonal antibody (diluted 1:200), anti-TLR4 polyclonal antibody (diluted 1:200) and β-actin (diluted 1:200) respectively. Thereafter they were incubated in the appropriate horseradish peroxidase-conjugated secondary antibody (diluted 1:5 000) for 2 h at room temperature. The blotted protein of every band was analyzed using GelPro32 software after enhanced chemiluminescence and later exposed to X-ray film. The ratio of every protein to β-actin product was considered as the relative strength of expression of every protein.

EMSA detection of NF-κB binding activity

Nuclear protein was extracted from brain tissue samples and protein concentration was determined using BCA kit. According to the LightShift® Chemiluminescent EMSA Kit instructions, 20 μl of nuclear protein (20 μg) was incubated for 20 min at room temperature after adding 1 μl of oligonucleotide probe. The mixture was resolved by electrophoresis on 6% nondenaturing polyacrylamide gel and transferred to membrane, which was then incubated using a UV crosslinker and reacted with Streptavidin-HRP conjugate. Shifted bands after imaging were analyzed using GelPro32 software.

ELISA detection of TNF-α, IL-1β, IL-6

According to the specific rat ELISA Kit instructions, the concentrations of inflammatory cytokines (TNF-α, IL-1β, IL-6) were determined in brain tissue samples. The ratio of every cytokine concentration to total protein concentration was considered as the level of cytokine in brain tissue.

Observation of localization and expression of HMGB1, TLR4 and RAGE by immunohistochemical staining

Peroxidase labeling method was used to detect immunohistochemical staining. The sections were incubated with anti-HMGB1 polyclonal antibody (diluted 1: 1 000), anti-RAGE polyclonal antibody (diluted 1:200) and anti-TLR4 polyclonal antibody (diluted 1:200) for 2 h at 37°C. The sections were incubated with strengthening agent for 30 min and horseradish peroxidase conjugated secondary antibodies for another 30 min at room temperature. After visualization by DAB kit and hematoxylin stain, the sections were observed under a light microscope (×200). The cells with HMGB1 transferred from nucleus to cytoplasm were considered HMGB1-positive. The average percentages of cells positive for HMGB1, RAGE and TLR4 within 0.5 mm area to the contusion cortex were calculated.

Detection of apoptotic cells by TUNEL staining

TUNEL staining was performed according to the manufacture's protocol for situ apoptosis detection kit. The average percentage of TUNEL-positive cells within 0.5 mm area to contusion cortex was calculated under a light microscope (×200).

Statistical analysis

All data were presented as mean±standard error ($\bar{x}\pm s$). SPSS 18.0 was used for statistical analysis. Data were subjected to one-way analysis of variance (ANOVA) followed by least-significant difference (LSD) post hoc test. Statistical significance was defined as $P<0.05$.

RESULTS

Beam walking test score after TBI

Before operation all the rats underwent training on the wooden beam for 3 days and all of them achieved a normal score of 7. The rats in sham group still kept the score of 7 at 24 h after sham operation. But the rats in TBI group had a score of 2.83±0.75 ($P<0.01$ compared with sham group), and those in TBI+Gly group had a score of 4.67±0.82 ($P<0.01$ compared with TBI group).

Brain water content after TBI

At 24 h after injury, brain water content of injured side was 79.97%±0.82% in sham group, 82.94%±0.65% in TBI group (*P*<0.01 compared with sham group), and 80.97%±0.49% in TBI+Gly group (*P*<0.01 compared with TBI group). Brain water contents of uninjured sides were 80.10%±0.69%, 80.38%±0.85% and 80.16%±0.72% for each group, which showed no statistically significant differences.

HMGB1, TLR4 and RAGE expression, NF-κB binding activity and inflammatory cytokines in injured brain tissue after TBI

Compared with sham group, in TBI group the mRNA and protein expression of HMGB1, TLR4 and RAGE around traumatic area were at a much higher level, NF-κB binding activity was significantly up-regulated, and productions of IL-1β, TNF-α and IL-6 were increased (all *P*<0.01). In TBI+Gly group compared with TBI group, the mRNA and protein expression of HMGB1, TLR4 and RAGE around trauma area were expressed at a much lower level, NF-κB binding activity was significantly down-regulated, and productions of IL-1β, TNF-α as well as IL-6 were decreased (all *P*< 0.05, Figures 1-4).

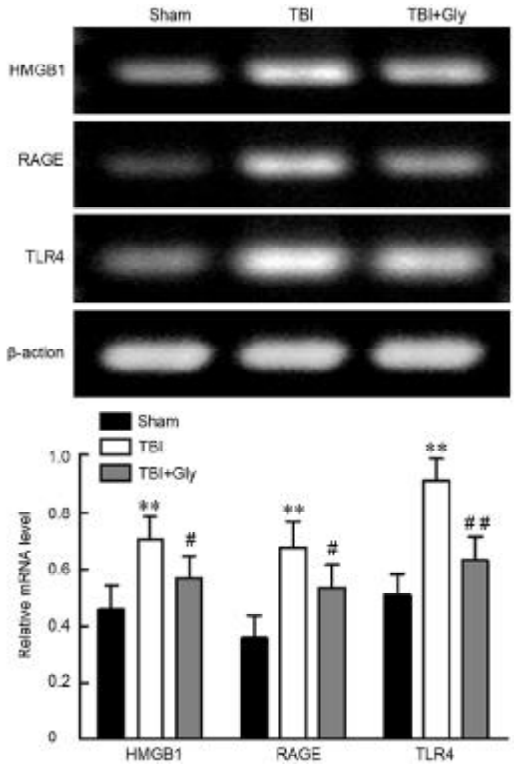

Figure 1. mRNA expression of HMGB1, RAGE and TLR4 by RT-PCR. ***P*<0.01 versus sham group, #*P*<0.05, ##*P*<0.01 versus TBI group.

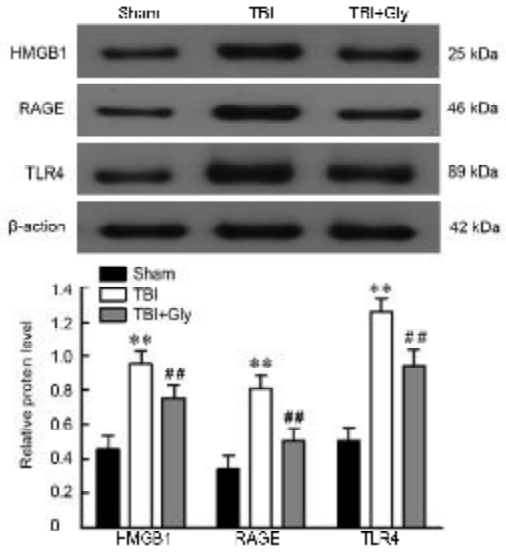

Figure 2. The relevant mRNA and protein expression of HMGB1, RAGE and TLR4. ***P*<0.01 versus sham group, ##*P*<0.01 versus TBI group.

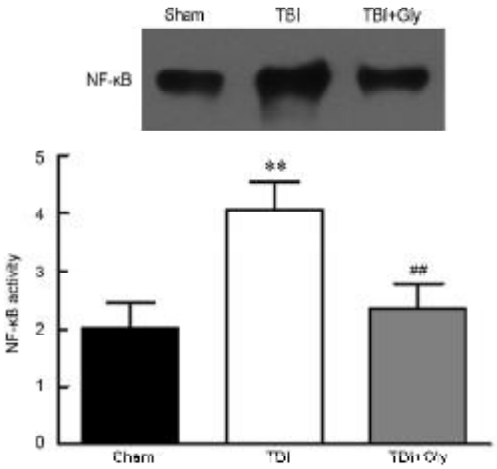

Figure 3. EMSA band and densitometric analysis of NF-κB binding activity. ***P*<0.01 versus sham group, ##*P*<0.01 versus TBI group.

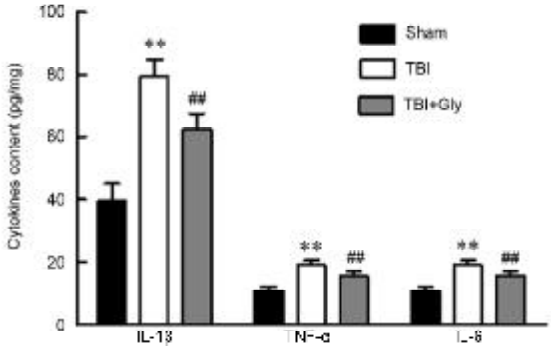

Figure 4. The levels of IL-1β, TNF-α and IL-6 measured by ELISA. ***P*<0.01 versus sham group, ##*P*<0.01 versus TBI group.

HMGB1, RAGE and TLR4-positive cells, apoptotic cells around cortical contusion after TBI

The percentages of HMGB1, RAGE and TLR4-positive cells and apoptotic cells were respectively 7.98%±1.44%, 5.60%±1.12%, 7.60%±1.29%, 8.19%±1.46% in sham group; 58.37%±5.06%, 54.15%±4.65%, 65.50%±4.83%, 52.02%±4.63% in TBI group (all P<0.01 compared with sham group); 39.99%±4.99%, 34.87%±5.02%, 43.33%±4.54%, 37.84%±5.16% in TBI+Gly group (all P<0.01 compared with TBI group, Figure 5).

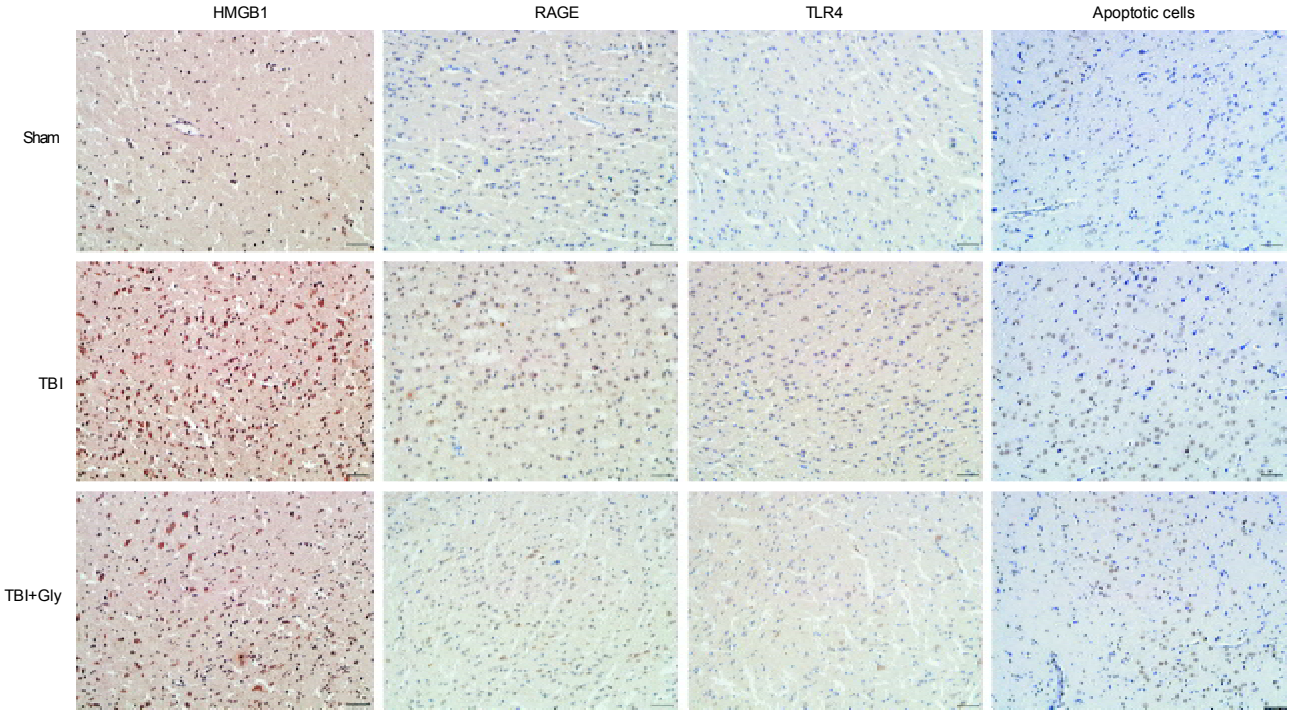

Figure 5. Immunohistochemistry staining showed that HMGB1 mainly distributed in the cell nucleus; few RAGE and TLR4-positive cells were observed in sham group. In TBI group, HMGB1 transferred from nucleus to cytoplasm and mostly redistributed in the cytoplasm; RAGE and TLR4-positive cells were obviously increased. In TBI+Gly group, HMGB1 cytoplasm expression reduced; RAGE and TLR4 positive cells were significantly decreased. TUNEL staining showed that there were few apoptotic cells in sham group. Lots of TUNEL apoptotic cells were showed in TBI group, comparatively less in TBI+Gly group. Scale bar=50 μm.

DISCUSSION

There is mass of evidence to suggest that inflammation plays a vital role in the pathophysiology of TBI. TBI initiates the inflammatory responses by blood brain barrier disruption, brain edema and inflammatory cell infiltration.[14] At the present time, there is no treatment with significant effect clinically. Therefore, exploration of new pharmacologic therapies that target the inflammatory process is necessary for TBI treatment.

Gly is shown to have anti-inflammatory and antiviral effects, and has been used in the treatment of patients with chronic hepatitis.[15] Recently Gly is reported to provide neuroprotective effects on cerebral ischemia[16], intracerebral hemorrhage[17] and ischemic spinal cord injury[18].

In the present experiment, we demonstrate that Gly exerts neuroprotective effects in a rat weight-dropping model of TBI. The results indicate that TBI leads to impair of beam walking balance, brain edema and cell apoptosis. Meanwhile, TBI induces inflammatory responses in the brain tissue, characterized by nucleus-cytoplasm translocation of HMGB1, up-regulated expression of HMGB1/HMGB1 receptors (TLR4 and RAGE), enhanced NF-κB activation, and promoted expression of inflammatory cytokines like IL-1β, TNF-α, and IL-6. Moreover, our results show that Gly administration (1) improves beam walking balance, (2) alleviates brain edema, (3) reduces cell apoptosis, (4) suppresses HMGB1 translocation, (5) inhibits mRNA and protein expressions of HMGB1/HMGB1 receptors (TLR4 and RAGE), (6) represses NF-κB DNA binding activity, and (7) decreases inflammatory cytokines expression. All of the effect support the viewpoint that Gly can reduce secondary inflammation to some extent and improve motor function recovery after TBI. And

to our knowledge, it is the first time that Gly is demonstrated to provide neuroprotective effects following TBI.

HMGB1 is a nonhistone nuclear protein with dual function. Intracellular HMGB1 is bound to DNA and plays an important role in transcriptional regulation. Extracellular HMGB1 conducts as a cytokine-like mediator of inflammation.[3] HMGB1 is a late inflammatory mediator in sepsis and is an early inflammatory mediator in ischemic or traumatic injury.[19] It has been reported that plasma level of HMGB1 increased within 30 min after severe trauma.[20] In our experiment, the expression of HMGB1 mRNA and protein also increased significantly and HMGB1 transferred obviously from nucleus to cytoplasm 24 h after TBI. Extracellular HMGB1 could trigger inflammation through its receptors and signaling pathway. Inflammatory cytokines would, in return, promote the secretion of HMGB1. They are able to form a positive feedback, starting and maintaining a cascade of inflammatory responses. HMGB1 might be the key factor to induce inflammation. Okuma et al[21] used anti-HMGB1 monoclonal antibody to treat TBI in rats and found that it can inhibit HMGB1 translocation, protect blood-brain barrier integrity, suppress inflammatory molecule expression, and improve motor function. Therefore, inhibition of HMGB1 secretion or release represents a novel and promising strategy for the therapy of TBI.

Gly has long been known to exhibit glucocorticiod-like anti-inflammatory actions by inhibiting 11 β -hydroxysteroid dehydrogenase.[22] Recently, Gly is shown to bind directly to HMGB1. Mollica et al[10] reported that Y15, F37, A16, V19, R23, and K42 on HMGB1 are important binding sites for Gly. These sites are very close to some of HMGB1 protein kinase C phosphorylation positions claimed by Oh et al[23]. The direct binding between HMGB1 and Gly may block the interaction between HMGB1 and the protein kinase C, inhibiting HMGB1 phosphorylation. Because phosphorylation of HMGB1 is the initial step for HMGB1 secretion[23], Gly may prevent HMGB1 translocation from nucleus to cytoplasm and break positive feedback loop between the HMGB1 and inflammatory cytokines. In this study, we showed that Gly treatment significantly prevented HMGB1 from cytoplasm translocation and inhibited HMGB1 inflammatory signaling pathway. Gly could retrain excessive inflammatory responses and possess strong neuroprotective effects. Honestly, the underlying molecular mechanism and interaction still needs further exploration.

Our study has some limitations. First, we began Gly intervention at 30 min after TBI. However, this is difficult in clinical practice. Fortunately, it has been reported that Gly suppresses infarct formation with an extended therapeutic window as late as 12 h after stroke.[16] In our further study, we will explore more practical and effective time of Gly intervention in rat TBI model. Second, we intravenously used single Gly dose of 10 mg/kg in this study. We will evaluate the effect of multiple Gly therapeutic doses for maximal neuroprotection in rats after TBI; meanwhile, we will detect the side-effects of Gly, such as pseudo-aldosteronism.[24]

In summary, TBI could induce activation of HMGB1/HMGB1 receptors (TLR4 and RAGE)/NF-κB signaling pathway and inflammatory cytokine expression, which would induce and/or aggravate the secondary brain injury. Gly intervention could down-regulate HMGB1/HMGB1 receptors (TLR4 and RAGE)/NF-κB signaling pathway and inflammatory cytokines expression, decrease brain edema, reduce cell apoptosis and probably improve neurological function after TBI. Gly can be expected to become a new drug for TBI treatment through affecting HMGB1.

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Licorice Compounds Glycyrrhizin and 18β-Glycyrrhetinic Acid Are Potent Modulators of Bile Acid-induced Cytotoxicity in Rat Hepatocytes*

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The accumulation of hydrophobic bile acids results in cholestatic liver injury by increasing oxidative stress, mitochondrial dysfunction, and activation of cell signaling pathways. Licorice root and its constituents have been utilized as antihepatotoxic agents. The purpose of this study was to evaluate the potential modulation by a primary component of licorice root, glycyrrhizin (GL), and its metabolite, 18β-glycyrrhetinic acid (GA), in a hepatocyte model of cholestatic liver injury. Preincubation of fresh rat hepatocyte suspensions with GL or GA reduced glycochenodeoxycholic acid (GCDC)-dependent reactive oxygen species generation, with GA more potent than GL. Interestingly, GL and GA had opposing effects toward GCDC-induced cytotoxicity; GA prevented both necrosis and apoptosis, whereas GL enhanced apoptosis. GCDC promoted activation of caspase 10, caspase 3, and PARP; all were inhibited by GA but not GL. Induction of apoptosis by GCDC was also associated with activation of JNK, which was prevented by GA. Activation of caspase 9 and dissipation of mitochondrial membrane potential were prevented by GA but not GL. In liver mitochondrial studies, GL and GA were both potent inhibitors of the mitochondrial permeability transition, reactive oxygen species generation, and cytochrome *c* release at submicromolar concentrations. Results from this study suggest that GL exhibits proapoptotic properties, whereas GA is a potent inhibitor of bile acid-induced apoptosis and necrosis in a manner consistent with its antioxidative effect.

Cholestatic liver disorders are characterized by impaired bile flow resulting in the retention of bile constituents and hepatocellular damage. Because there are few effective therapies available, the development of cirrhosis and the need for liver transplantation is a frequent outcome in cholestatic children and adults (1). The accumulation of hydrophobic bile acids within the liver is an important factor in the pathogenesis of cholestatic liver disorders (2). Higher concentrations (≥250 μM) of hydrophobic bile acids, such as glycochenodeoxycholic acid (GCDC), promote hepatocyte death by necrosis, and lower concentrations cause apoptosis (3–5). Mechanistic studies have revealed that several factors, including physicochemical properties (6) and death receptor activation (7), account for the pro-apoptotic effects of bile acids. Activation of cell stress signaling pathways, including caspases and mitogen-activated protein kinases (MAPK),[1] are strongly implicated in both the initiation and execution of events culminating in apoptotic cell death. However, the toxicity of bile acids is not uniform; for example, taurochenodeoxycholic acid not only stimulates apoptotic pathways but also activates cell survival proteins, such as phosphatidylinositol 3-phosphate kinase or MAPK extracellular signal-regulated kinase (ERK1/2) (8). Thus, there is a complex interplay between cell death and survival signals in bile acid-induced cytotoxicity that determines ultimate cell fate.

Extensive evidence also supports the involvement of mitochondrial pathways in bile acid-induced hepatocyte toxicity, including induction of the mitochondrial permeability transition (MPT) (9). Upon MPT induction, there is a loss of mitochondrial polarization, onset of mitochondrial swelling, release of soluble proteins such as cytochrome *c* and apoptosis-inducing factor from the intermembrane space, and activation of caspase 9. Furthermore, oxidative stress generated by mitochondria plays a role in bile acid-induced cellular toxicity, as demonstrated in liver mitochondria (10, 11) and rat hepatocytes (12, 13) as well as in *in vivo* studies with whole animals exposed to bile acids (14). Moreover, a variety of diverse antioxidants reduce both oxidative stress and bile acid-induced hepatocyte toxicity (11–15).

Licorice root is an herbal preparation that has been used for decades to reduce liver injury in a number of clinical disorders. In 1977, Suzuki *et al.* (16) reported that the principal triterpene component of licorice root, glycyrrhizin (GL), benefits patients with chronic hepatitis C infection. Derivatives of licorice root have been used in Asia to treat children with biliary atresia (17), a cholestatic liver disease, although no clinical trials have been reported. Increasing evidence supports the hypothesis that GL, or its hydrolyzed metabolite 18β-glycyrretinic acid (GA), protects against several models of oxidant-mediated tox-

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; DAPI, 4,6-diamidino-2-phenylindole; MPT, mitochondrial permeability transition; CsA, cyclosporin A; DCF-DA, 2′,7′-dichlorofluorescin diacetate; DCFein, dichlorofluorscein; GCDC, glycochenodeoxycholic acid; GL, glycyrrhizin; GA, glycyrrhetinic acid; KRH, Krebs-Ringer HEPES (buffer); ROS, reactive oxygen species; MOPS, 4-morpholinepropanesulfonic acid; TRAIL, tumor necrosis factor apoptosis-inducing ligand; PARP, poly(ADP-ribose) polymerase.

icity, including exposure to CCl_4 (18), *t*-butyl hydroperoxide (19), and ischemia-reperfusion injury (20), with GA generally exhibiting greater hepatic protection than GL. Although several hypotheses have been offered to account for the hepatoprotective effects of GL and GA, the effects of these compounds on molecular and biochemical pathways of cell injury have not been well characterized. Therefore, the purpose of this study was to examine the effects of GA and GL on cell pathways of bile acid-induced cytotoxicity in both freshly isolated rat hepatocyte suspensions and purified liver mitochondrial fractions.

EXPERIMENTAL PROCEDURES

Materials—Sodium glycochenodeoxycholate, lactate dehydrogenase kits, ammonium glycyrrhizin, and 18β-glycyrrhetinic acid were obtained from Sigma. Cyclosporin A (CsA) was purchased from Alexis Biochemicals (San Diego, CA). 2′,7′-Dichlorofluorescin diacetate (DCF-DA) and bovine serum albumin (fraction V) were from Eastman Kodak Co. and Calbiochem, respectively. The fluorescent probe JC-1 was obtained from Molecular Probes (Eugene, OR). Primary antibodies against caspase 3, cleaved caspase 9, caspase 10, native and cleaved PARP, phosphorylated and unphosphorylated p38 MAPK, and phosphorylated and unphosphorylated stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) were purchased from Cell Signaling Technology (Beverley, MA). All other chemicals were reagent grade or better.

Isolation of Rat Hepatocytes—Hepatocytes were isolated by a recirculating collagenase technique from male Sprague-Dawley rats (175–225 g) (Sasco, Inc., Omaha, NE) maintained on a 12-h light-dark cycle and fed standard laboratory rat chow, as described previously (15). Initial hepatocyte viability measured by trypan blue exclusion was always >94%. Fresh hepatocytes were resuspended in a Krebs-Ringer HEPES (KRH) buffer containing 0.2% bovine serum albumin (KRH/BSA) to a concentration of $\approx 1 \times 10^6$/ml. This study was approved by the Institutional Animal Care and Use Committee, University of Colorado Health Sciences Center.

Measurement of Reactive Oxygen Species in Rat Hepatocytes—Generation of reactive oxygen species (ROS) was measured spectrofluorometrically using the ROS-detecting probe, 2′,7′-dichlorofluorscein (DCFein), as described previously in detail (21). Briefly, hepatocytes were loaded with DCF-DA for 30 min at 37 °C prior to a 30 min preincubation with graded concentrations of GL or GA. DCF-DA is trapped within cells and deesterified, yielding nonfluorescent dichlorofluoroscin, which is oxidized to the fluorescent DCFein by several ROS. Hepatocytes were exposed to GCDC (0 or 100 μM) for 4 h in a 37 °C shaking water bath at room temperature, and aliquots were removed for analysis of ROS by measuring DCFein fluorescence at 490 nm excitation and 520 nm emission. The results were expressed as fluorescence units/10^6 cells.

Determination of Hepatocyte Apoptosis and Necrosis—Hepatocyte apoptosis was quantitated by determining the percentage of hepatocytes with nuclear morphologic changes of apoptosis (fragmentation and margination of chromatin) detected by fluorescence microscopy of DAPI-stained fixed hepatocytes (13). Necrosis was assessed by the release of lactate dehydrogenase activity from cells and expressed as the percentage of total cellular activity released into the medium (13).

Mitochondrial Depolarization—Flow cytofluorometric analysis was performed to determine the effect of GL and GA upon GCDC-dependent mitochondrial depolarization in hepatocytes, as described previously (12). Briefly, freshly isolated hepatocytes were pretreated with 25 μM GL or 10 μM GA for 30 min and then incubated with 100 μM GCDC for 4 h. Aliquots of cells were removed hourly, loaded with 7.6 μM JC-1 or 3 μM propidium iodide for 15 min at 22 °C in the dark, and washed with KRH buffer at 4 °C prior to flow cytometry on a BD Biosciences FACSCalibur using Flojo software. In actively respiring mitochondria within cells, JC-1 aggregates form, and the intensity of their fluorescence at 590 nm is proportional to the mitochondria $\Delta\psi$ and indicative of a closed MPT pore. Neither GL or GA alone affected JC-1 fluorescence. For each time point and treatment 10,000 cells were analyzed. The fluorescence of JC-1 aggregates was determined only in live cells identified through gating for propidium iodide fluorescence.

SDS-PAGE and Immunoblotting—Total cell lysates were obtained from hepatocytes for immunoblot analysis as follows. Hepatocytes (3×10^6) were pelleted by centrifugation at 2500 rpm for 5 min, resuspended in KRH buffer (no bovine serum albumin), and recentrifuged to obtain a washed cell pellet. Cells were lysed in 1 ml of a buffer containing 62.5 mM Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.00125% bromphenol blue followed by a 15-s sonication on ice, as described by Shah *et al.* (22). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes, and nonspecific proteins were blocked by a 1-h incubation in fresh 5% nonfat dry milk. The blots were probed against the appropriate primary antibody by an overnight incubation at 4 °C followed by a 1-h incubation with rabbit anti-horseradish peroxidase with biotinylated horseradish peroxidase utilized as a size marker. On selected blots, β-actin (Oncogene Research, Boston, MA) was probed to demonstrate equal protein loading.

Mitochondrial ROS Generation—Fresh rat liver mitochondria were isolated by differential centrifugation through a Percoll gradient as described previously (11). ROS were quantitated spectrofluorimetrically using DCFein (11). Briefly, purified mitochondria were resuspended in a buffer containing 5 mM HEPES, 50 mM KCl, 2 mM KH_2PO_4, 125 mM sucrose, pH 7.4, treated with 1% Chelex 100 (wash buffer), and loaded with 8 μM DCF-DA at 28 °C for 30 min. The loaded mitochondria were washed twice with wash buffer, centrifuged at 10,000 × *g* for 10 min, and finally resuspended in 20 ml of a buffer containing 10 mM MOPS, 100 mM NaCl, 125 mM sucrose, pH 7.4, treated with 1% Chelex 100. Mitochondria were then preincubated with graded concentrations of GL or GA, or Me_2SO solvent vehicle. Solvent alone had no effect on any measurements and was at a concentration of ≤0.1%. Mitochondria were then incubated with GCDC, and aliquots were removed at specified time points for DCFein fluorescence measurements at 490 nm excitation and 520 nm emission. The results were expressed as DCFein fluorescence/mg of mitochondrial protein.

MPT and Cytochrome c Content in Liver Mitochondria—MPT induction was quantitated in purified mitochondria spectrophotometrically at 540 nm as described previously (11). Briefly, hepatic mitochondria were preincubated at 25 °C for 5 min alone or in the presence of GL, GA, or the MPT blocker CsA prior to the addition of 100 μM $CaCl_2$, 5 mM sodium succinate, and 5 μM rotenone. Following this 5-min incubation, the MPT was induced by addition of 100 μM GCDC. Mitochondrial swelling was quantitated by the reduction in absorbance at 540 nm during the 5-min incubation with GCDC. After the MPT experiment, mitochondrial samples were centrifuged at 13,000 × *g* for 30 min at 4 °C to isolate the mitochondrial pellet for immunoblot analysis of cytochrome *c* using anti-mouse cytochrome *c* antibody and anti-mouse horseradish peroxidase secondary antibody (BD Biosciences).

Statistical Analysis—Statistical analysis between groups was conducted by analysis of variance using the Scheffe test or by *t* test for comparing means from two groups. A *p* value of <0.05 was considered significant. Values were expressed as means ± S.E.

RESULTS

GL and GA Reduce GCDC-stimulated ROS Generation—To determine the effect of licorice compounds on bile acid-induced ROS generation, freshly isolated rat hepatocyte suspensions were incubated for 4 h with 100 μM GCDC, and DCF fluorescence was measured. GCDC increased DCF fluorescence linearly in a time-dependent manner when compared with control hepatocytes (35.8 ± 1.5 *versus* 9.7 ± 0.9 fluorescence units/10^6 cells at 4 h) (Fig. 1*a*). Pretreatment with GL decreased ROS generation modestly at all concentrations (0.5–25 μM) (Fig. 1*a*). GA treatment reduced ROS generation to a greater extent, with all concentrations reducing ROS generation by >60% at 4 h (Fig. 1*b*). Concentration-effect relationships of the two compounds after 4 h of incubation with GCDC (Fig. 1*c*) showed that both GA and GL function at low concentrations to reduce bile acid-induced oxidative stress, that maximal effect was achieved at low concentrations, and that GA was superior to GL in this effect.

GL and GA Differentially Regulate GCDC-induced Cytotoxicity—We next determined the effects of the licorice compounds on cell death pathways. Hepatocytes exposed to 100 μM GCDC underwent a time-dependent increase in necrosis, as evident by the percent of lactate dehydrogenase leakage (38.2 ± 2.2% *versus* 15.9 ± 1.3% for control cells at 4 h) (Fig. 2). Pretreatment with GL afforded no protection against cell necrosis (Fig. 2*a*), whereas all concentrations of GA reduced cell necrosis by >60% at 4 h (Fig. 2*b*). A concentration-effect comparison of GL and GA (Fig. 2*c*) demonstrates the superior protection afforded at all concentrations by GA.

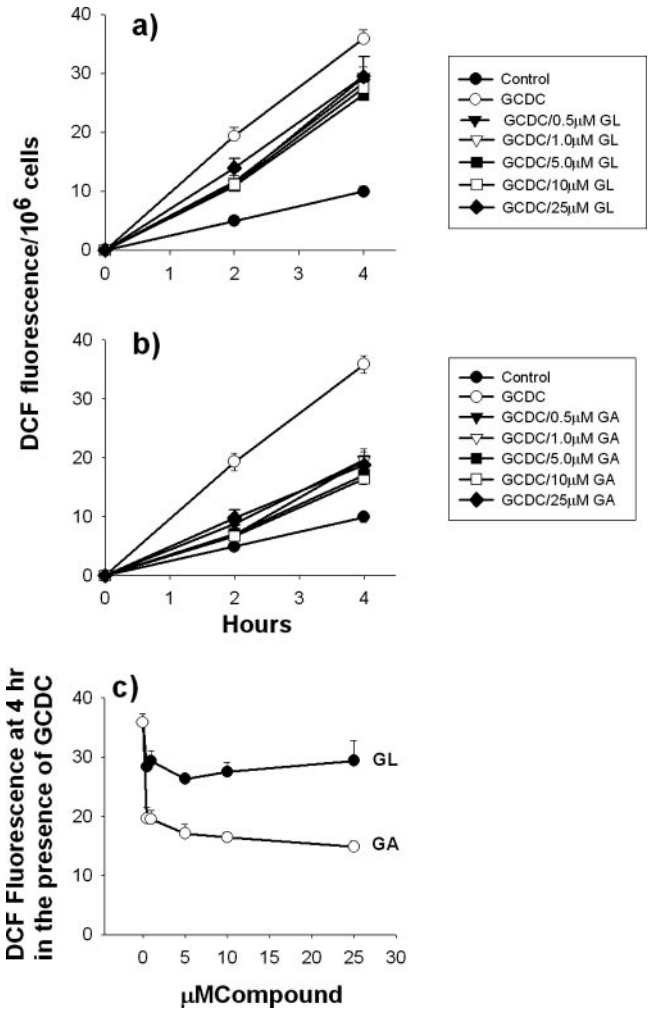

FIG. 1. **Effects of GL and GA on ROS generation in isolated rat hepatocyte suspensions exposed to GCDC.** Hepatocytes (10^6/ml) were loaded with 10 μM DCF-DA for 30 min prior to preincubation with various concentrations of GL (*a*) or GA (*b*) followed by exposure to 100 μM GCDC. Aliquots were removed at 2 and 4 h for DCF fluorescence, expressed as DCFein fluorescence/10^6 cells, as described under "Experimental Procedures." The 4 h DCFein values for GCDC-exposed hepatocytes are plotted *versus* concentrations of GL and GA (*c*). Results are from at least six separate experiments and are expressed as mean ± S.E.

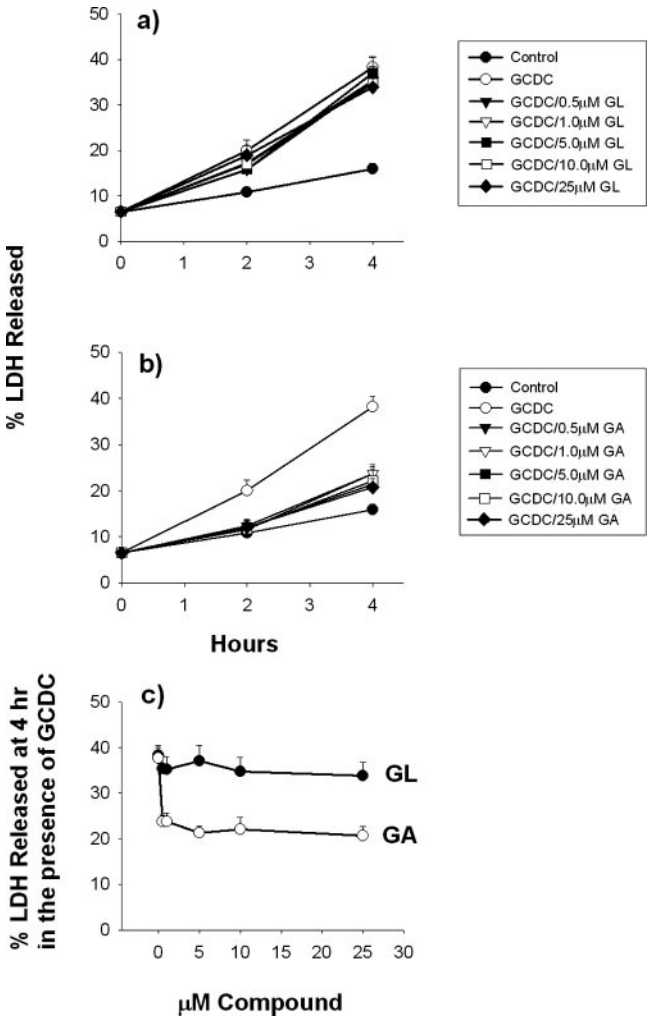

FIG. 2. **Effects of GL and GA on cellular necrosis in isolated rat hepatocyte suspensions exposed to GCDC.** Hepatocytes were preincubated with various concentrations of either GL (*a*) or GA (*b*) for 30 min prior to the addition of 100 μM GCDC. Aliquots were removed at 2 and 4 h and necrosis assessed as percent lactate dehydrogenase (*LDH*) released, as described under "Experimental Procedures." The 4-h percent lactate dehydrogenase released values for GCDC-exposed hepatocytes were plotted *versus* concentrations of GL and GA (*c*). Results are from at least six separate experiments and expressed as mean ± S.E.

The differential effects of GL and GA on hepatocytes were more dramatic when DAPI-stained hepatocyte nuclei were examined for apoptosis (Fig. 3). Hepatocytes treated with 100 μM GCDC underwent significant apoptosis by 4 h (31.5 ± 11.9% *versus* 1.6 ± 0.7% for control cells) (Fig. 4). Preincubating hepatocytes with 25 μM GL enhanced apoptosis by ~170–210% after 2 h incubation (Fig. 4*a*). In contrast, 25 μM GA significantly inhibited GCDC-induced apoptosis by >70% throughout the course of the experiment (Fig. 4*b*). A concentration-effect analysis of the effects of GL and GA on GCDC-induced apoptosis at 3 h (Fig. 4*c*) demonstrated that GL enhanced apoptosis at concentrations of 0.5 μM and above, whereas GA protected against apoptosis at ≥10 μM.

Previous experiments have demonstrated that GCDC causes a reduction of mitochondrial membrane potential (indicating the MPT) that precedes induction of apoptosis in hepatocytes (11, 15). Therefore, JC-1 fluorescence was used to determine the effects of GL and GA on bile acid induction of MPT in live hepatocytes. Hepatocytes exposed to GCDC for 3 h (Fig. 5*a*, *red* data line) showed a decrease in mitochondrial membrane potential (shift to left of JC-1 aggregate fluorescence curve) when compared with control cells (*blue* data line). The time course (Fig. 5, *b* and *c*) demonstrated a decrease in JC-1 fluorescence by 1 h. Preincubation with 10 μM GL (Fig. 5*, a* and *b, black* data line) failed to prevent the dissipation of membrane potential at any time point. However, pretreating cells with 25 μM GA (Fig. 5, *a* and *c*, *green* data line) significantly prevented the reduction in membrane potential for at least 3 h, commensurate with the protection offered against necrosis and apoptosis. GL and GA alone (Fig. 5, *a* and *c*, *gold* data line) had only a slight effect on membrane potential.

Effects of GL and GA on Changes in Caspase and MAPK Activation—Immunoblots of whole cell lysates indicated that procaspase 10 was reduced after 3 h of incubation with GCDC (Fig. 6*a*). This apparent activation of caspase 10 was prevented by 25 μM GA but not by 1 or 10 μM GL. Activation of caspase 10 previously has been shown to process executioner caspases 3 and 7 (23). As shown in Fig. 6*b*, caspase 3 existed predominantly in the native, uncleaved form (procaspase 3) in the absence of GCDC (*lane 1*). However, after 3 h GCDC reduced levels of procaspase 3. Preincubation of hepatocytes with GA prevented loss of procaspase 3, whereas GL yielded no protection at 1 μM, and at 10 μM GL increased the appearance of the

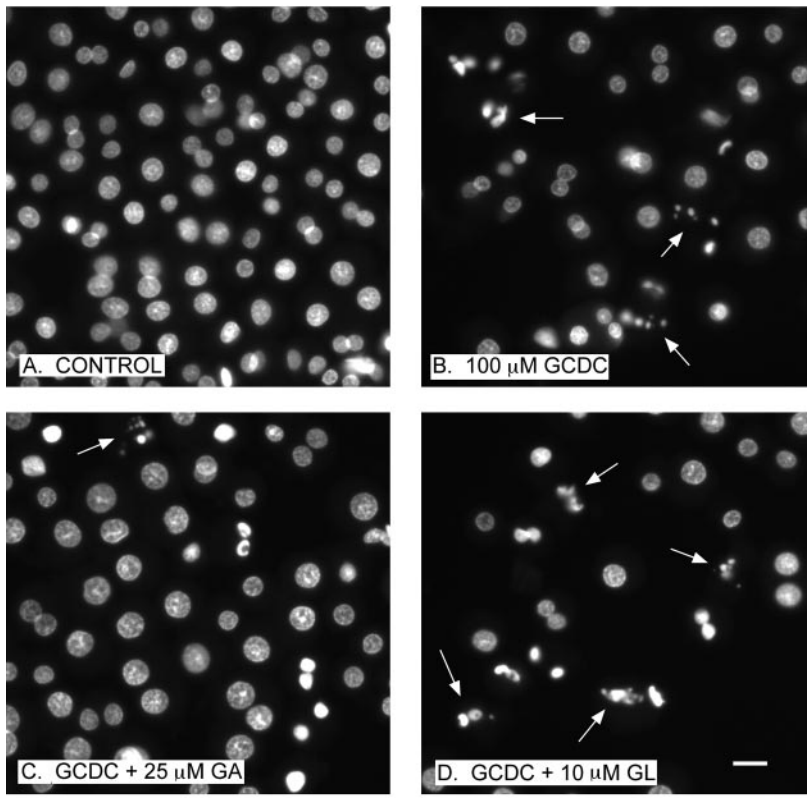

FIG. 3. **Fluorescence microscopy of hepatocyte nuclei labeled with DAPI.** Hepatocytes were incubated with no additions (*A*), with 100 μM GCDC alone (*B*), or in the presence of 25 μM GA (*C*) or 10 μM GL (*D*) for 3 h. After the incubation, cells were fixed, cytofuged onto a slide, and stained with DAPI for fluorescence microscopy. Only those cells that had fragmented nuclei or marginated chromatin (depicted by *arrows*) were considered apoptotic. *Scale bar* (in the *lower right corner*) = ~10 μm.

cleaved caspase 3. Similar results were observed by examining the cleavage of PARP, where GCDC promoted PARP cleavage, which was prevented by GA and potentiated by GL (Fig. 6*c*).

Caspase 9 is activated following cytochrome *c* release from mitochondria. Immunoblot analysis of cleaved caspase 9 revealed an increase of caspase 9 after incubation with GCDC (Fig. 6*d*), which was only mildly reduced by GA and was potentiated by 10 μM GL (Fig. 6*d*). In selected experiments, β-actin was probed to demonstrate equal loading of all lanes (Fig. 6*e*). Taken together, these data support the hypothesis that the anti-apoptotic effects of GA is primarily through a caspase 9-independent mechanism.

Activation of MAPK has been recently reported to be involved in cell signaling cascades involved in bile acid cytotoxicity (24, 25). We examined activation of the two members of the MAPK family implicated in bile acid toxicity, p38 MAPK and JNK; the latter is also a member of the SAPK family (Fig. 7). Band densities of phosphorylated MAPK were expressed relative to total MAPK levels and then adjusted to control samples, which were normalized to a value of 1.0. The effects of GL and GA on phosphorylation of p38 MAPK in GCDC-treated cells are shown in Fig. 7*a*. The ratio of phosphorylated to total p38 MAPK remains relatively unchanged by treatment with GCDC (Fig. 7*a*, *lane 2*), GA (*lane 3*), or GL (*lanes 4* and *5*), suggesting that apoptosis was not p38 MAPK-dependent. In Fig. 7*b*, the ratio of phosphorylated to total JNK density was increased by exposure to GCDC alone (1.9-fold, *lane 2*). Pretreatment with GA (Fig. 7*b*, *lane 3*) prevented JNK phosphorylation, whereas GL (*lanes 4* and *5*) had no significant effect. These data support a role for JNK activation during GCDC-induced apoptosis, suggesting that the anti-apoptotic effect of GA may be mediated by inhibition of the SAPK/JNK pathway.

Effects of GL and GA on Liver Mitochondria—ROS generation and induction of the MPT in liver mitochondria by bile acids have been implicated in hepatocellular death by necrotic and apoptotic mechanisms. Therefore, we next examined the direct effects of GL and GA on mitochondrial function. Incubation of purified liver mitochondria with 100 μM GCDC resulted in a linear increase in ROS generation (Fig. 8). Preincubation with GL inhibited ROS generation in a dose-dependent manner at low concentrations (0.1–1 μM), reaching a plateau at 1.0 μM GL, which reduced ROS generation by >60% at 10 min (Fig. 8*a*). GA reduced ROS generation >60% at all concentrations (0.1–10 μM) (Fig. 8*b*). These treatment effects were comparable with the cytotoxicity responses observed in Figs. 1 and 2, with GA exhibiting a greater antioxidative and protective effect than GL.

Next, the effects of GL and GA on GCDC-induced MPT in purified mitochondria were compared. As shown previously (11, 13), GCDC induced the MPT when incubated for 5 min with succinate-energized mitochondria (Fig. 9). In the current study, a dose-dependent decrease in the magnitude of the MPT was observed when mitochondria were preincubated with 0.1–1.0 μM GL (Fig. 9*a*), with a reversal of this effect as the concentration of GL was increased to 5 or 10 μM. GA inhibition of the MPT was almost identical to that observed with GL, including the reversal of protection observed above 1 μM (Fig. 9*b*). Neither GL nor GA (up to 25 μM) incubated alone with mitochondria induced the MPT (data not shown). Because of the magnitude of MPT inhibition by low (including submicromolar) concentrations of GL and GA, we compared these compounds against CsA, a direct blocker of the MPT (Fig. 9*c*). On an equimolar basis, both GL and GA offered protection against the GCDC-induced MPT almost equal to that provided by CsA.

Subsequent to induction of the MPT by GCDC, mitochondria released substantial amounts of cytochrome *c* (Fig. 9*d*). Preincubation with graded concentrations of GL and GA showed similar protection against loss of cytochrome *c*, paralleling the reduction of MPT magnitude (Fig. 9, *a* and *b*).

DISCUSSION

Licorice root has long been utilized as an herbal remedy against a variety of ailments in Asian cultures (26). Although the precise biological mechanisms responsible for these clin-

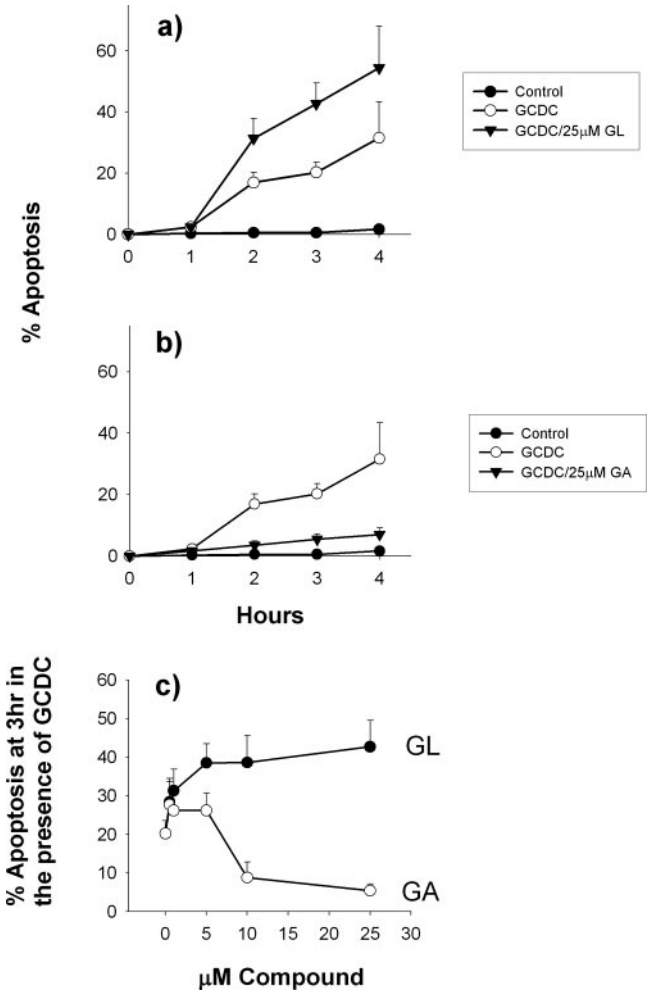

FIG. 4. **Effects of GL and GA on GCDC-induced hepatocyte apoptosis.** Hepatocytes were exposed to 100 μM GCDC for 4 h in the absence or presence of 25 μM GL (*a*) or GA (*b*). Aliquots were removed hourly for quantitation of apoptosis as described under "Experimental Procedures." The percent of apoptotic cells after 3 h of exposure to GCDC was plotted *versus* concentrations of GL and GA (*c*). Results are from at least six separate experiments and expressed as mean ± S.E.

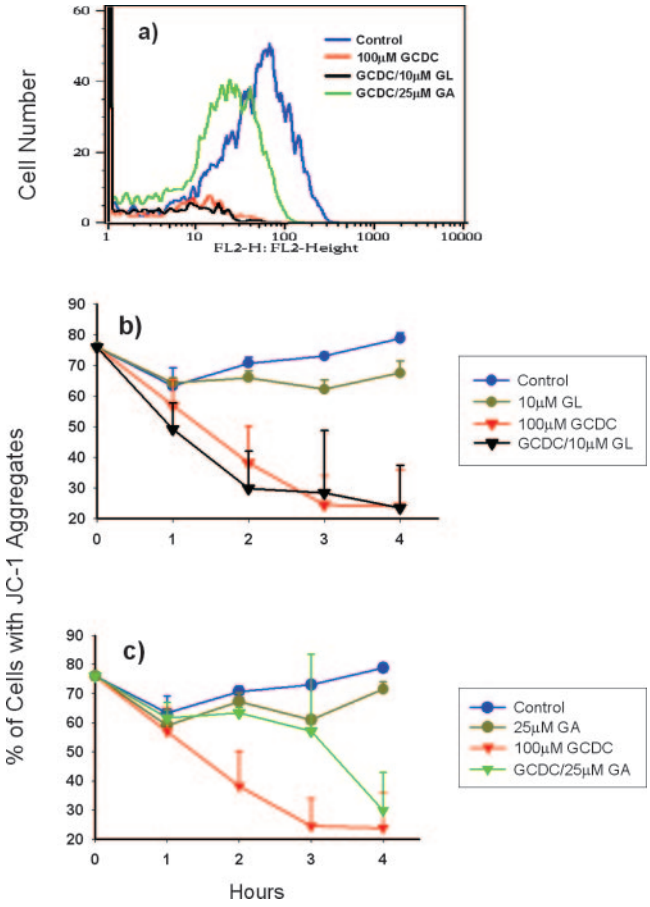

FIG. 5. **Effects of GA and GL on GCDC-induced mitochondrial depolarization in rat hepatocytes.** Isolated rat hepatocytes were treated with 100 μM GCDC alone (*red line*), or combined with 10 μM GL (*black line*) or 25 μM GA (*green line*). Aliquots were removed hourly and loaded with JC-1 and propidium iodide as described under "Experimental Procedures." In *a*, a representative plot of JC-1 aggregate fluorescence is shown after 3 h, indicating mitochondrial depolarization in GCDC-treated cells compared with control hepatocytes (*blue line*), which was prevented by GA but not GL. The time course of JC-1 aggregate formation is depicted in *b* and *c*, demonstrating protection by GA but not GL. Neither GL nor GA alone (*gold lines*) affected mitochondrial depolarization. Results are from three separate experiments and expressed as mean ± S.E.

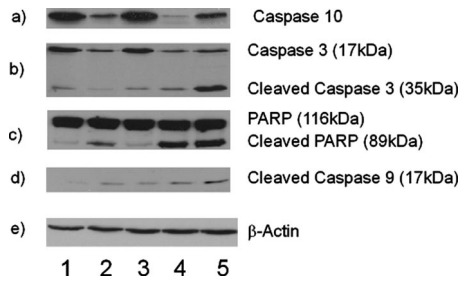

FIG. 6. **Immunoblot analysis of caspases and PARP from rat hepatocytes treated with GCDC.** Whole cell lysates obtained from hepatocytes after 3 h of incubation were separated by SDS-PAGE and immunoblotted as described under "Experimental Procedures." For each blot, the lane assignments were as follows: *lane 1*, control; *lane 2*, 100 μM GCDC; *lane 3*, 100 μM GCDC + 25 μM GA; *lane 4*, 100 μM GCDC + 1 μM GL; and *lane 5*, 100 μM GCDC + 10 μM GL. Blots were probed with antibodies raised against caspase 10 (*a*), caspase 3 (*b*), native and cleaved PARP (*c*), cleaved caspase 9 (*d*), and β-actin (*e*). These are representative results from 2–4 separate hepatocyte preparations.

ical benefits are unknown, evidence from experimental studies document that GL and its major metabolite by intestinal metabolism, GA, are protective in whole animals and cultured hepatocytes (18, 27). Several hypotheses have been put forward to account for the hepatic protection offered by these compounds including stimulation of cytochrome P-450 and glutathione *S*-transferase activities (27) or their activity as an antioxidant through glutathione preservation (18). Although these compounds are commonly used in herbal preparations purported to be of benefit in cholestatic liver disease (17), their biological effects in cholestatic liver injury have not been characterized. Therefore, the current study was performed to determine the effects of GL and GA on pathways involved in bile acid-induced cytotoxicity.

The results of this study reveal GL and GA to be potent modulators of bile acid-induced cytotoxicity with GL enhancing GCDC-induced apoptosis and GA significantly inhibiting both necrotic and apoptotic cell death. Micromolar concentrations of GL enhanced GCDC-induced activation of several pro-apoptotic pathways, including caspase 10 and JNK signaling. In contrast, GA inhibited these signaling pathways and afforded significant protection against cytotoxicity. This protective role of GA was consistent with its antioxidative effect, although other potential effects of GA (28, 29) were not explored. Interestingly, the potentiation of GCDC-induced apoptosis by GL was independent of its modest reduction of ROS generation. Importantly, the protective effect of GA in hepatocytes was

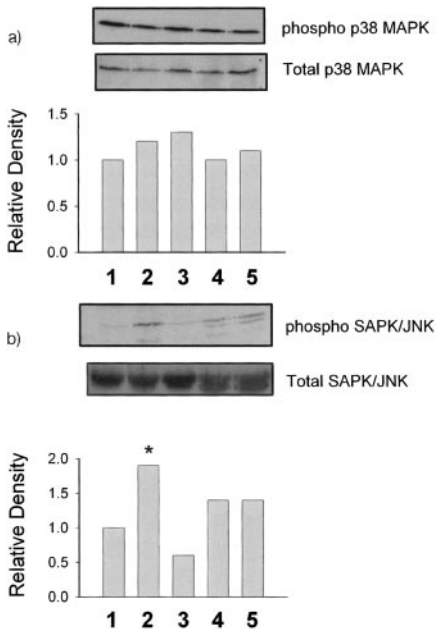

FIG. 7. **Immunoblot analysis of MAPK in rat hepatocytes treated with GCDC.** Whole cell lysates obtained from control hepatocytes after 3 h of incubation were separated by SDS-PAGE and immunoblotted as described under "Experimental Procedures." For each blot, the lane assignments were as follows: *lane 1*, control; *lane 2*, 100 μM GCDC; *lane 3*, 100 μM GCDC + 25 μM GA; *lane 4*, 100 μM GCDC + 1 μM GL; and *lane 5*, 100 μM GCDC + 10 μM GL. Blots were probed with antibodies raised against total and phosphorylated p38 MAPK (*a*) and total and phosphorylated SAPK/JNK (*b*). Levels of activation by GCDC in the absence or presence of GL or GA are graphically depicted in *bar graphs* and are expressed as the ratio of the relative density of phosphorylated/total protein, with control cells standardized to 1.0. The results are from three separate hepatocyte preparations. *, denotes statistical significance ($p < 0.05$) of control hepatocytes *versus* GCDC-treated cells.

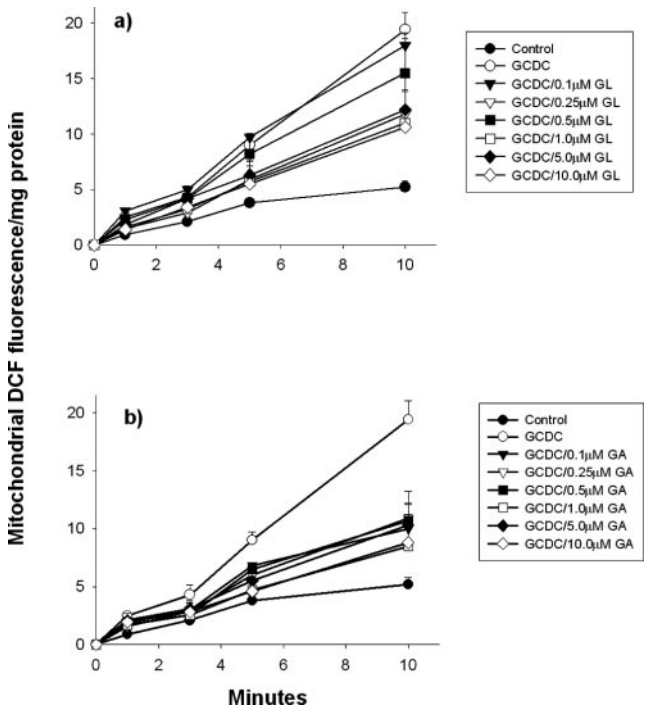

FIG. 8. **Effects of GL and GA on ROS generation in purified liver mitochondria treated with GCDC.** Percoll gradient-purified liver mitochondria were loaded with DCF-DA, preincubated with various concentrations of GL (*a*) or GA (*b*), and then incubated with 100 μM GCDC. Aliquots were removed at designated time points, and DCF fluorescence was expressed as DCFein fluorescence/mg of protein. Results are from 3–4 separate experiments and expressed as mean ± S.E.

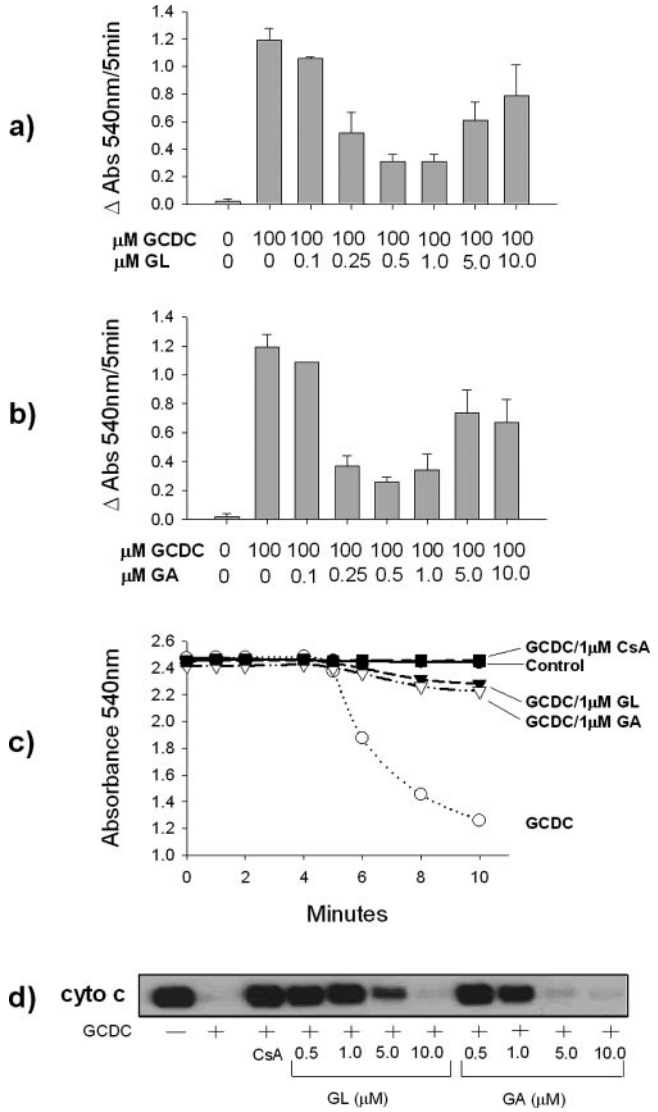

FIG. 9. **Effects of GL and GA on GCDC-induced MPT and cytochrome *c* release from purified mitochondria.** Rat liver mitochondria were preincubated for 10 min with 0–10 μM GL (*a*) or GA (*b*) prior to induction of MPT by 100 μM GCDC. Mitochondrial swelling was monitored at 540 nm as described under "Experimental Procedures." Results are from 3–4 separate experiments and expressed as mean ± S.E. In *panel c*, the inhibitory potency of GA and GL are compared with CsA in a representative experiment. After the 5-min incubation of mitochondrial with GCDC, mitochondria were isolated and immunoblotted for cytochrome *c* content (*d*) as described under "Experimental Procedures."

accompanied by inhibition of the MPT in live cells, ROS generation, cytochrome *c* release from mitochondria, and caspase 9 activation.

Previous studies have associated increased oxidative stress with the severity of bile acid-induced cytotoxicity in hepatocyte suspensions (15, 25) and in whole animals receiving parenterally administered bile acids (14). Correspondingly, oxidative stress and cytotoxicity were attenuated by antioxidants including α-tocopherol, β-carotene, or the coenzyme Q analog, idebenone (13, 15, 30). The relative degree of antioxidant activity of GL and GA (Fig. 1) correlated well with the capacity of each compound to suppress GCDC-induced cellular necrosis (Fig. 2). However, the potentiation of apoptosis by GL (Fig. 3) implicates activation of other apoptotic signaling pathways or inhibition of cell survival cascades. In contrast, GA continued to demonstrate marked anti-apoptotic effects even at concentrations as low as 0.5 μM. In prior studies of hepatocyte apoptosis,

Yoshikawa *et al.* (31) reported that GL inhibited tumor necrosis factor-α- but not Fas-dependent apoptosis in HepG2 cells at concentrations that significantly enhanced apoptosis in our study. However, ROS generation, mitochondrial function, and caspase activation were not addressed in that study (31). In the current study, the reduction of oxidant stress and the cytoprotective effect by GA were consistent with another study that reported an antioxidative role of GA in the amelioration of carbon tetrachloride-induced liver injury (18).

One well characterized caspase-dependent pathway responsible for bile acid-induced hepatocyte apoptosis requires death receptor activation of caspase 8 as a response to formation and aggregation of a death-induced signaling complex (DISC) (32). Upon activation of caspase 8, signaling through the mitochondrial pathways results in downstream caspase 3 cleavage, activating the nuclear enzyme PARP, which is responsible for nuclear degradation. A closely related homolog to caspase 8, caspase 10, is another target of Fas ligand and TRAIL-induced activation (33, 34) and has been reported to promote apoptosis in certain cell types (35, 36). Caspase 10 exists as four known isoforms and is expressed in many tissues including liver and skeletal muscle (23). Despite its being implicated as an inducer of apoptosis, the role of caspase 10 in bile acid-induced cytotoxicity has not been examined. In our studies, procaspase 10 levels in rat hepatocyte suspensions underwent increased proteolysis in the presence of GCDC, which was prevented by GA. In a recent study, Higuchi *et al.* (37) found no activation of caspase 10 by GCDC alone but found that co-incubation of GCDC with TRAIL, a death receptor cell signaling agent, promoted caspase 10 cleavage. There are differences between our study and that of Higuchi *et al.* (37) that could account for the differing effects of bile acids on caspase 10; those authors used HuH-7 cells transfected with a sodium-dependent transporting polypeptide in culture for 12 h, and our study utilized freshly isolated rat hepatocytes. The role of caspase 10 in bile acid-induced cytotoxicity requires further study.

Previous studies indicate that activation of p38 and JNK, via the SAPK pathway, is associated with bile acid-induced apoptosis (24, 25, 38), whereas activation of ERK may suppress apoptosis (39, 40, 44). Both p38 and JNK, activated by stress and inflammatory stimuli, regulate AP-1 transcription factor and its component, c-Jun, by phosphorylation reactions (41). In our study, JNK activation, commensurate with oxidative stress, was a key signal in GCDC cytotoxicity. It has been proposed that oxidative stress itself is responsible for JNK activation (25, 42). This sequence of events is supported by the differential effects of GA and GL on ROS generation and JNK activation. In addition, in our model the dependence of caspase 10 on ROS generation and JNK activation is consistent with the observation of Chaudhary *et al.* (43), suggesting that increased ROS generation may be the upstream event that triggers JNK and caspase activation in bile acid-induced hepatocyte toxicity.

In addition to death receptor-initiated cytotoxicity, bile acids also promote cell death by direct effects on mitochondrial structure and homeostasis (12, 15, 45, 46). Consistent with previous findings (15, 25), GCDC increased generation of ROS, caused mitochondrial depolarization, and activated caspase 9 in rat hepatocytes. GA, providing a more robust antioxidative effect than GL, prevented this apoptotic pathway, whereas GL failed to afford protection. However, in contrast to their dichotomous effects on GCDC toxicity in hepatocytes, submicromolar concentrations of GL and GA similarly blocked the MPT and release of cytochrome *c* in purified liver mitochondria exposed to GCDC. Thus the differential effects of GA *versus* GL on cytotoxicity do not appear to be related to direct effects on mitochondria. However, it must be emphasized that although cellular uptake of GL and GA has been characterized previously (47, 48), mitochondrial uptake, transport, and metabolism have not been examined.

Several factors must be considered prior to examining the possible benefical or modulatory roles of these compounds in hepatobiliary disorders, including achievable tissue concentrations, mode of administration, metabolism and potential toxicity. Orally ingested GL undergoes hydrolysis by β-glucuronidase in the intestine resulting in GL monoglucuronide and, ultimately, GA, which is absorbed into the bloodstream (49). Intravenous administration of GL-containing compounds results in the appearance of both GL and GA in the plasma of animals (50), although conversion of GL to GA is believed to occur primarily in the mucosa of the small intestine (51). Intravenous administration of GL in healthy men and in hepatitis C patients achieved maximal plasma concentrations of 29 μg/ml (approx 36 μM) (52) and 120 μM (53), respectively, whereas orally administered GL yielded no detectable plasma GL and only very low (<200 ng/ml) concentrations of GA (52). In another study, plasma GA levels reached 10 μM in humans ingesting licorice (54), similar to the protective concentrations of GA in our study.

In conclusion, low concentrations of GL resulted in enhanced bile acid-induced apoptosis of isolated hepatocytes through activation of caspases and the SAPK pathway member, JNK. Conversely, GA inhibited these pathways, prevented bile acid-induced mitochondrial depolarization, reduced oxidative stress, and protected against apoptosis and necrosis. Further evaluation of these compounds are warranted in regard to a potential role in treating cholestatic liver disease and other liver diseases associated with increased oxidative stress.

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Licorice Compounds Glycyrrhizin and 18β-Glycyrrhetinic Acid Are Potent Modulators of Bile Acid-induced Cytotoxicity in Rat Hepatocytes

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Acute effects of a superoxide radical-generating system on DNA double-strand stability in Chinese hamster ovary cells

Determination by a modified fluorometric procedure *

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Summary

The rate of oxyradical generation by a xanthine oxidase–xanthine system to acutely cause DNA strand breakage in Chinese hamster ovary cells was studied in a phosphate-buffered saline system. DNA strand breakage, measured by a fluorometric procedure, was found to increase curvilinearly as a function of oxyradical generation. Results of studying the ability of 5 mM mannitol, 10 mM dimethylthiourea, 300 μg superoxide dismutase/ml, or 1 mg catalase/ml to interfere with DNA damage at a high rate of oxyradical production best supported a hydrogen peroxide-promoted mechanism for DNA breakage.

Evidence presented by Weitzman et al. (1985) using human neutrophils as oxyradical generators and supported by studies of Zimmerman and Cerutti (1984) suggests that the production of active oxygen species in the presence of living cells can promote the evolution of cell transformation processes which lead to neoplasia. Since microbial infections have been associated with higher incidences of cancer in humans (Mackowiak, 1987) and since normal body defense mechanisms are well known to involve phagocytes which produce active oxygen species used to inactivate these microbes, we have undertaken studies to define the DNA damage produced by the generation of oxyradicals in a system of living cells. We have also attempted to determine what active oxygen species may be primarily involved in the DNA-damaging process.

Recent studies by Weitberg et al. (1983, 1985)

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and Sofuni and Ishidate (1984) have suggested that oxyradical generation in systems containing Chinese hamster ovary (CHO) cells or Chinese hamster fibroblasts, respectively, can produce DNA damage in these cells as evidenced by increased numbers of sister-chromatid exchanges and various other chromosomal aberrations. In these studies, either stimulated human granulocytes or xanthine oxidase systems were used as oxyradical generators; the effect of these radicals on cell chromosomes was measured 24 or more hours later. Although effects on cellular chromosomes were reported in these studies, the time periods used, lack of available information regarding actual quantities of oxyradicals produced, and failure to use purified xanthine oxidase make it difficult to distinguish what acute effects oxyradical may have had on cellular DNA.

In this investigation we have applied a fluorometric procedure to study the acute effects of oxyradicals on DNA strand breakage in CHO cells. Commercially available xanthine oxidase was preliminarily chromatographed to remove contaminating proteases and standardized by spectrophotometric methods.

Materials and methods

Ham's F-10 medium, fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (PBS), and antibiotics were products of Gibco Laboratories; ethidium bromide (CAS 1239-45-8), xanthine oxidase (buttermilk), xanthine (CAS 69-89-6), mercaptoethanol (CAS 60-24-2), cytochrome *c* type VI (CAS 9007-43-6), superoxide dismutase, catalase, cyclohexane diaminetetraacetate, sodium laurylsulfate (CAS 151-21-3), urea (CAS 57-13-6), and sodium dithionite (CAS 7775-14-6) were products of Sigma Chemical Co. Pentex bovine serum albumin (BSA) was from Miles Laboratories, Sephadex from Pharmacia, and all other chemicals used were reagent grade. All solutions were prepared in high purity (Milli-Q) water.

CHO cells were grown in modified Ham's F-10 medium containing 10% FBS with 100 μg kanamycin sulfate/ml. Culture flasks (75 cm^2) were seeded with $2–3 \times 10^5$ cells that had a doubling time of 12–14 h. All experiments were carried out at the time cells reached confluence.

Xanthine oxidase was freed of protease activity by the method of Waud et al. (1975). This was undertaken not only to prevent the effects of the protease on subunit structure and activity of xanthine oxidase, but also to prevent possible effects of protease activity on cell morphology and function that have been described by Agar and Gordon (1984) and might otherwise contribute to apparent damage caused by active oxygen. The final preparation was suspended in Dulbecco's PBS containing 0.1% BSA. Preparation activity was standardized at 25 °C by the method of Hart et al. (1970). Superoxide anion production by the xanthine oxidase–xanthine system used for exposing cells to oxyradicals was determined by measuring initial reduction rates of ferricytochrome *c* in the same system under conditions used for cell treatment. The reaction mixture of 3.0 ml contained 2×10^{-5} M ferricytochrome *c*, 0.1 mM xanthine, and xanthine oxidase in Dulbecco's PBS. Cytochrome reduction was followed at 550 nm, and the rate of superoxide production was carried out by using a millimolar absorption coefficient of 29.5 for cytochrome *c*. The reaction was begun by adding substrate to the system pre-equilibrated to a temperature of 37 °C.

Monolayers of confluent CHO cells were washed with Dulbecco's PBS and incubated in Dulbecco's PBS for 30 min at 37 °C in the presence of varying concentrations of xanthine and/or xanthine oxidase. After incubation, the reaction mixture was poured off, and the cell layer was washed 3 times with PBS. After washing, cells were harvested by trypsinization for 2 min. Cells were finally washed in growth media containing 10% FBS followed by 2 washes in PBS. The final cell pellets were placed on ice and resuspended in ice-cold 0.25 M sucrose–10 mM sodium phosphate–1 mM magnesium chloride (pH 7.4). This preparation was used for analyzing DNA double-strand content.

The fluorometric analysis of cellular double-stranded DNA was conducted by a modification of the method of Birnboim and Jevcak (1981). Under the conditions reported by these authors, we found that only 20% of the cellular double-stranded DNA remained. As a result, their procedure was re-examined and modified to yield approximately 74% double-stranded DNA after al-

kali diffusion-induced DNA unwinding. This degree of recovery could be attained provided that 0.17 M NaOH was substituted for the 0.2 M NaOH originally reported. In addition, the incubation conditions were modified; a single, 30-min diffusion period at 0 °C was used to promote uncoiling of short-length DNA strands produced by cellular oxyradical exposure. We found 0.25 M sucrose–10 mM sodium phosphate–1 mM magnesium chloride (pH 7.4) to be adequate for initial cellular suspension. Cell suspensions used for the uncoiling procedure and all subsequent steps used for uncoiling were carried out in a darkened room. The final fluorescence was read by using an Aminco–Bowman fluorometer with an excitation wavelength of 520 nm; emission was determined at 590 nm.

The effects of various radical removing or quenching agents were studied in a system identical to that described for studying oxyradical effects on confluent CHO cells. Catalase (1 mg/ml), superoxide dismutase (300 μg/ml), mannitol (5 mM), or dimethylthiourea (10 mM) were incorporated into PBS before oxyradical formation was induced by adding xanthine to the xanthine oxidase system. Incubation times and other conditions and manipulations were identical to those described above.

Tests for statistical significance were carried out using the Student's *t*-distribution analysis. Calculations were performed during a Compucorp 344 computer.

Results

Fig. 1 shows the percentage of double-stranded DNA remaining in CHO cells as a function of the initial rate of superoxide radical generated by the xanthine oxidase–xanthine system used. Points in the figure represent the mean values of 8 determinations for control or for systems containing 0.1, 0.25, or 0.5 units of xanthine oxidase ml. Span limits above and below each point represent the standard deviation. Xanthine, 0.1 mM, served as substrate in a system that contained 1 μmole of total xanthine. A 30-min incubation period was allowed so that approximately the same quantity of superoxide anion was produced by each enzyme system used.

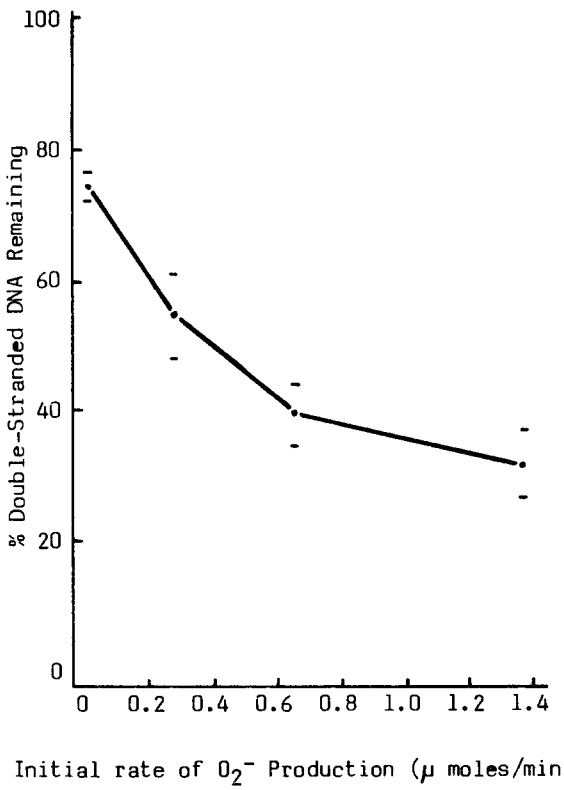

Fig. 1. Recovery of double-stranded DNA from CHO cells exposed to increasing rates of superoxide anion production in PBS.

The amount of DNA damage produced increased curvilinearly as a function of the initial rate of superoxide anion production. The control cell system contained no xanthine or xanthine oxidase and yielded a recovery of $74.3 \pm 2.09\%$ (mean $\pm$ 1 S.D.) double-stranded DNA. At the highest level of superoxide anion generation used, only $32.0 \pm 4.01\%$ double-stranded DNA was recovered from the CHO cell system. At each rate of superoxide anion production used there was a significantly lower recovery of double-stranded DNA ($P < 0.001$) than for the control. DNA recoveries from control systems containing 0.1 mM xanthine, 0.1 unit xanthine oxidase/ml, or 0.5 unit xanthine oxidase/ml in PBS were $65.5 \pm 3.97\%$, $67.9 \pm 2.56\%$, and $66.4 \pm 3.09\%$, respectively. Each of these systems showed a significantly lower recovery than that noted for the buffer control ($P < 0.001$) and a significantly higher recovery than that noted for complete systems containing

xanthine and xanthine oxidase ($P < 0.001$). There were no differences in DNA recoveries found in the controls containing xanthine or xanthine oxidase in PBS. Complete systems containing PBS with xanthine and xanthine oxidase each showed DNA recoveries that were significantly different from the others ($P < 0.001$, $P < 0.01$ for system containing 0.25 unit xanthine oxidase/ml vs. system containing 0.5 unit xanthine oxidase/ml). The initial rate of superoxide anion production could not be further increased by increasing xanthine concentration in the system. This was shown spectrophotometrically with the ferricytochrome assay system and the temperature used for carrying out the cell studies. We did not use higher concentrations of xanthine oxidase in our studies because attempts to use xanthine oxidase at concentrations of 1 unit/ml were associated with signs of cellular damage as noted by the trypan blue exclusion test.

To establish conditions for studying the effects of radical-trapping agents on the recovery of double-stranded DNA from CHO cells, we first examined these agents' effects on ferricytochrome reduction in the system described previously. We slowed the reaction rate to a more easily examined one by performing the studies at 25°C. When ferricytochrome reduction rate was studied in the standard system, the rate of reduction appeared to be counteracted by a back reaction of oxidation apparently catalyzed by a product resulting from the reaction occurring between xanthine and xanthine oxidase (Fig. 2, C). Incorporating 5 mM mannitol (M) or 10 mM dimethylthiourea (D) into the system caused an increase in the total reduction of ferricytochrome measured. Each agent was, in turn, associated with a delay in ferrocytochrome back-oxidation. Dimethylthiourea produced a greater effect than did mannitol.

We presumed that the back-oxidation was caused by the build up of hydrogen peroxide generated in the system from superoxide anion dismutation occurring at rates similar to the reduction of ferricytochrome *c*. To test this we examined the same system after adding 50 μg of catalase. When this was done, identical curves for reducing ferricytochrome *c* were noted for the control system, with or without the addition of either 5 mM mannitol or 10 mM dimethylthiourea. The kinetics of the reaction with catalase in the system are illustrated in Fig. 3. When catalase was present, there was no ferrocytochrome *c* back-oxidation, and all of the ferricytochrome *c* was found to be relatively quickly reduced.

Ferricytochrome *c* reduction by the xanthine–xanthine oxidase system in the presence of catalase was almost completely inhibited when sufficient superoxide dismutase was added. Maximum inhibition was noted when 150 μg of superoxide dismutase were added, and the reduction curve it

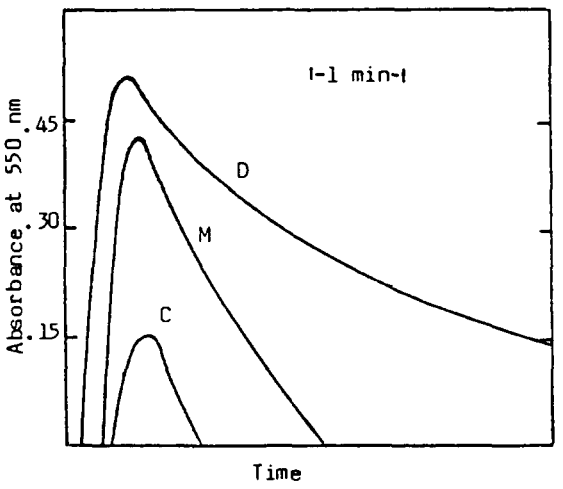

Fig. 2. Reduction–reoxidation of ferri-cytochrome *c* by a xanthine–xanthine oxidase system (C) and effects of 5 mM mannitol (M) or 10 mM dimethylthiourea (D) on the reaction in PBS.

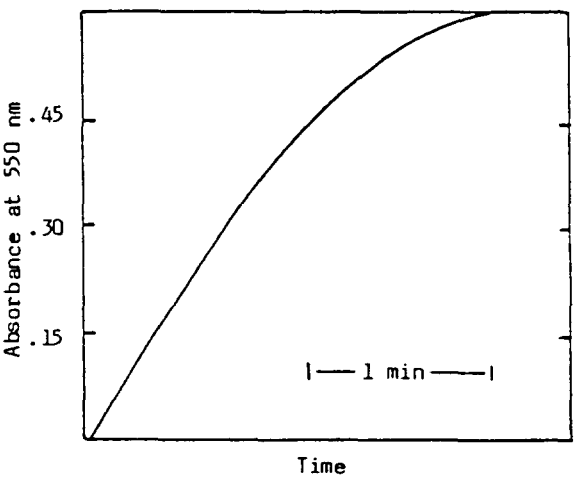

Fig. 3. Reduction of ferricytochrome *c* by a xanthine–xanthine oxidase system containing 50 μg of catalase in PBS.

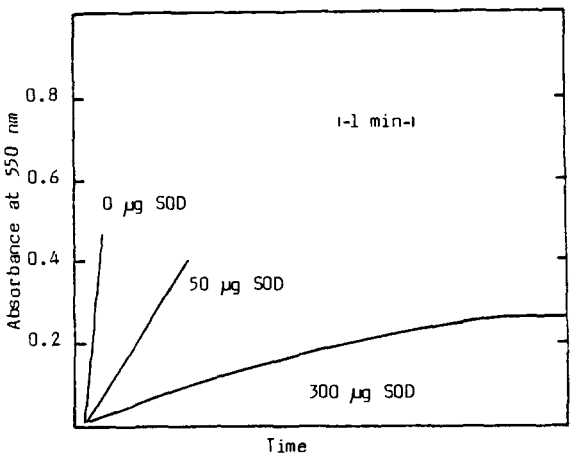

Fig. 4. Effect of superoxide dismutase (SOD) on the reduction of ferricytochrome *c* by a xanthine–xanthine oxidase system containing 50 μg catalase in PBS.

produced was identical to that seen when either 300 μg or 600 μg of superoxide dismutase was added (cf. Fig. 4). Residual reduction of ferricytochrome in the xanthine × xanthine oxidase system is caused by the presence of deflavo xanthine oxidase within these preparations. This was recognized by McCord and Fridovich (1969).

We next utilized these various systems to examine the effect of the radical-trapping agents on the recovery of double-stranded DNA from CHO cells. To ensure adequate catalase activity, we further increased the amount of catalase used to 1 mg/ml. The results of these studies are given in Table 1. In each case 6 separate studies were done, and the results represent the means ± 1 S.D. for each condition. To place maximum stress on each protective agent used, we used the maximum quantity of xanthine oxidase used previously (0.5 unit/ml). Once again a marked drop in recoverable double-stranded DNA similar to that previously seen, was noted. Each addition of the various agents to the system caused a significant protective effect, with maximum protection being granted by the system containing catalase. In this case, the percentage of recoverable double-stranded DNA was not different from that seen in the control ($P > 0.2$). Superoxide dismutase gave the poorest protection of all agents used; however, its effect was still significant ($P > 0.001$, < 0.01). The order of protective effect was catalase greater than catalase plus superoxide dismutase ($P > 0.1$)

TABLE 1

THE EFFECT OF VARIOUS AGENTS ON THE RECOVERY OF DOUBLE-STRANDED DNA FROM CHO CELLS INCUBATED WITH 0.5 UNIT OF XANTHINE OXIDASE (XO)/ml AN 0.1 mM XANTHINE (X) IN PBS

System	Double-stranded DNA recovered (%) (mean ± S.D.)
Control	75.3 ± 3.07
XO + X	35.1 ± 4.44
XO + X + 5 mM mannitol	63.3 ± 8.98
XO + X + 10 mM dimethylthiourea	54.1 ± 6.90
XO + X + 300 μg superoxide dismutase	51.3 ± 9.31
XO + X + 1 mg/ml catalase	71.6 ± 9.43
XO + X + 300 μg superoxide dismutase + 1 mg/ml catalase	68.0 ± 3.34

greater than mannitol ($P > 0.2$) greater than dimethylthiourea ($P > 0.05$, < 0.1) equals superoxide dismutase. Mannitol's effect was significantly greater than that produced by superoxide dismutase ($P < 0.05$) as were the effects of catalase with or without superoxide dismutase ($P < 0.01$). Systems containing catalase were also significantly more effective in protecting cellular double-stranded DNA than was the system containing dimethylthiourea ($P < 0.01$).

Discussion

The method employed in these studies for measuring DNA damage relies on the principle that ethidium bromide binds selectively to double-stranded DNA and not to single-stranded DNA produced when short duplex regions of double-stranded DNA are destabilized by alkali. When applied to a study of CHO cells exposed to varying rates of superoxide anion production caused by xanthine interaction with varying amounts of xanthine oxidase, a curvilinear decrease in the amount of remaining double-stranded DNA occurred as the rate of oxyradical generation was increased. The recovery of double-stranded DNA decreased significantly at each rate of superoxide radical generation used. Because each system gave

sufficient time for all xanthine to be utilized, the increased damage to DNA observed was not the result of total superoxide anion generated but rather resulted from the rate of its generation within the system. These results imply that cell defense mechanisms were operative in protecting cellular DNA and that their ability to do so was a function of the cells' exposure rate to oxyradical species involved. This interpretation is consistent with the observation of Cantoni et al. (1986), who recently observed that CHO cells can effectively remove the DNA strand breakage activity of hydrogen peroxide when cells are incubated with this oxidizing agent. Hydrogen peroxide or a derivative thereof could be the DNA strand-breaking agent here, because superoxide anion generated in the system is known to readily disproportionate in aqueous systems to yield hydrogen peroxide (cf. McCord and Fridovich, 1969). Unlike studies previously mentioned, we purified commercially available xanthine oxidase to remove contaminating proteases; therefore, protease action during superoxide anion generation by the xanthine–xanthine oxidase system could not have contributed to the results.

Although previous studies have indicated that DNA damage in CHO cells can be produced by oxyradical generation external to the cells, relating the damage produced to the actual rate of oxyradical generated was not attempted. McLean et al. (1982) studied this response effect using increasing quantities of radiation exposure to acutely generate oxyradicals in a human white blood cell system and showed a curvilinear effect on leukocyte DNA similar to that noted here. Our studies and those of McLean et al. (1982) imply that approximately 30% of the DNA contained within both human leukocytes and CHO cells is not susceptible to DNA damage at radical generation rates that do not cause appreciable cell death. The effects of oxidizing agents may induce cellular changes that eventually limit the oxidizing agent's access to cell DNA. Schraufstatter et al. (1986), reporting hydrogen peroxide's effects on DNA strand breakage in P388D_2 murine macrophages and human lymphocytes, found that the maximum DNA strand breakage induced by increasing concentrations of hydrogen peroxide approached a level of approximately 80% of the total double strands available for ethidium bromide binding.

The xanthine oxidase–xanthine system used in these studies generates superoxide anion as its primary product; hydrogen peroxide is produced as a secondary product by disproportionation in the aqueous system. We used the system of McCord and Fridovich (1969) to show this and to demonstrate that a reoxidation of ferrocytochrome *c* occurs after the initial ferricytochrome *c* reduction by superoxide anion. To prepare for our studies on radical quenching agent's effects on DNA strand breakage in CHO cells, we first looked at the kinetics of the ferricytochrome *c* reduction in the presence of xanthine oxidase at the maximum concentration of xanthine oxidase used for producing DNA damage. Both mannitol and dimethylthiourea were capable of delaying cytochrome reoxidation, and dimethylthiourea showed greater activity in doing so than did mannitol at the concentrations to be used. Catalase completely prevented the reoxidation, thereby implicating hydrogen peroxide as the active agent in this reoxidation. As expected, superoxide dismutase, at sufficient concentration, completely prevented ferricytochrome *c* reduction.

To obtain some information on the active oxygen species probably responsible for the DNA damage induced by the xanthine oxidase–xanthine system, we utilized the aforementioned agents at the concentrations used in our spectrophotometric system to determine their effects, if any, on preventing cellular DNA damage. All agents proved to be effective, but only catalase appeared to completely prevent DNA damage. Superoxide dismutase, at a concentration that completely prevented ferricytochrome *c* reduction in a soluble system, showed the least effect in preventing DNA damage and appeared to slightly promote DNA damage when it was incorporated into the system with catalase. These findings strongly suggest that hydrogen peroxide, or some derivative thereof, is a principal contributor to the DNA damage observed.

Because it has been well recognized that both superoxide anion and hydrogen peroxide can interact in the presence of a metal catalyst to form hydroxy radical via a Haber–Weiss-type reaction (Halliwell and Gutteridge, 1986), we investigated the effect of two known hydroxy radical quenchers

on this system. We chose these two agents primarily because one – namely, mannitol – shows comparatively low reactivity toward hydroxyl radical (Dorfman and Adams 1973), whereas the other (dimethylthiourea) not only shows marked reactivity toward hydroxyl radical but also appears to be relatively nontoxic to cells (Fox, 1984). These two agents were studied at concentrations that, by our spectrophotometric findings, also retarded reoxidation of ferrocytochrome *c* to the oxidized form. In the latter case, dimethylthiourea appeared to be more effective in retarding the reoxidation than did mannitol.

When the effect of 5 mM mannitol was compared with the effect of 10 mM dimethylthiourea in the xanthine–xanthine oxidase system that produced the greatest DNA damage, the mannitol apparently gave the best protection, although its protection level was not significantly greater than that seen for dimethylthiourea ($P > 0.05$, < 0.10). However, its effect was significantly greater than that seen for superoxide dismutase, whereas that of dimethylthiourea was not. Dimethylthiourea's effect was significantly less than that given by catalase ($P > 0.001$, < 0.01), whereas mannitol's effect, although less than that seen with catalase, did not reach a level of significance ($P > 0.1$). These results apparently indicate that mannitol expresses its effect via a mechanism other than reaction with hydroxyl radical. This may be related to mannitol's ability to bind to hydrogen peroxide (Halliwell and Gutteridge, 1986), which could conceivably impair hydrogen peroxide's ability to diffuse into the cell, and produce its effect on arrival.

These data best support a role for hydrogen peroxide, rather than for superoxide anion in producing of DNA strand breakage in CHO cells. The rate of hydrogen peroxide production appears to be an important factor and this is related to the rate of superoxide anion production from the xanthine oxidase–xanthine system used. The complete inhibition by catalase, the partial inhibition by a dithiourea compound that can react with hydrogen peroxide (Halliwell and Gutteridge, 1986), and the partial inhibition of the effect by mannitol, which can bind hydrogen peroxide, are consistent with this conclusion. However, because superoxide dismutase did cause some strand breakage inhibition, the concept that superoxide anion may play a contributory role — perhaps through an effect via a Haber–Weiss-type reaction — is supported. Hydroxyl radical derived either from the intracellular metabolism of hydrogen peroxide and superoxide anion or the intracellular metabolism of hydrogen peroxide via iron-linked mechanisms (Halliwell and Gutteridge, 1986) may be the primary functional agents involved here in DNA strand breakage. These are also generated by high-energy radiation, i.e., X-rays or γ-rays, which are known to induce DNA damage. In view of these results, it follows that acute DNA damage caused by active oxygen species may be the inciting event that can lead to observed chromosome damage and realignment described for a variety of neoplastic tissues. The relationship of the oxyradical production rate to DNA strand breakage and its relationship to observable chromosome damage, is to be the subject of investigation.

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TOPIC HIGHLIGHT

2016 Alcoholic Liver Disease: Global view

Relationships among alcoholic liver disease, antioxidants, and antioxidant enzymes

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Abstract

Excessive consumption of alcoholic beverages is a serious cause of liver disease worldwide. The metabolism of ethanol generates reactive oxygen species, which play a significant role in the deterioration of alcoholic liver disease (ALD). Antioxidant phytochemicals, such as polyphenols, regulate the expression of ALD-associated proteins and peptides, namely, catalase, superoxide dismutase, glutathione, glutathione peroxidase, and glutathione reductase. These plant antioxidants have electrophilic activity and may induce antioxidant enzymes *via* the Kelch-like ECH-associated protein 1-NF-E2-related factor-2 pathway and antioxidant responsive elements. Furthermore, these antioxidants are reported to alleviate cell injury caused by oxidants or inflammatory cytokines. These phenomena are likely induced *via* the regulation of mitogen-activating protein kinase (MAPK) pathways by plant antioxidants, similar to preconditioning in ischemia-reperfusion models. Although the relationship between plant antioxidants and ALD has not been adequately investigated, plant antioxidants may be preventive for ALD because of their electrophilic and regulatory activities in the MAPK pathway.

Key words: Electrophile; Mitogen-activating protein kinase; Plant antioxidants; Reactive oxygen species; Preconditioning

Core tip: The metabolic process of ethanol generates reactive oxygen species, which play a significant role in the deterioration of alcoholic liver disease (ALD). Antioxidant phytochemicals, such as polyphenols, upregulate the expression of antioxidant enzymes and peptides *via* the Kelch-like ECH-associated protein

1-NF-E2-related factor-2 pathway, which leads to antioxidant responsive elements in animal models. Furthermore, these antioxidants alleviate cell injury caused by oxidants or inflammatory cytokines *via* impairment of hyperactivation of mitogen-activating protein kinase pathways, similar to preconditioning in ischemia-reperfusion models. Although the relationship between plant antioxidants and ALD has not been adequately investigated, plant antioxidants may be preventive for ALD.

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INTRODUCTION

Humans are surrounded by many chemicals, including nutrients, phytochemicals, food additives, pharmaceuticals, and drugs. Although the intestine and liver absorb and metabolize many types of chemicals[1] for utilization or detoxification[2], some become more toxic once metabolized[3]. Ethanol, which is a component of alcoholic beverages, is one of the most common and abundant chemicals in daily life. Consuming ethanol can be relaxing and provides other benefits, but excessive drinking can be harmful physically and mentally and may decrease quality of life. Moderate consumption of alcohol has been shown to reduce the risks of cardiovascular disease[4] and non-alcohol fatty liver disease[5]. With moderate intake, most ethanol is oxidized by alcohol dehydrogenase and catabolized to acetaldehyde, which is subsequently catabolized to acetate *via* aldehyde dehydrogenase in the mitochondria. However, with binge drinking, ethanol is predominately metabolized to acetaldehyde *via* cytochrome P450, family 2, subfamily E, polypeptide 1 (CYP2E1), which comprises a microsomal ethanol-oxidizing system[6] that is involved in the generation of reactive oxygen species (ROS)[7-9]. Despite much evidence demonstrating a role for CYP2E1 in alcoholic liver disease (ALD), several of our studies have demonstrated that consumption of ethanol-containing diets significantly increased hepatic CYP2E1 levels without significantly affecting plasma alanine aminotransferase (ALT) activity (unpublished data). These findings support the existence of a potent endogenous antioxidant system that can prevent potential damage *via* the excessive expression of CYP2E1[10].

Binge drinking may cause liver injury, as demonstrated by increased blood levels of ALT, aspartate aminotransferase (AST), and/or lactate dehydrogenase (LDH)[11-14] and lipid accumulation in the liver-alcoholic fatty liver[12,13,15,16]. Hepatic functions are gradually lost with the progression of ALD[11], which is one of the most critical causes of cirrhosis[11,17]. Three mechanisms have been proposed to cause alcoholic liver injury: (1) acetaldehyde toxicity[18]; (2) metabolic generation of ROS or exposure to oxidative stress[10,19-21]; and (3) provocation of an immune response that causes oxidative stress in hepatocytes[13,22-24]. ALD patients appear to exhibit oxidative stress[11]; thus, increasing defense activities against this stress is important in the prevention of ALD.

In mammals, ROS is scavenged by antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, and antioxidant substances, such as vitamins and glutathione (GSH) in collaboration with glutathione peroxidase (GPx) and glutathione reductase (GR)[25]. In previous studies, the induction and/or restoration of these substances and enzymes, which are reduced by ethanol administration, appeared to ameliorate ALD[12,13,23,26]. Some vitamins exhibit antioxidant activity and are reduced in the ALD model[27-29]. They are also deficient in ALD patients, although if present in sufficient quantities, may contribute to the prevention of oxidative stress[30]. Vitamin E is not only a lipophilic antioxidant but also may improve lipid metabolism *via* interaction with lipid accumulation-related proteins, namely patatin-like phospholipase domain containing 3 (PNPLA3) and microsomal triglyceride transfer protein[31]. However, several clinical studies have identified only partial effects of vitamin E in ALD[32,33]. Therefore, the induction of antioxidant enzymes may be more effective than vitamin supplementation in the prevention of ALD.

A trend in gastronomic culture is the exclusion of low molecular weight phytochemicals during plant breeding or processing because of their toxicity, taste, or deteriorating color. However, phytochemicals have recently received attention for their physiological activities in mammals. Many types of phytochemicals abundant in fruit and vegetables are known to have antioxidant activity. Although research efforts have focused on phenolic compounds due to their direct scavenging activity of ROS[34,35], their direct activity towards endogenous ROS appears limited in mammals because of their relatively low concentrations in the bloodstream[2,36,37]. However, many types of polyphenols, non-phenolic phytochemicals, and antioxidant-rich plant fractions have recently been reported to elicit an antioxidant defense system against liver damage induced by ethanol[34,35,38,39], other chemicals[40-43], or abnormal metabolism[21,44] to reduce oxidative stress and cell death[34,42,43,45] and to improve lipid metabolism[12,16,44,46] in various organs. In addition, some phytochemicals change both phase Ⅰ and phase Ⅱ enzymes of drug metabolism, including CYP2E1[7,13,16,47]. Recent reports indicated that some polyphenols can improve epithelial cell junctions[48-51], indicating a role for the hepatic immune response. These findings

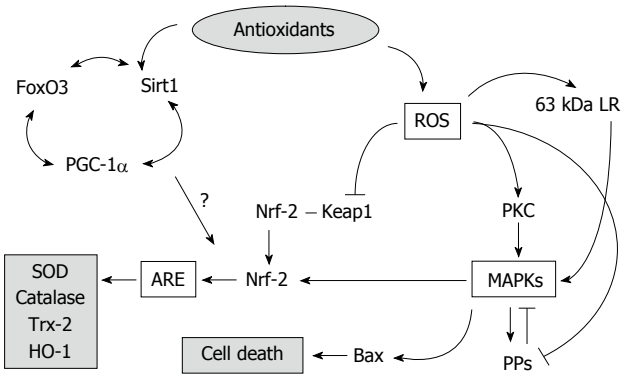

Figure 1 Oxidative stress-stimulating signaling pathways. The oval with the gray indicates the start point; gray boxes indicate consequences; other boxes indicate key substances. ARE: Antioxidant responsive element; FoxO3: Forkhead winged-helix box class O3 transcription factor; HO-1: Heme oxygenase-1; Keap1: Kelch-like ECH-associated protein 1; LR: Laminin receptor; MAPK: Mitogen-activating protein kinase; Nrf2: NF-E2-related factor-2; PGC-1α: Peroxisome proliferator-activated responsive element γ coactivator-1α; PKC: Protein kinase C; PP: Protein phosphatase; ROS: Reactive oxygen species; Sirt1: Sirtuin 1; SOD: Superoxide dismutase; Trx: Thioredoxin.

suggest that phytochemicals could potentially have a comprehensive preventive effect on ALD. However, the physiological activities of phytochemicals in the prevention of ALD have not been well recognized.

In this review, we discuss the physiological activities of phytochemicals and the mechanisms for cell injury, the regulation of antioxidant and pro-oxidant enzyme expression, and concomitant intestinal permeability. Herein, "antioxidants" are defined as the phytochemicals that elicit or enhance the antioxidant defense system, regardless of their radical scavenging activity. Because information regarding the effects of antioxidants in ALD patients or animal models is insufficient for discussion, various oxidative stress models in animals and cells are included. In particular, the mechanisms of non-alcoholic fatty liver disease (NAFLD) may comprise, in part, the mechanisms of ALD because these two diseases likely share many common pathways[31].

MECHANISMS OF LIVER INJURY FROM ALCOHOL CONSUMPTION

As a cause of oxidative stress, ROS are generated by pro-oxidant enzymes, such as CYP2E1 in hepatocytes[7,52,53] and NADPH oxidase (NOX) in Kupffer cells (liver-dwelling macrophages)[25]. In addition, populations of intestinal bacteria that comprise the intestinal environment have been suggested to be involved in ALD *via* stimulation of the immune system. For example, lipopolysaccharides (LPS) derived from intestinal bacteria[15,24,54] activate NOXs and produce inflammatory cytokines[55-58] in macrophages. Acetaldehyde increases the permeability of LPS between intestinal epithelial cells[15,59,60], which is also involved in the deterioration of ALD. Dietary polyunsaturated fatty acids are also thought to enhance oxidative stress[15,29] and are a source of prostaglandins[61]. In a previous study, ethanol administration increased the plasma prostaglandin E_2 level[62], and some prostaglandins are thought to cause inflammation in NAFLD[61,63]. These data suggest that prostaglandins enhance deterioration of ALD; however, the influence of antioxidants on prostaglandins will not be detailed here.

As shown in Figure 1, oxidative stress stimulates intracellular events *via* the mitogen-activating protein kinase (MAPK)[64] pathway, as initiated by the activation of protein kinase C (PKC)[30,65,66] or the degradation of protein phosphatases (PPs)[67]. These signals activate the Kelch-like ECH-associated protein 1 (Keap1)-NF-E2-related factor-2 (Nrf2) pathway, which leads to antioxidant responsive element (ARE)[45,68-70]. However, MAPK hyperactivation also leads to cell death *via* activation of the Bax/Bcl-2 pathway[71,72]. In addition, antioxidant enzymes have been reported to be induced *via* several intracellular pathways, such as the Keap1-Nrf2-ARE pathway[45,69,70,73] and the Sirt1 (sirtuin-1)-FoxO3 (forkhead winged-helix box class O3 transcription factor)-PGC-1a (PPARγ coactivator-1a) pathway[45,68]. The regulation of Sirt1 and Nrf2 levels has also been reported[45], which implies cross-talk between both pathways, whereas the activation of Sirt1 and resveratrol, an activator of Sirt1, have been reported to inhibit the DNA-binding activity of Nrf2 *via* deacetylation *in vitro*[74]. Taken together, substances that deactivate or normalize MAPKs and/or activate ARE or Sirt1[45,75] are potential candidates for the prevention of ALD, but the mechanisms are unknown.

Antioxidant enzymes and peptides

In mammals, SOD generates hydrogen peroxide, which is catabolized to a hydroxyl radical by catalase and detoxified by GSH in collaboration with GPx[25]. The oxidized glutathione form is recruited to GSH by GR with NAD(P)H[76]. Heme oxygenase-1 (HO-1) contributes to the antioxidant system because of the production of bilirubin as a redox substance.

It has been suggested that the hepatic catalase level is negatively associated with the severity of alcoholic liver injury[10] and that SODs scavenge hydroxyl peroxides generated in the cytosol and mitochondria, thereby terminating autoxidation. Thus, catalase and SODs are essential for the antioxidant system. There are three isozymes of SOD in the cytosol, mitochondria, and extracellular matrix: CuZn-SOD, Mn-SOD, and extracellular SOD. SOD levels have been shown to be regulated by MAPK activity[77]. GSH is not an enzyme but a redox tripeptide that acts as a proton donor. GSH levels, GPx content, and/or GR content were reduced in rats fed ethanol diets and, in some cases, ALD animals[16,23,62] or under other oxidative conditions[3,78]. The FoxO transcriptional factor is involved in GPx and Sirt1 protein expression[79]. These findings indicate that in addition to catalase and SOD, GSH is essential for

reducing hepatic oxidative stress.

Under oxidative conditions, HO-1 appears to be rapidly induced *via* the Keap1-Nrf2 pathway[45,69,80,81]. This enzyme may also be involved in the immune response[55]. Furthermore, in ALD model animals, HO-1 levels have been reported to be reduced[13,16,82]. Adiponectin has received recent attention because of its anti-inflammatory functions *via* Sirt1 activation, HO-1 induction, and NOX suppression in Kupffer cells[55]. However, the blood concentration of this adipokine was higher in ALD patients compared with controls[83] or equal to the controls in ALD animals[84], which suggests that adiponectin may be less effective against ALD than antioxidants.

Thioredoxin (Trx) is a ubiquitous scavenger of oxidative species. Endogenous Trx is reported to be reduced by ethanol ingestion; however, the levels can be restored by supplementation with exogenous Trx, which has been demonstrated to ameliorate the symptoms of ALD[84]. Because Trx is a peptide, it must be digested in the digestive system, indicating that it is difficult for exogenous Trx to directly scavenge hepatic ROS.

Pro-oxidant enzymes

In microsomes, CYP2E1 is a phase Ⅰ enzyme of drug metabolism that adds a hydroxyl residue to chemicals to increase hydrophilia and may generate ROS[7-9]. Chronic ingestion of ethanol and other small chemicals increase hepatic CYP2E1. CYP2E1 induction has also been demonstrated in animals with NAFLD[52,85] and hepatic insufficiency. Insulin signaling may suppress CYP2E1 expression[53] *via* the Akt pathway but not the MAPK pathway[86], with subsequent expression of certain microRNAs[87].

Macrophage-like cells, including Kupffer cells, express NOXs and generate ROS with the consumption of NAD(P)H[24] to eliminate xenobiotics[25]. Many isoforms of NOXs have been identified, and NOX-2 is uniquely expressed in phagocytes. NOX expression was regulated *via* the Keap1-Nrf2 pathway in a mouse glial-neural co-cultured system[88] in which NOX-2 predominantly caused oxidative stress. In ALD animals, NOX-2 in Kupffer cells was activated by LPS[55]. In addition, Kupffer cells produce inflammatory cytokines[13,24,55], such as tumor necrosis factor alpha (TNF-α) and interleukin-6. Thus, the reduction of NOXs and inflammatory cytokines are important for ALD.

Given the gut-liver axis in ALD, intestinal conditions play a considerable role in ALD severity, particularly conditions mediated by LPS[15,60]. In the large intestine in humans (or the cecum in animals), an enormous number of intestinal bacteria live and ferment undigested food matter, flaked epithelial cells, and digestive fluid[25]; some of these species generate LPS, which provokes the host's immune system[15]. Small amounts of LPS can pass through gaps in the epithelial cells into the intestine. Ethanol or its metabolites are reported to widen this gap[15,59]. Therefore, improving intercellular junctions or reducing LPS-producing bacteria may have a partial preventive effect on ALD[15].

PLANT ANTIOXIDANTS

Classification of plant antioxidants

Figure 2 shows the structures of representative antioxidants abundant in fruit and vegetables. Polyphenol is a generic name for compounds that have a mono- or polycyclic structure with hydroxyl residues. Flavonoids, including anthocyanins, catechins, and flavonols, form one of the largest groups of polyphenols. Anthocyanins have a red, purple, or blue color in grapes[42], berries[34], seed coats[89], and root crops[37,77]. Catechins include epicatechin, epigallocatechin, and epigallocatechin galate (EGCG) and are sometimes referred to as "tannins"[35]. Proanthocyanidins are polymers of catechins (but not anthocyanin despite the similarity in names); they are categorized as catechins and are widely abundant in crops, particularly tea[27,90], apples[91], and grapes[92]. Quercetin, kaempferol, and isorhamnetin belong to the flavonol group and are ubiquitous in plants. Narirutin and hesperidin belong to the flavanone group and are abundant in the albedo of citrus peel[14,23]. Resveratrol is categorized as a stilbenoid, a phytoalexin, and is present in wine[93] and grapes[42]; it has recently received substantial attention for its physiological functions. Chlorogenic acid is a caffeic acid derivative and one of the most widely consumed polyphenols because of its abundance in coffee and other plants. Alkaloids, such as berberine[46], are also included in the polyphenol group. Curcumin, a curcuminoid present in turmeric, has a yellow color and also belongs to polyphenols.

Lignans, a terpenoid whose metabolites exert estrogenic activity in the lumen, as well as isoflavones and coumestans possess antioxidant activity. Sulfide and thiocyanate compounds are present in garlic[12,82], onions[47], and *Brassicaceae* plants[16] and are reported to be chemopreventive.

PROVOCATION OF THE ANTIOXIDANT SYSTEM BY PLANT ANTIOXIDANTS AND PLANT EXTRACTS

Flavonoids

In animal models, quercetin ameliorated lipid metabolism and ethanol-induced liver damage by inducing antioxidant enzymes, increasing GSH levels, and reducing CYP2E1 activity[20,39]. Quercetin also inhibited the activity and expression of CYP2E1 in human hepatocytes[20,94], which was consistent with *in vivo* findings. In non-alcoholic steatohepatitis animals, quercetin ingestion increased hepatic catalase, SOD, GPx, and GR activities and the GSH level[21] and reduced hepatic lipid accumulation and CYP2E1

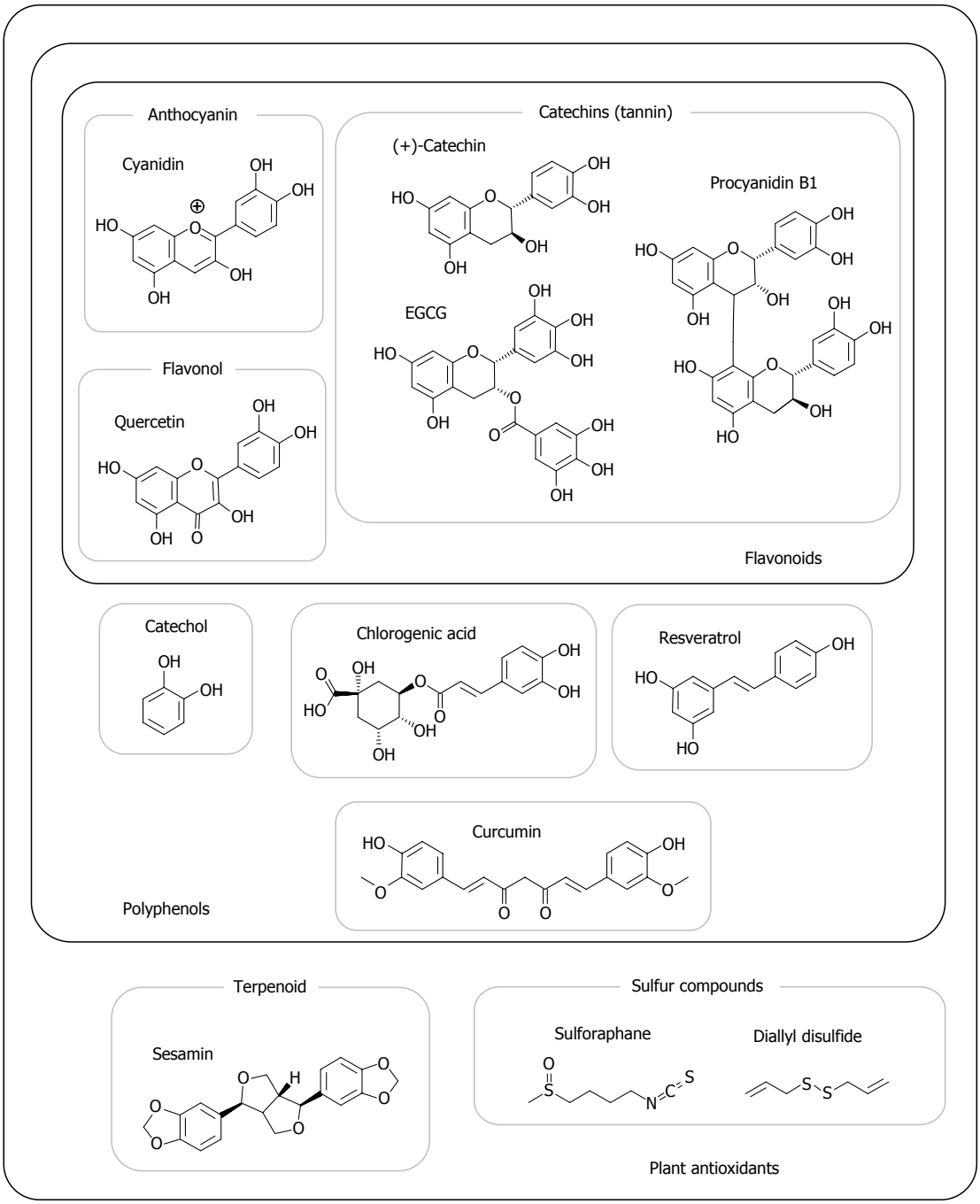

Figure 2 Structures of representative plant antioxidants and their classification.

expression[21,85]. A computer simulation predicted the involvement of quercetin in PGC1a and PNPLA3[31]. Furthermore, hyperoside (quercetin-3-O-galactoside) has been reported to increase cell viability and HO-1 activity *via* MAPKs and ARE[95] in L-02 cells.

Pigments from grapes[42], colored potatoes[77], and black soybean seed coats[89] that contain abundant anthocyanin have been reported to induce antioxidant enzymes *via* the alteration of MAPK activities in cells in other oxidative conditions. An anthocyanin fraction from bilberries appears effective in improving lipid metabolism *via* the AMP-activated protein kinase pathway[96]; however, its involvement in ALD has not been assessed. Alcohol-free red wine increased the blood antioxidant capacity in a human study[97], which suggests a preventive function of the polyphenol fraction in red wine against ALD. However, other studies have demonstrated that alcohol-free red wine worked with ochratoxin A to increase the intercellular permeability in Caco-2/TC7 cells[98], and alcohol-containing red wine increased hepatic and renal CYP2E1 expression in rats, whereas ethanol did not[99]. Malvidin, an anthocyanin in red wine, has been reported to attenuate MAPK activity, which was

promoted by LPS[64], and to enhance PP activity in RAW 264.7 macrophage cells. An anthocyanin-rich extract from colored potato increased Mn-SOD expression *via* extracellular signal regulated kinase (ERK) activation in HepG2 cells[77]. It has also been reported that an ethanol-induced acute gastric lesion was prevented by the ingestion of strawberry extract rich in anthocyanin prior to ethanol treatment *via* the induction of gastric antioxidant enzymes[34].

In animal studies, catechin- and tannin-rich extracts from pecan nut shells improved ALD symptoms by restoring antioxidant enzymes[35,38]. A tea extract rich in catechins reduced CYP2E1 expression and hepatic lesion *via* paracetamol injection[92], and a diet that contained EGCG improved hepatic injury; although there was no reduction in hepatic CYP2E1 levels[100]. In a clinical study, EGCG-rich green tea and its extract also increased the blood GSH level[90]. The ingestion of green tea extract also restored antioxidant activity in the brain that had been decreased by ethanol and aging[28]. Furthermore, catechins have been reported to suppress the expression of NOX and inflammatory cytokines in macrophages[56], dendrocytes[57], and human cerebral microvascular endothelial cells (hCMEC)[101] as well as restore antioxidant enzymes in human neuroblastoma cells[102]. Catechins have both antioxidant and pro-oxidant activities. They have recently been reported to stimulate the 63 kDa laminin receptor[56,57,101,103], which ROS may initiate[104], and consequently to calm over-activation of the immune system *via* the inactivation of the Toll-like receptor (TLR) 2 and 4 pathways. TLR 4, in particular, plays a central role in Kupffer cell stimulation with LPS and the induction of ALD deterioration[57]. Dietary catechins may thus contribute to the impairment of ROS generation *via* LPS and the prevention of ALD.

Citrus flavonoids, narirutin, and glycosylated citrus flavonoids also improved ALD and reduced inflammatory cytokine levels[14,23].

Other phenolic antioxidants and non-phenolic antioxidants

Resveratrol (Figure 2) restores or induces antioxidant enzymes in ALD model rats[93], lung fibroblasts[105], and rats with spontaneous hypertension[75] and diabetes[44,73] *via* the activation of sirtuins in some cases. *In vitro*, resveratrol stimulated HO-1 induction *via* the MAPK-Nrf2 pathway in PC12 cells[81]. Thus, red wine consumption is likely to be superior to other alcoholic beverages in the prevention of ALD. Resveratrol concentrations in wine may be insufficient to prevent ALD; however, it may be responsible for the "French paradox"[106]. Resveratrol has been reported to activate monocytes and produce inflammatory cytokines *in vitro*, which indicates that provoking the immune system with resveratrol may not prevent the deterioration of ALD[107]. Thus, excessive red wine consumption should not be recommended. Polydatin, a resveratrol glycoside, stimulates Sirt1 and Nrf2 and induces antioxidant enzymes in glomerular cells[45].

Chlorogenic acid (Figure 2) and caffeic acid restored the hepatic activity of SOD and GPx and hepatic injuries promoted by methamphetamine injection for 7 d[43].

Honokiol, identified in *Magnolia officinalis*[19], improved ALD, restored the hepatic GSH content and SOD activity, and reduced inflammatory cytokine levels in an ALD animal model[19].

Hispidin, a fungal polyphenol with PKC-inhibitory activity, increased HO-1 and catalase activities in H9c2 cardiomyoblast cells[65].

Berberine is a benzyl isoquinoline alkaloid in the *Coptis* genus that has been reported to reduce ALD symptoms, increase levels of GSH and PGC1a, and normalize CYP2E1 expression in the livers of animals fed an alcohol-containing diet[46].

The sulfur-containing compounds (Figure 2) diallyl disulfide and garlic oil have been reported to improve alcoholic hepatic injury[12] by increasing HO-1 levels *via* the Nrf2 pathway and increasing the GSH level *in vivo*[82] and *in vitro*[94]. A similar preventive effect has also been identified in diallyl sulfide treatment in astrocytes[30]. Sulforaphane has been reported to act as an inducer of HO-1[16], which suggests that these compounds may be useful in the treatment of ALD. In addition to restoring HO-1 levels, sulforaphane improved hepatic lipid accumulation in ALD animals[16]. The consumption of onion powder, which is rich in sulfide compounds and flavonols, has also been reported to reduce hepatic CYP2E1 levels in normal rats[47].

Oleanolic acid, a triterpenoid, restored antioxidant enzymes and increased nucleic Nrf2 levels and improved ALD[13]. Sesamin (Figure 2) is a well-characterized terpenoid in sesame seeds that may contribute to the reduction of fatty liver by promoting β-oxidation of fatty acids and inducing hepatic aldehyde dehydrogenase[108,109]. Maslinic acid, a triterpenoid rich in basil, brown mustard, and other plants, has been reported to protect hepatic injury *via* acute ethanol toxicity[62]. These data suggest that some types of terpenoids may improve the symptoms of ALD.

Curcumin (Figure 2), but not resveratrol, has been reported to restore hepatic antioxidant enzymes reduced by aflatoxin in rats[110]. Curcumin also increased antioxidant enzymes as well as Nrf2 and HO-1 levels in quails under heat stress[111].

Mangiferin, identified in mango[112], is a xanthine derivative that has been reported to restore pulmonic and hepatic antioxidant enzyme levels reduced by benzo(a)pyrene in mice[3].

Plant extracts that contain significant amounts of antioxidants also prevent oxidative damage in various other organs. An extract from black tea[27] improved ALD symptoms in rats. The extracts from apples[91], *Amorphophallus commutatus*[40], cinnamon[113], and hibiscus[22,41] partially normalized hepatic oxidative

stress induced by chemical toxins.

Improvement of fatty acid accumulation

Alcoholic fatty liver is a predictive symptom of ALD, and hepatic inflammation is also present in non-alcohol steatohepatitic animals[21,41,52]. Moreover, a computer simulation predicted many common pathways between alcoholic fatty liver and NAFLD that were associated with inflammation, lipid metabolism, and some immunity[31]. These data suggest that a reduction in lipids in the liver may lead to an improvement in liver injuries[16,19,100]. In addition to the induction of antioxidant enzymes, some plant antioxidants have recently been reported to improve lipid metabolism and reduce hepatic lipid accumulation[19,39,46], which may also contribute to the amelioration of ALD.

Improvement of intestinal permeability by plant antioxidants and plant extracts

Antioxidants, such as quercetin, resveratrol, EGCG, and naringenin, prevent the downregulation of junction proteins, namely, Zo-1 and/or Occludins, and consequently enhance intercellular barrier functions *in vitro*[49] and *in vivo*[50]. In contrast, EGCG has been reported to disturb the barrier function of hepatic epithelial cells[114] because of ROS-induced ERK activation. In addition to intestinal cell models, cocoa polyphenol extract improved barrier functions disturbed by a high glucose condition in retinal pigment epithelium cells[51]. Cocoa polyphenol extract and resveratrol also attenuated the permeability of renal cell junctions *in vitro*[48,115], and EGCG increased the adhesion of hCMEC[101]. The tightness of cellular junctions regulated by antioxidants may be involved in the severity of ALD and should be elucidated.

Mechanisms for ALD prevention via plant antioxidants

Cellular oxidative stress is caused by many factors, such as exposure to humoral factors[22,75], enzymatic generation of ROS[7-9,24], metabolites of chemicals[41,91,102,116], or the mitochondrial respiratory chain[39]. Two major mechanisms may be proposed for hepatic injury prevention *via* oxidation: (1) the impairment of oxidative signaling that leads to cell death; and (2) the activation of the Keap1-Nrf2 pathway, which results in the induction of antioxidant enzymes.

As a leading mechanism, "preconditioning" in ischemia-reperfusion models has been proposed to alleviate tissue damage. In ischemia-reperfusion models, excessive ROS are present following reperfusion, whereas slight ischemic-reperfusion pretreatment to tissues or cells alters MAPK activities and interferes with cellular damage[117-119]. It has been reported that ROS stimulate PKC, MAPKs, and subsequent events that lead to cell death[89] or induce an antioxidant system (Figure 1). MAPKs appear to activate both PPs[66,120] and Nrf2[69]. Once activated, PPs may deactivate not only MAPKs but also other phosphorylated proteins related to the MAPK signaling pathways[66], which may lead to a comprehensive impairment of MAPK signaling. Despite their antioxidant activity, polyphenols also have a slight pro-oxidant activity[72,121]. This impact may increase MAPK and PP activity[103] or PP stability[120] prior to crucial oxidative stress by ROS. At minimum, PPs activated by antioxidants may partially inhibit MAPK pathway activation. Following pretreatment with plant antioxidants, the hyperactivation of MAPKs by injuring stimuli appears to decrease[22,41,48,64]. These findings may support the preconditioning hypothesis[1]. Taken together, ROS and/or MAPK are key regulators of both cell injury and antioxidant enzyme induction.

In addition, this mechanism can explain the effects of antioxidants on the barrier functions of epithelial cells. Junction proteins and the intercellular barrier function are disturbed by oxidative stress[48,114]. Antioxidants have been reported to exhibit minimal activity to generate ROS[114,121] and subsequently activate MAPKs, which disturbs barrier function *in vitro*[114]. However, antioxidant pretreatment may diminish excessive oxidative stress, as previously discussed, which leads to the protection of barrier function[49,50].

It has been suggested that ROS (and electrophilic reagents) directly activate the Keap1-Nrf2 pathway. Keap1 is a sensor of intracellular oxidative stress and couples with Nrf2[122]. Once Keap1 is oxidized, Nrf2 is released, moves to the nuclei, and activates ARE. Regarding the relationship between chemical structures and antioxidant activities, it has been suggested that electrophilic compounds, such as flavonoids, curcumin, and thiocyanate-related compounds, stimulate the Keap1-Nrf2 pathway[122]. Satoh *et al*[123] proposed the importance of ortho- or para-positions of hydroxyl residues in the benzene structure, which result in hydroquinone and catechol, respectively (Figure 2), because of their electrophilic residue. Some flavonoid compounds have a catechol structure (Figure 2), which indicates an interaction between flavonoids and Keap1. These results may support the hypothesis proposed by Satoh *et al*[123].

This hypothesis suggests that antioxidants directly activate Keap1. However, some antioxidants appear to induce antioxidant enzymes *via* MAPK activation despite the upper proteins of Keap1 (Figure 1), as demonstrated with specific inhibitors of MAPKs that diminished the induction[77] or activation of Nrf2[81]. Antioxidants may contribute to the induction of antioxidant enzymes *via* MAPK pathways rather than through direct activation of Keap1. Moreover, resveratrol has a resorcinol structure rather than a catechol structure. Resorcinol has less electrophilic activity than catechol[123]; however, it appears to stimulate Nrf2[122]. This mechanism must also be elucidated.

In *in vivo* studies, the ingestion of antioxidants induces (or tends to induce) antioxidant enzymes in the lung[3], thymus[124], brain[28,125], and kidney[45], despite very low concentrations in the bloodstream[2,36,37].

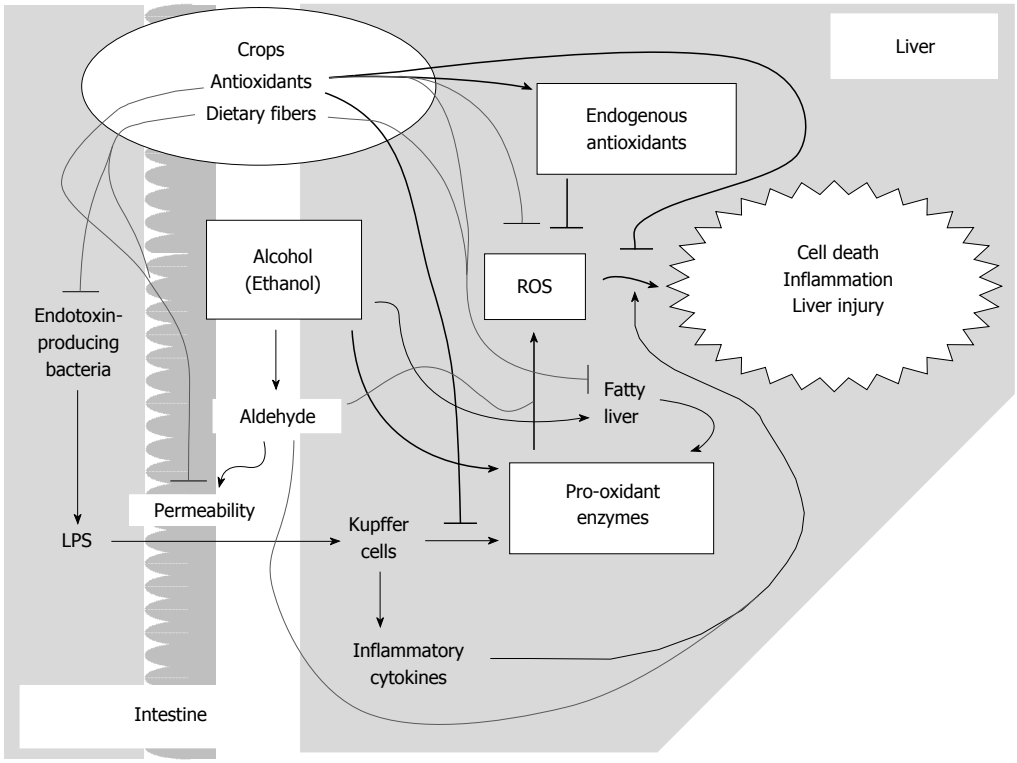

Figure 3 Potential multiple effects of crop components on alcoholic liver disease. LPS: Lipopolysaccharide; ROS: Reactive oxygen species.

These reports imply that there is an intermediate signal by polyphenols, such as nerve and/or humoral pathways, rather than direct stimulation of cells or organs; they may also be explained by remote ischemic preconditioning[117]. This preconditioning suggests that some types of stimuli can regulate MAPK activities in remote organs.

PERSPECTIVE

Even ubiquitous plant antioxidants, such as anthocyanins and flavonols, appear to have many physiological activities, indicating that botanical substances can provoke the antioxidant system. Apart from oxidative stress *via* lipid accumulation, lipids also appear to be a central cause of ALD. For example, prostaglandins, which are initiated by phospholipase (PL) A_2 and activated by cyclooxygenases[61], are involved in inflammatory events, and PNPLA3 has been suggested to have PLA_2 activity and to regulate hepatic lipid accumulation[63]. Therefore, the regulation of prostaglandins and/or expression of their related proteins may be critical for the improvement of ALD.

Fruits and vegetables are great sources of antioxidants as well as dietary fibers (DFs)[126], which were once considered to be unwanted materials or non-nutrients. It is now well established that the ingestion of DFs improves lipid metabolism and reduces hepatic lipids[127,128]. Some types of DFs, particularly water-soluble fibers, promote the excretion of lipids into feces and the synthesis of short-chain fatty acids (SCFA) in the intestine[126,129], which are proposed as prebiotics. Oral ingestion of butyrate, a type of SCFA produced from DF, promotes junction protein expression and an increase in intestinal barrier function[130]. These findings also suggest the potential of DFs in the prevention of ALD. Thus, intact fruits and vegetables, including both antioxidants and DF, are worthy of consideration for ALD prevention.

Mammals often intrinsically treat plant chemicals as xenobiotics and have developed metabolic systems against phytochemicals[1]. The human body evolved with environmental factors, including phytochemicals and DFs. The data reviewed here imply the necessity for the unwanted materials to elicit an accomplished defense system, a barrier function in the intestine and a chemical metabolizing system in the intestine, and liver against xenobiotic substances.

However, most of these data are derived from animal and cell studies. In these studies, antioxidants may, in some cases, be overdosed[75], which makes it difficult to justify their effectiveness in humans, particularly ALD patients who may have impaired liver functions[11]. As previously reported, vitamin E supplementation only partially improved ALD[32,33] despite its effectiveness in cell studies. Thus, it is important for future studies to accumulate clinical data regarding the relationships among ALD, antioxidants, and antioxidant enzymes.

In conclusion, plants have a potential role in the prevention of ALD (Figure 3). Although most individuals are aware that abstinence from alcohol is the most

effective way to prevent ALD, it is recognized that this is not easy. Therefore, it is important to improve our defense system against ALD. Many types of plant antioxidants with electrophilic activity may activate antioxidant enzymes or peptides under oxidative conditions and alleviate ALD, which may occur *via* a mechanism that is somewhat similar to preconditioning in ischemia-reperfusion models[117-119]. The antioxidants reviewed here are common in vegetables and fruits, which can be easily consumed. Moreover, plants contain abundant amounts of DF and vitamins. Vitamins are wasted by binge drinking[27,28], and DFs can improve lipid metabolism and intestinal conditions[127,128] in mammals. Therefore, non-processed food materials may have considerable intrinsic potential. Clearly, ALD patients should be administered appropriate medications to facilitate recovery from crucial damage. However, fresh vegetables and fruits may be more effective than processed foods in comprehensively preventing hepatic damage induced by alcohol. Antioxidants commonly taste bitter, and DFs appear to exhibit a bad texture; thus, they have been eliminated from foods over centuries. However, humans have evolved alongside phytochemicals and DFs to overcome these issues. Thus, an approach that elicits the intrinsic potential of the human body to prevent ALD and other lifestyle-related disorders should be reconsidered.

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Chapter 22
Transgenic Mouse Models for Alcohol Metabolism, Toxicity, and Cancer

Claire Heit, Hongbin Dong, Ying Chen, Yatrik M. Shah, David C. Thompson, and Vasilis Vasiliou

Abstract Alcohol abuse leads to tissue damage including a variety of cancers; however, the molecular mechanisms by which this damage occurs remain to be fully understood. The primary enzymes involved in ethanol metabolism include alcohol dehydrogenase (ADH), cytochrome P450 isoform 2E1, (CYP2E1), catalase (CAT), and aldehyde dehydrogenases (ALDH). Genetic polymorphisms in human genes encoding these enzymes are associated with increased risks of alcohol-related tissue damage, as well as differences in alcohol consumption and dependence. Oxidative stress resulting from ethanol oxidation is one established pathogenic event in alcohol-induced toxicity. Ethanol metabolism generates free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), and has been associated with diminished glutathione (GSH) levels as well as changes in other antioxidant mechanisms. In addition, the formation of protein and DNA adducts associated with the accumulation of ethanol-derived aldehydes can adversely affect critical biological functions and thereby promote cellular and tissue pathology. Animal models have proven to be valuable tools for investigating mechanisms underlying pathogenesis caused by alcohol. In this review, we provide a brief discussion on several animal models with genetic defects in alcohol-metabolizing enzymes and GSH-synthesizing enzymes and their relevance to alcohol research.

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22.1 Introduction

The Centers for Disease Control and Prevention reported that the annual number of alcohol-related deaths was 88,000 in the United States from 2006 to 2010. Alcohol is a causal factor in more than 60 human diseases and places a significant burden on the economy with healthcare costs estimated in 2006 as surpassing 223 billion dollars in the United States alone [1]. A comprehensive understanding of the mechanisms mediating alcohol toxicity is essential because it facilitates the development of therapies that prevent and/or treat the pathologies associated with alcohol consumption. The cellular and molecular mechanisms leading to alcohol-induced tissue damage are not fully understood. However, emerging evidence indicates that common mechanisms of cell injury, such as stress, inflammation, and alterations in signaling (including apoptosis) pathways, are all involved in the deleterious effects of alcohol.

Animals in which expression of specific proteins are repressed, the so-called knockout animals, represent an innovative and powerful research tool for scientific discovery. Genetic manipulation of proteins involved in the metabolism of ethanol or in the cellular defense mechanisms against alcohol-induced oxidative stress have allowed the exploration of their roles in alcohol-related pathologies, such as alcoholic liver disease, pancreatitis, cardiovascular disease, and diabetes mellitus, as well as in various cancers, including oral, colorectal, liver, pancreatic, aerodigestive, breast, and colon [2–7]. Currently available animal models will be outlined in the following review. In addition, double and triple knockout strains of these mice are currently being produced in our laboratory.

22.2 Clinical Significance of Human Polymorphisms of Genes Involved in Ethanol Metabolism

Ethanol is metabolized primarily *via* oxidation to acetaldehyde through the enzymatic activity of alcohol dehydrogenases (ADH), catalase (CAT), and cytochrome p450 2E1 (CYP2E1) (Fig. 22.1). Acetaldehyde is then oxidized to acetate by the aldehyde dehydrogenases (ALDHs). The role of ADH in ethanol metabolism is well established [8–10]. The human genome contains three Class I *ADH* genes (*ADH1A, ADH1B, ADH1C*); in contrast, rodents have only one *Adh1* gene [11]. Genetic polymorphisms in *ADH1* genes are associated with colon and breast cancers [4, 5, 12]. The role of CYP2E1 in ethanol metabolism, oxidative injury, and cancer is also well established [13–17] and genetic polymorphisms are associated with increased cancer risk [18–20]. Catalase appears to play an important role in ethanol metabolism in the brain [14, 15]. Nevertheless, polymorphisms in the catalase gene are associated with diabetes mellitus, hypertension, and vitiligo [21, 22]. ALDHs are a family of 19 human proteins that metabolize aldehydes, in which three isoforms are responsible for metabolizing acetaldehyde. Mitochondrial ALDH2 is the primary enzyme involved in the metabolism of acetaldehyde ($Km \leq 5$ μM). The ALDH2*2 allele

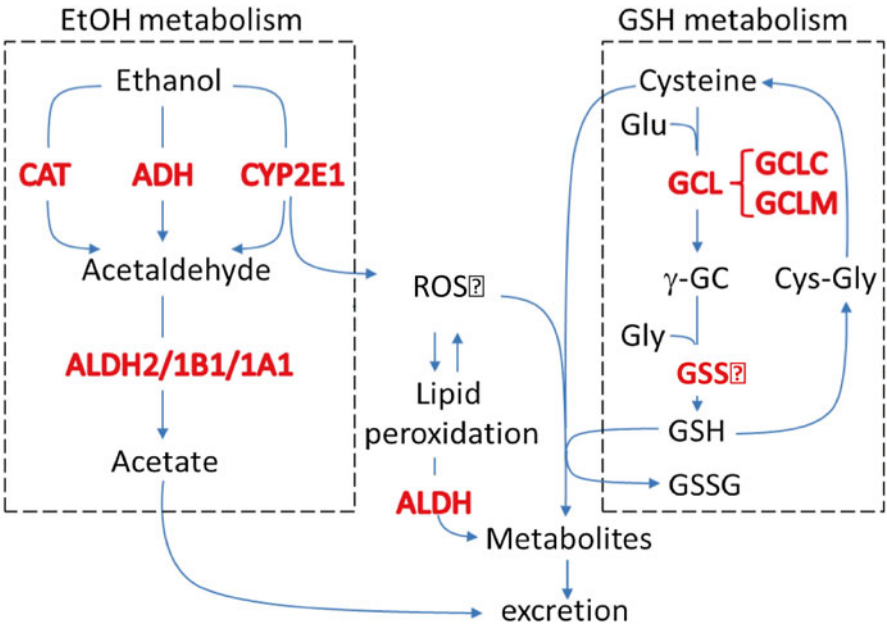

Fig. 22.1 Major enzymatic pathways involved in ethanol and glutathione metabolism. Ethanol (EtOH) is subject to metabolism by catalase (CAT), cytochrome P450 isoform 2E1 (CYP2E1), and alcohol dehydrogenase (ADH). Acetaldehyde is metabolized by aldehyde dehydrogenase (ALDH) isoforms 1A1, 1B1, and 2. In the glutathione (GSH) pathway, glutamate cysteine ligase (GCL), which includes two subunits the catalytic subunit (GCLC) and the modifier subunit (GCLM), catalyzes the synthesis of γ-glutamylcysteine (γ-GC). γ-GC is then coupled to glycine by glutathione synthetase (GSS) to form GSH. During oxidative processes, reactive oxygen species (ROS) form which can cause lipid peroxidation. ROS can be reduced by GSH, in the process forming GSSG, the oxidized form

(which appears restricted to an Asian genetic background) causes marked reductions in acetaldehyde metabolism that manifest clinically as flushing syndrome and ethanol avoidance in heterozygous and homozygous individuals. This polymorphism is also associated with alcohol-related cancers [3, 23]. ALDH1B1 has the next lowest Km for acetaldehyde (Km = 55 μM), implicating a role in acetaldehyde metabolism secondary to ALDH2. ALDH1B1 has been proposed as a biomarker for colon cancer [24]. Several ALDH1B1 polymorphisms have been found in humans and recent studies have linked these polymorphisms to drinking aversion, elevated systolic blood pressure, and frequent hypersensitivity reactions in Caucasians [25, 26]. ALDH1A1 has a role in acetaldehyde metabolism and drinking preference [27, 28] and a deficiency in this enzyme is associated with ethanol hypersensitivity in Caucasian subjects [29]. Polymorphisms in alcohol- and acetaldehyde-metabolizing enzymes have been closely linked with alcohol-related cancers. In a Japanese population, p53 accumulation, esophageal neoplasia, and esophageal squamous cell carcinomas were increased in subjects whose genes included the inactive heterozygous allele ALDH2 *1/*2 and the less active ADH1B *1/*1 [30, 31]. These polymorphisms also exhibited more frequent acetaldehyde-induced DNA damage [32].

Taken together, the association between human polymorphisms of ethanol-metabolizing genes and alcohol-related diseases implicates a significant pathogenic role of ethanol metabolism in alcohol toxicity. Various research groups have developed animal models that harbor genetic ablations of ethanol-metabolizing enzymes. These models can serve as important experimental tools to elucidate the mechanistic roles of specific enzymes or pathways in alcohol-related diseases and therefore have direct relevance to alcohol research.

22.3 Animal Models for Alcohol-Induced Cancer

Low to moderate consumption of alcohol has tissue-protective properties [33]. However, heavy alcohol consumption increases the risk of several diseases, including cancer. A comprehensive review of epidemiological data demonstrated a significant increase in cancer risk for several epithelial-derived tumors associated with ethanol consumption [3]. Studies using experimental animals, however, support the notion that ethanol acts as a cocarcinogen or tumor promoter rather than being a carcinogen itself [5]. The mechanisms by which alcohol promotes tumorigenesis remain unclear due primarily to a lack of good animal models that can recapitulate the increased risk of alcohol in carcinogenesis. Animal models analogous to inflammation-promoted cancers, such as the azoxymethane (AOM)/dextran sulfate sodium (DSS) model, are needed [34]. In this model, the carcinogen AOM by itself is administered at a dose that causes no dysplasia; however, when administered in combination with DSS (which induces inflammation), a synergistic increase in the number of tumors is observed. The creation of a similar model for alcohol will rely on a better understanding of the interactions between the genetic mutations (or carcinogen) with the duration, route, and concentration of ethanol for the epithelial tumor that is to be modeled. Moreover, diets that better mimic heavy alcohol consumption in humans are required. The interaction of tumor-promoting dietary components, such as alcohol, high-fat, and iron, may lead to more robust and precise models. Lastly, experimental evidence indicates that the metabolism of ethanol leading to the generation of acetaldehyde and free radicals is intimately involved in alcohol-associated carcinogenesis [3]. Therefore, a more comprehensive understanding of the enzymes required for alcohol metabolism in cancer are needed and the genetic animal models discussed in this review could represent unique opportunities to identify their roles in alcohol-induced cancers.

22.4 Glutathione in Alcoholic Tissue Injury

In the development of alcohol-induced tissue injury, it is apparent that numerous pathways in target organs are modulated by ethanol [35, 36]. Oxidative stress appears to play a central role in many of these pathways [37]. Ethanol metabolism, CYP2E1

induction, compromised antioxidant defense, mitochondrial injury, inflammation, hypoxia, and iron overload can all contribute to the alcohol-induced oxidative environment. Accumulation of the reactive molecules (including reactive oxygen species and electrophilic products, such as acetaldehyde and lipid peroxidation products) can be harmful to a biological system due to their propensity to inactivate enzymes and cause DNA damage, loss of protein functions and cell death [38].

Glutathione (GSH) plays an important role as an antioxidant by serving as a cofactor for antioxidant enzymes, such as glutathione peroxidase and glutathione *S*-transferases, or by directly scavenging free radicals [39]. It is the most abundant nonprotein thiol, attaining a concentration in the high millimolar range in the liver [40]. Because of its abundance, GSH plays a key role in maintaining cellular redox homeostasis and, therefore, enzymes that help generate GSH are critical in protecting cells against oxidative stress. GSH is a tripeptide composed of glutamate, cysteine, and glycine. It is synthesized in most types of cells by two successive enzymatic reactions. The first reaction couples glutamate and cysteine and is catalyzed by glutamate-cysteine ligase (GCL), resulting in the formation of γ-glutamylcysteine (γ-GC) [41] (Fig. 22.1). The second reaction, catalyzed by GSH synthetase, couples γ-GC with glycine. The formation of γ-GC by GCL is considered rate-limiting in GSH biosynthesis, and GCL has been the principal target of drugs designed to inhibit GSH biosynthesis [41] and to generate mice with GSH deficiency [42].

In higher eukaryotes, GCL in its most catalytically efficient form is a heterodimer composed of a catalytic (GCLC) and a modifier (GCLM) subunit, each of which is encoded by separate genes on different chromosomes. GCLC possesses all of the catalytic activity of γ-GC formation; GCLM optimizes the kinetic properties of the holoenzyme, thereby regulating tissue GSH levels [43]. GSH is exclusively synthesized in the cytoplasm [39] and further distributed into mitochondria, endoplasmic reticulum, and nuclei, where it plays a pivotal role in the normal functioning of these subcellular organelles [44]. During detoxication of free radicals, GSH is oxidized to glutathione disulfide (GSSG). Both GSH and GSSG can be transported outside the cell where it is broken down in sequence by γ-glutamyl transferases and dipeptidase, producing free cysteine and glycine for intracellular reutilization [45]. Depletion of hepatic GSH, particularly mitochondrial GSH, occurs as a result of excessive GSH consumption by free radicals and acetaldehyde generated during alcohol metabolism [46]. Given the above considerations, animal models exhibiting a GSH deficiency will serve as important tools to study GSH-regulated redox biology in ethanol metabolism and ethanol-induced tissue damage. As such, they are of direct relevance to alcohol research.

22.5 Mouse Models with Genetic Deficiencies in Ethanol-Metabolizing Enzymes

ADH1 global knockout: The *Adh1*$^{-/-}$ mouse line has been generated by Duester [47] (Table 22.1). It should be noted that human *ADH1* gene family consists of three genes, *viz. ADH1A*, *ADH1B*, and *ADH1C*, whereas the mouse genome has a single

Table 22.1 Transgenic mouse models

Strain	Genetic background	Phenotype	References
Adh1$^{-/-}$	C57BL6	• No gross abnormality • Reduction in blood ethanol clearance	Deltour et al. [9]
Cat$^{-/-}$	C57BL6	• No gross abnormality • Deficiency in brain mitochondrial respiration • Has not been used in ethanol toxicity studies	Ho et al. [49]
Cyp2e1$^{-/-}$	129/Sv	• No gross abnormality • Decreased sensitivity to acetaminophen hepatotoxicity • Resistance to ethanol-induced fatty liver and oxidant stress	Lee et al. [50], Lu et al. [54]
Cat$^{-/-}$ *Cyp2e1*$^{-/-}$ *double knockout*	C57BL6/129 mixed	• No gross abnormality • Has not been used in ethanol toxicity studies	Unpublished
Aldh2$^{-/-}$	C57BL6	• No gross abnormality • High susceptibility to ethanol toxicities by oral administration • High sensitivity to inhalation toxicities of acetaldehyde	Isse et al. [55], Oyama et al. [59, 61]
Aldh1a1$^{-/-}$	C57BL6	• Viable and fertile • Decreased susceptibility to diet-induced obesity and insulin resistance • Cataract development at age of 6-month • Has not been used in ethanol toxicity studies	Fan et al. [74], Ziouzenkova et al. (2007)
Aldh1b1$^{-/-}$	C57BL6	• No gross abnormality • Reduction in blood acetaldehyde clearance	Unpublished

A variety of transgenic strains are available for research. For each strain, the genetic background and phenotypes are provided

Adh1 gene [11]. *Adh1*$^{-/-}$ *mice* have limited capacity to oxidize ethanol and retinol. Pharmacokinetic studies show a reduction in blood ethanol clearance in these animals [9]. Following parenteral administration of ethanol, these mice displayed an increased sleep time and embryonic resorption was increased threefold [9]. While ADH1 is thought to be responsible for the majority of ethanol metabolism in the liver, new pharmacokinetic evidence suggests a role for other ADH isoforms as well [48]. Therefore, this model may be useful in determining the pathophysiological importance of compensatory ADH isoforms as well as elucidating the kinetics of these enzymes for ethanol.

Catalase global knockout: The *Cat*$^{-/-}$ mouse strain was developed and characterized by Ho and colleagues [49] (Table 22.1). These mice do not express catalase and

develop normally, i.e., exhibit no gross abnormalities. However, brain mitochondria of these animals show deficiencies in mitochondrial respiration. To date, this knockout strain has not been subjected to ethanol toxicity studies. Given that earlier studies have shown a significant role of catalase in modulating ethanol sensitivity in the brain [14, 15], the *Cat*$^{-/-}$ mice would be anticipated to be a valuable animal model for examining ethanol drinking preference as well as alcohol toxicities.

CYP2E1 global knockout: CYP2E1 is an ethanol-inducible enzyme with a role in hepatic ethanol oxidation. By genetically ablating exon 2 of *Cyp2e1* gene, Gonzalez and colleagues developed *Cyp2e1*$^{-/-}$ mice [50] (Table 22.1). These mice do not express CYP2E1 enzyme and develop normally [50]. They also show lower sensitivity to the deleterious hepatic effects of acetaminophen [50]. As one of the primary xenobiotic/endobiotic-metabolizing p450s, CYP2E1 is a contributor to a variety of cellular toxicities induced by endogenous or exogenous pathogens. Using the *Cyp2e1*$^{-/-}$ mouse model, CYP2E1 has been shown to play a pivotal role in mediating hepatotoxicity making this an interesting model for alcohol-related liver toxicity [51, 52] *Cyp2e1*$^{-/-}$ and *Cyp2e1* knock-in mice have been used to examine the potentiation of ethanol-induced hypoxia. *Cyp2e1*$^{-/-}$ mice exhibited the lowest levels of hypoxia and HIF1-α [53]. Similarly, ethanol-induced fatty liver and oxidant stress are blunted in these mice [54]; this study confirmed the important role of CYP2E1 in ethanol-induced liver toxicities. *Cyp2e1*$^{-/-}$ mice also display longer ethanol-induced sleep time than do wild-type mice [15], confirming the relevance of the *Cyp2e1*$^{-/-}$ mouse line for the study of the CYP2E1 enzyme in ethanol toxicities and alcohol-induced drinking preference.

22.6 Mouse Models with Genetic Deficiencies in Acetaldehyde-Metabolizing Enzymes

ALDH2 global knockout: The *Aldh2*$^{-/-}$ strain was originally developed and characterized by Isse and colleagues [55, 56] (Table 22.1). *Aldh2*$^{-/-}$ mice do not express ALDH2 protein and have no detectable capacity to oxidize acetaldehyde, propionaldehyde, or methoxyacetaldehyde in liver mitochondrial fractions. Following oral administration of ethanol, *Aldh2*$^{-/-}$ mice exhibit higher ethanol and acetaldehyde levels and lower acetate levels in the blood, brain, and liver than *Aldh2*$^{+/+}$ mice [57, 58]. Further, they are more susceptible to ethanol-induced body weight loss [59], but show no change in mortality [60]. *Aldh2*$^{-/-}$ mice are more sensitive to the toxic effects of inhaled acetaldehyde [61] and exhibit more frequent mutations in the T cell receptor site than their corresponding wild-type [62]. A single oral dose of ethanol in *Aldh2*$^{-/-}$ downregulates the alcohol-metabolizing CYP2E1 mRNA [63], which suggests that there is compensation due to an abundance of acetaldehyde. This treatment has also been shown to decrease hepatic malondialdehyde and increase hepatic glutathione, both markers of oxidative stress, in *Aldh2*$^{-/-}$ mice [64]. Acetaldehyde adducts are also increased in *Aldh2*$^{-/-}$ mice. These mice have been

used to determine ethanol- and acetaldehyde-induced cholinergic changes in the hippocampus. The null mice exhibit decreases in choline acetyltransferase mRNA and protein; however, neurotrophins (nerve growth factor or brain-derived neurotrophic factor) remain unaffected [65], indicating that aldehydes have a selective effect in the brain. *Aldh2*$^{-/-}$ mice also exhibit alcohol avoidance in a test of preference and difference in liver or brain acetaldehyde levels [55]. ALDH2 also appears to influence bone growth and cardiac function, as demonstrated by reductions in trabecular bone formation and cardiomyocyte function in *Aldh2*$^{-/-}$ mice treated with alcohol [66, 67]. Stomach DNA adducts are dramatically increased after chronic ethanol feeding of *Aldh2*$^{-/-}$ mice [68, 69] and acute ethanol treatment increases hepatic oxidative DNA adducts in null mice [70, 71]. The *Aldh2*$^{-/-}$ strain represents a valuable strain that can be used to identify functions of ALDH2 in ethanol metabolism and toxicity.

ALDH2 conditional knockout: A "knockout-first" conditional allele for *Aldh2* has been developed by Skarnes and colleagues [72]. These mice have been crossed with *FLP* mice to generate *Aldh2* floxed conditional knockout (*Aldh2*$^{f/f}$) mice, which can be further crossed with specific *CRE* mouse lines to generate cell-specific *Aldh2* knockout mice. As expected, *Aldh2*$^{f/f}$ mice develop normally and exhibit no observed phenotype (unpublished observation). To date, no ethanol studies have been conducted in these mice. This strain can be used to study tissue-specific contributions of ALDH2 in ethanol metabolism and toxicity.

ALDH1B1 global knockout: The *Aldh1b1*$^{-/-}$ strain was recently generated by Vasiliou and coworkers (*Singh S, Vasiliou V et al.*, manuscript in preparation) (Table 22.1). The *Aldh1b1*$^{-/-}$ mice develop normally and show no overt phenotype. In agreement with the catalytic properties of ALDH1B1 (i.e., the second lowest Km for acetaldehyde oxidation [73]), these mice exhibit higher blood concentrations of acetaldehyde following acute ethanol administration [manuscript in preparation]. The *Aldh1b1*$^{-/-}$ strain represents the first animal model for the study of ALDH1B1 enzyme in ethanol-induced tissue injury.

ALDH1A1 global knockout: The *Aldh1a1*$^{-/-}$ mouse line has been generated by Fan et al. [74] (Table 22.1). These mice are fertile and exhibit no overt phenotype, except that aged *Aldh1a1*$^{-/-}$ mice display ~2.4-fold higher cataract incidence than wild-type mice [75]. While ALDH1A1 primarily metabolizes retinaldehyde, it also plays a role in acetaldehyde metabolism. Genetic variants of *ALDH1A1* (that result in low enzyme activity) have been associated with increased alcohol sensitivity in Caucasians [29]. Therefore, the *Aldh1a1*$^{-/-}$ mouse line represents a useful animal model for investigation of the ALDH1A1 enzyme in ethanol toxicities.

22.7 Mouse Models with GSH Deficiency

GCLC conditional (Gclc $^{f/f}$*) knockout*: The global gene knockout of *Gclc* results in embryonic lethality, indicating an essential role of GSH in mouse development [76]. The *Gclc*$^{f/f}$ strain was developed and originally characterized by Chen and

colleagues [77]. The in vivo role of hepatic GSH has been investigated using the hepatocyte-specific *Gclc* knockout ($Gclc^{h/h}$) mice created by intercrossing $Gclc^{f/f}$ and *Alb-Cre* mice [77]. $Gclc^{h/h}$ mice experience almost complete loss of hepatic GSH (~5 % of normal) and die from acute liver failure when mitochondrial failure occurs [77]. Chronic administration of *N*-acetylcysteine, a treatment that promotes only a mild increase in liver GSH levels (to 8 % of normal) but partially preserves mitochondrial function, allows $Gclc^{h/h}$ mice to survive to adulthood, albeit with the serious liver pathologies fibrosis and cirrhosis [78]. These studies demonstrate an essential role of GSH in normal functioning of the liver. The $Gclc^{f/f}$ mice represent a unique model that can be used to elucidate cell-specific functions of GSH in ethanol metabolism and toxicity.

GCLM global knockout: The $Gclm^{-/-}$ strain was developed and originally characterized by Yang and coworkers [79]. $Gclm^{-/-}$ mice are viable and fertile, despite having only 9–16 % of the normal GSH levels in liver, lung, pancreas, erythrocytes, and plasma [79]. Except when challenged with oxidant stress [80, 81], $Gclm^{-/-}$ mice exhibit no overt phenotype, making them a useful model for studying chronic GSH depletion. Interestingly, these mice show accelerated clearance of ethanol and acetaldehyde and are protected from alcohol-induced steatosis (*Chen Y, Vasiliou V et al, manuscript in preparation*). Thus, $Gclm^{-/-}$ mice represent a model wherein significant GSH depletion in the liver is associated with beneficial metabolic and stress responses to ethanol.

22.8 Concluding Remarks

Alcohol use is widespread and related to numerous diseases, including oral, colorectal, liver, pancreas, aerodigestive, breast, and colon cancers. Ethanol metabolism and resultant oxidative stress are primary pathogenic events mediating alcohol-induced organ damage and neurobehavioral changes, the molecular details of which are not yet fully understood. The knockout mouse models for enzymes metabolizing ethanol (ADH1, CAT, and CYP2E1), acetaldehyde (ALDH2, ALDH1A1, and ALDH1B1) and enzymes involved in GSH synthesis (GCLC and GCLM), which we have discussed briefly in this review, represent unique and highly relevant animal models for alcohol research. Utilization of these models will deliver valuable information about the fundamental mechanisms underlying ethanol toxicity. Such knowledge should accelerate the development of more effective, targeted therapies to both prevent and treat health issues associated with excessive alcohol consumption.

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Article

The Protective Effect of Glycyrrhizic Acid on Renal Tubular Epithelial Cell Injury Induced by High Glucose

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Abstract: The aim of this study was to determine the beneficial effect of glycyrrhizic acid (GA) on type 2 diabetic nephropathy using renal tubular epithelial cell line (NRK-52E). The cells are divided into normal group (NG), high glucose group (HG), and treatment group (HG + GA). The methylthiazoletetrazolium (MTT) assay was used to detect the cell proliferation. Cell cycle analysis was performed using flow cytometry. Model driven architecture (MDA), reactive oxygen species (ROS) and superoxide dismutase (SOD) were also measured. Electron microscopy and histological were used to detect the changes in cell ultrastructure. The phosphorylation of AMP-activated protein kinase (AMPK), silent information regulator T1 (SIRT1), manganese-superoxide dismutase (Mn-SOD) and transforming growth factor-β1 (TGF-β1) were assessed by immunohistochemistry, immunofluorescence, and western blotting. Real-time fluorescent quantitative PCR (RT-qPCR) was used to measure Mn-SOD and PPARγ co-activator 1α (PGC-1a) mRNA. We find that high glucose increases NRK-52E cell proliferation and TGF-β1 expression, but decreases expression of AMPK, SIRT1 and Mn-SOD. These effects are significantly attenuated by GA. Our findings suggest that GA has protective effects against high

glucose-induced cell proliferation and oxidative stress at least in part by increasing AMPK, SIRT1 and Mn-SOD expression in NRK-52E cells.

Keywords: diabetic nephropathy; glycyrrhizic acid; NRK-52E

1. Introduction

Diabetic nephropathy (DN), one of the most severe microvascular complications of type 1 and type 2 diabetes, is a major cause of end-stage renal disease [1]. It seriously affects patients' life quality. Pathological manifestations of DN include glomerular hypertrophy, basement membrane thickening and accumulation of extracellular matrix, tubulo-interstitial disease, and glomerulosclerosis. The pathogenesis of DN is very complex and has not been fully elucidated. High glucose is the foundation and key of DN, and can induce an abnormal increase of reactive oxygen species (ROS) and oxidative stress occurrence. In addition, high glucose can induce abnormal glucose metabolism and hemodynamic changes. High glucose-induced renal tubular epithelial cells have been used as an *in vitro* model for studies of early-stage DN.

Tubular epithelial cells account for 90% of the total kidney volume. Recent studies showed that the degree of seriousness of tubular interstitial disease is closely related to the DN renal dysfunction. The early-stage DN mainly results in glomerular proteinuria, but its long-term prognosis depends on the severity of tubulointerstitial damage. Tubular epithelial cell hypertrophy is a major factor in causing kidney hypertrophy. Therefore, the early hypertrophy inhibition for diabetic renal tubular epithelial cells helps to control the DN disease.

Functions of tubular epithelial cells can be affected by many external factors such as transforming growth factor-β (TGF-β). In the normal situation, TGF-β can inhibit the cell proliferation and inflammation. However, over-expression of TGF-β may cause pathological changes and promote cell proliferation and extracellular matrix accumulation. The increase of ROS and model driven architecture (MDA) generation, and decrease of antioxidant enzyme superoxide dismutase (SOD) activity are the result of oxidative stress. Studies have shown that high glucose-induced oxidative stress and renal cortical injury is related to down-regulation of PPARγ co-activator 1α (PGC-1α) expression [2].

AMP-activated protein kinase (AMPK) is a serine/threonine kinase evolutionarily conserved with a catalytic α-subunit and regulatory β- and γ-subunits, forming a heterotrimeric complex. It is abundantly expressed in the kidney [3]. AMPK has become a hot research subject for type 2 diabetes. Previous studies have shown that manganese superoxide dismutase (Mn-SOD) can reduce high glucose induced increase of ROS, thereby activate AMPK [4].

Glycyrrhizic acid (GA) is a triterpenesaponin glycoside, which is the primary bioactive component of jor plant root extract of *Glyccyrhiza Glabra* (Liquorice), a shrub from the Leguminosae family [5,6]. Recently, a study showed that GA was able to protect rabbits from renal ischemia reperfusion injuries [7]. Another report shows that after treating diabetic rats with glycyrrhizin for 60 days, TGF-β1 expression in renal tissue was decreased [8].

However, little information is available about the effect of GA on the proliferation of tubular epithelial cells induced by high glucose. The aim of this study was to test the hypothesis if GA has a protective effect

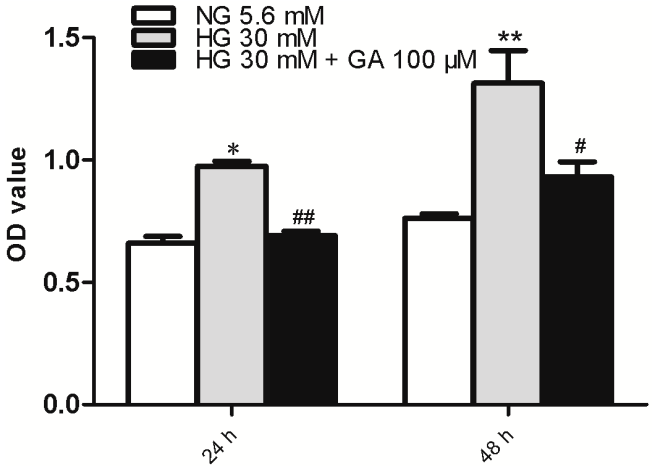
against high glucose-induced tubular epithelial cells damage by reducing cell proliferation and oxidative stress. We employed multiple approaches to examine the expression of factors such as AMPK, SIRT1 (silent information regulator T1), Mn-SOD and TGF-β1 in NRK-52E cells in the absence or presence of high glucose, GA, or both. Our data are consistent with our hypothesis.

2. Results and Discussion

2.1. GA (Glycyrrhizic Acid) Reverses the High Glucose-Induced Effect on Cell Proliferation in NRK-52E Cells

NRK-52E cell proliferation was evaluated using MTT (methylthiazoletetrazolium) analysis. The results showed that compared with the NG (normal group) group, 30 mM glucose alone increased NRK-52E cell proliferation at both 24 and 48 h time points ($p < 0.05$). We tested the effect of GA at 25, 50, 100, 200 μmol/L and found GA at 100 μmol/L can inhibit NRK-52E cell proliferation induced by HG ($p < 0.05$) (Figure 1).

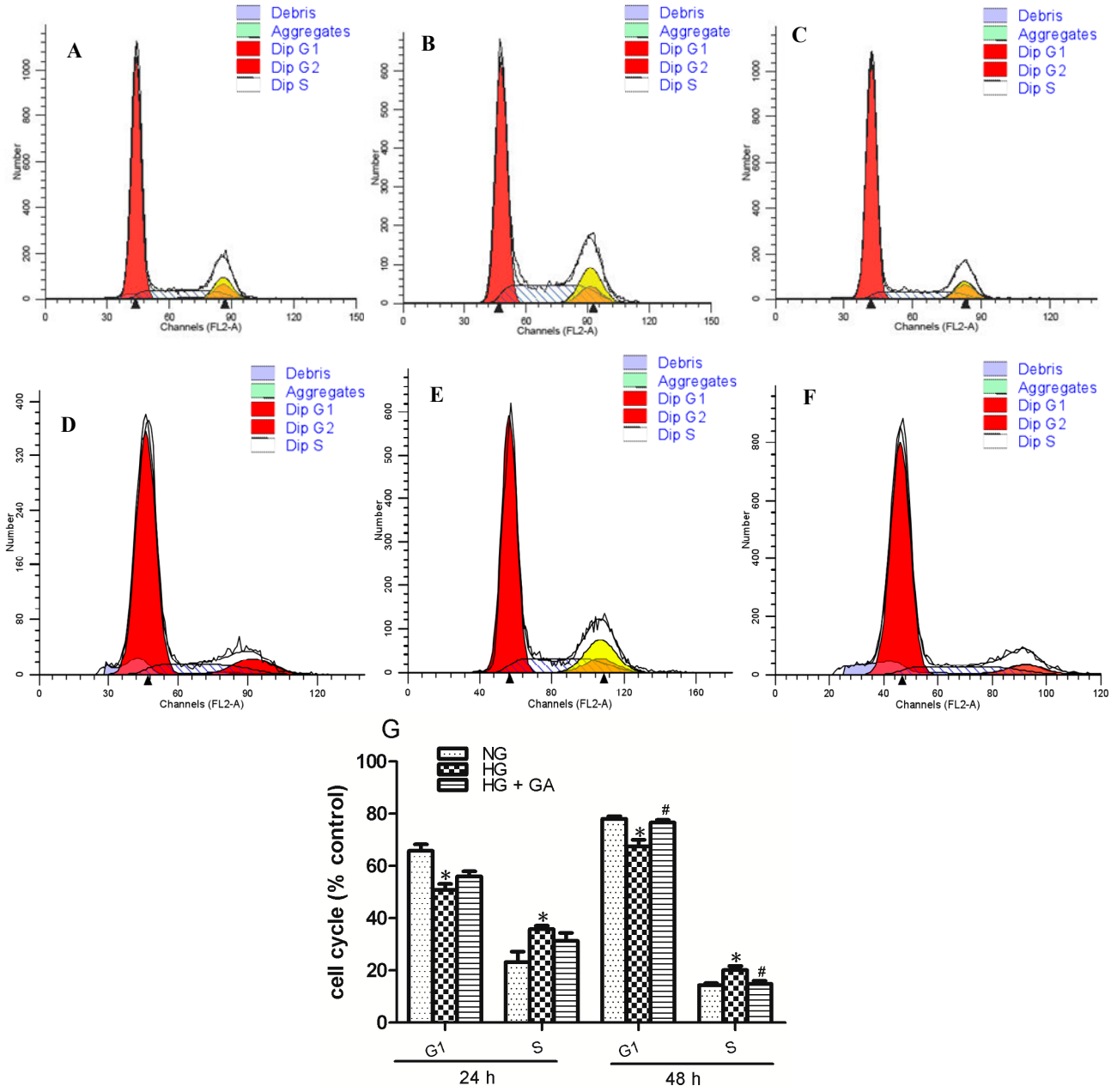
Figure 1. Proliferation assay. NRK-52E cells were treated with high glucose (HG) with or without glycyrrhizic acid (GA) as indicated for 24 or 48 h, followed by MTT (methylthiazoletetrazolium) analyses. Cells receiving normal glucose (NG) were included as control. $n = 3$. * $p < 0.05$ *vs.* NG; ** $p < 0.01$ *vs.* NG; $^{\#}$ $p < 0.05$ *vs.* HG; $^{\#\#}$ $p < 0.01$ *vs.* HG. OD (optical density).



2.2. Effect of GA on Cell Cycle Induced by HG (High Glucose) in NRK-52E Cells

A flow cytometry was used to evaluate the effect of GA treatment upon cell cycle profiles (Figure 2A–F). After 24 and 48 h incubation in HG group, the proportion of G1 phase decrease and S phase increase in NRK-52E cells ($p < 0.05$). In contrast, more cells in G1 phase and fewer cells in S phases were significantly obtained in GA group after 48 h incubation. ($p < 0.05$). At 24 h time point, GA did not significantly increase the number of cells in G1 ($p > 0.05$), or decrease the cells number in S phase ($p > 0.05$), compared with HG group (Figure 2G).

Figure 2. Flow cytometric analysis of the cell cycle. Cells were treated for 24 or 48 h as indicated, (**A**) NG group; (**B**) HG group; (**C**) HG + GA group (treated with 24 h); (**D**) NG group; (**E**) HG group; (**F**) HG + GA group (treated with 48 h); and (**G**) Followed by flow cytometric analysis. * $p < 0.05$ *vs.* NG; $^{\#}$ $p < 0.05$ *vs.* HG.



2.3. Histopathological Findings

For the NG group, most of NRK-52E cells were round or oval-shaped. Cilia existed in membrane. Organelles were normal. Membrane integrity was good. For the HG group, some cells increased volumes significantly. The number of round cells also increased. Cells tended to change fusiform (Figure 3). The cells incubated in HG exhibited injury features, including an irregular nucleus, chromatin condensation, nuclear envelope shrinkage, organelle shortage, rough endoplasmic reticulum slight expansion, cilias fusion and shortage. The situation became much better in the groups treated with GA (final concentration of 100 μmol/L) in comparison with the HG group (Figure 4).

Figure 3. Effect of GA on cell morphology induced by HG in NRK-52E cells. The cells were incubated in the three groups for 24 h. They were stained with hematoxylin-eosin. Original magnification: ×200.

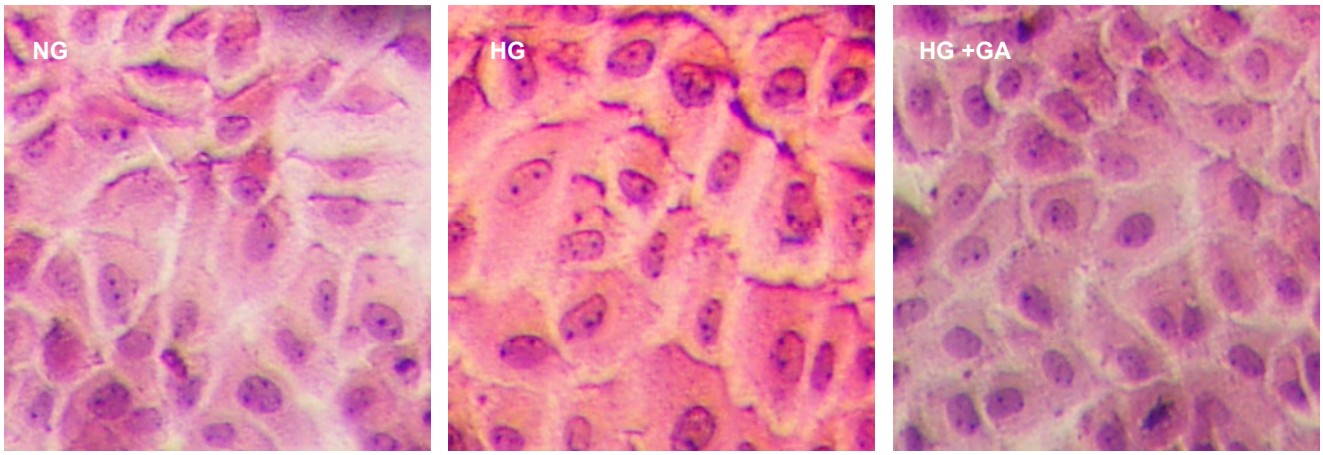
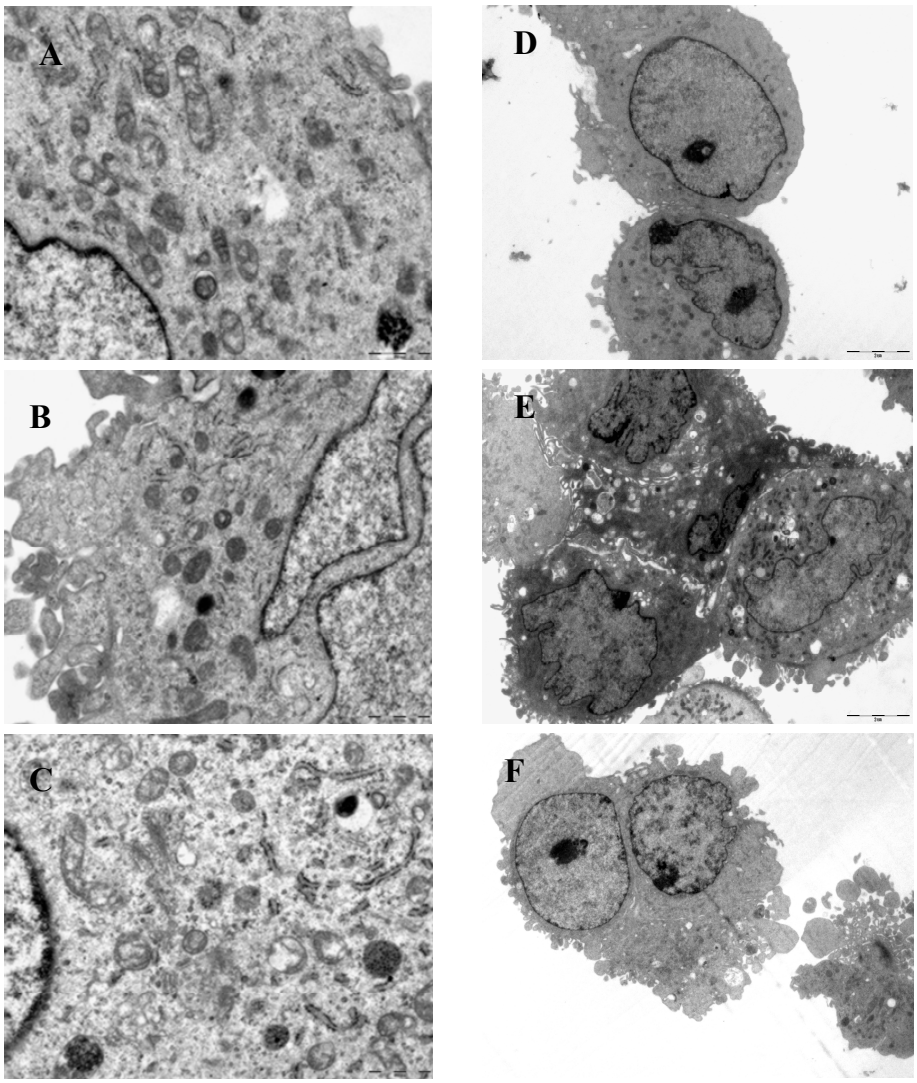


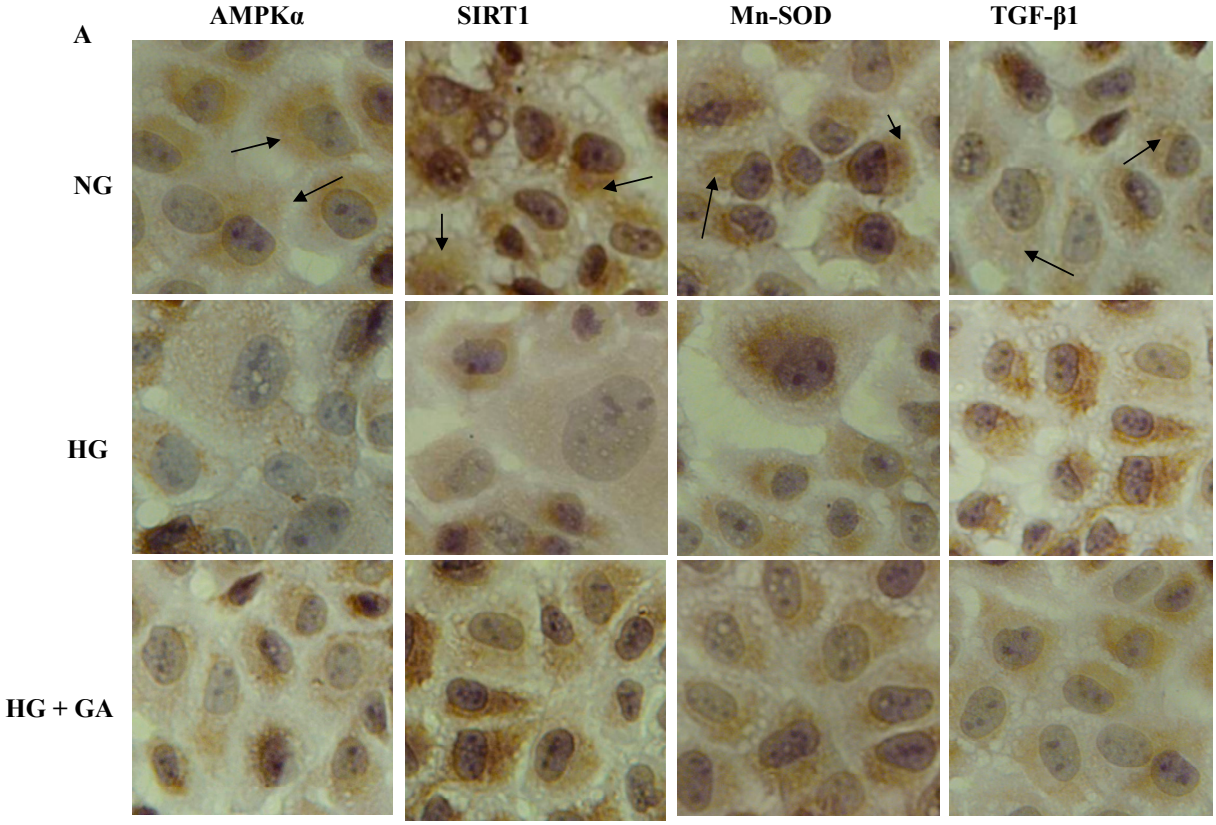
Figure 4. Electron microscopy analyses. NRK-52E cells were grouped and treated as in Figure 1 and examined under an electron microscopy after 48 h. (**A**,**D**) NG group; (**B**,**E**) HG group; (**C**,**F**) HG + GA group. **A**–**C**: ×4000, **D**–**E**: ×1000.



2.4. Effect of GA on SIRT1 (Silent Information Regulator T1), AMPKα (AMP-Activated Protein Kinase α), Mn-SOD (Manganese-Superoxide Dismutase) and TGF-β1 (Transforming Growth Factor-β1) Proteins Expression

SIRT1, AMPKα and Mn-SOD proteins were detected by immunohistochemistry (Figure 5A and Table 1) and immunofluorescence (Figure 5B,C). TGF-β1 was detected by immunohistochemistry. The results showed that the fluorescence intensity and optical density in the HG group were lower than that in the control group for SIRT1, AMPKα and Mn-SOD ($p < 0.05$). SIRT1 and Mn-SOD increased in the GA group ($p < 0.05$). However, for AMPKα, it increased only at 48 h but not at 24 h ($p > 0.05$). Immunohistochemical experiment showed that HG increased TGF-β1 protein expression, which was reversed in the GA group ($p < 0.05$).

Figure 5. Immunohistochemistry and immunofluorescence analyses. (**A**) NRK-52E cells were grouped and treated as in Figure 1 for 24 h, followed by immunohistochemistry to assess expression of proteins as indicated. Original magnification: ×400; (**B**) As in **B**, except that cells were analyzed by immunofluorescence staining. Original magnification: ×200; and (**C**) Relative levels of protein expression. * $p < 0.05$ *vs.* NG; # $p < 0.05$ *vs.* HG. Apoptosis is labeled with arrow.



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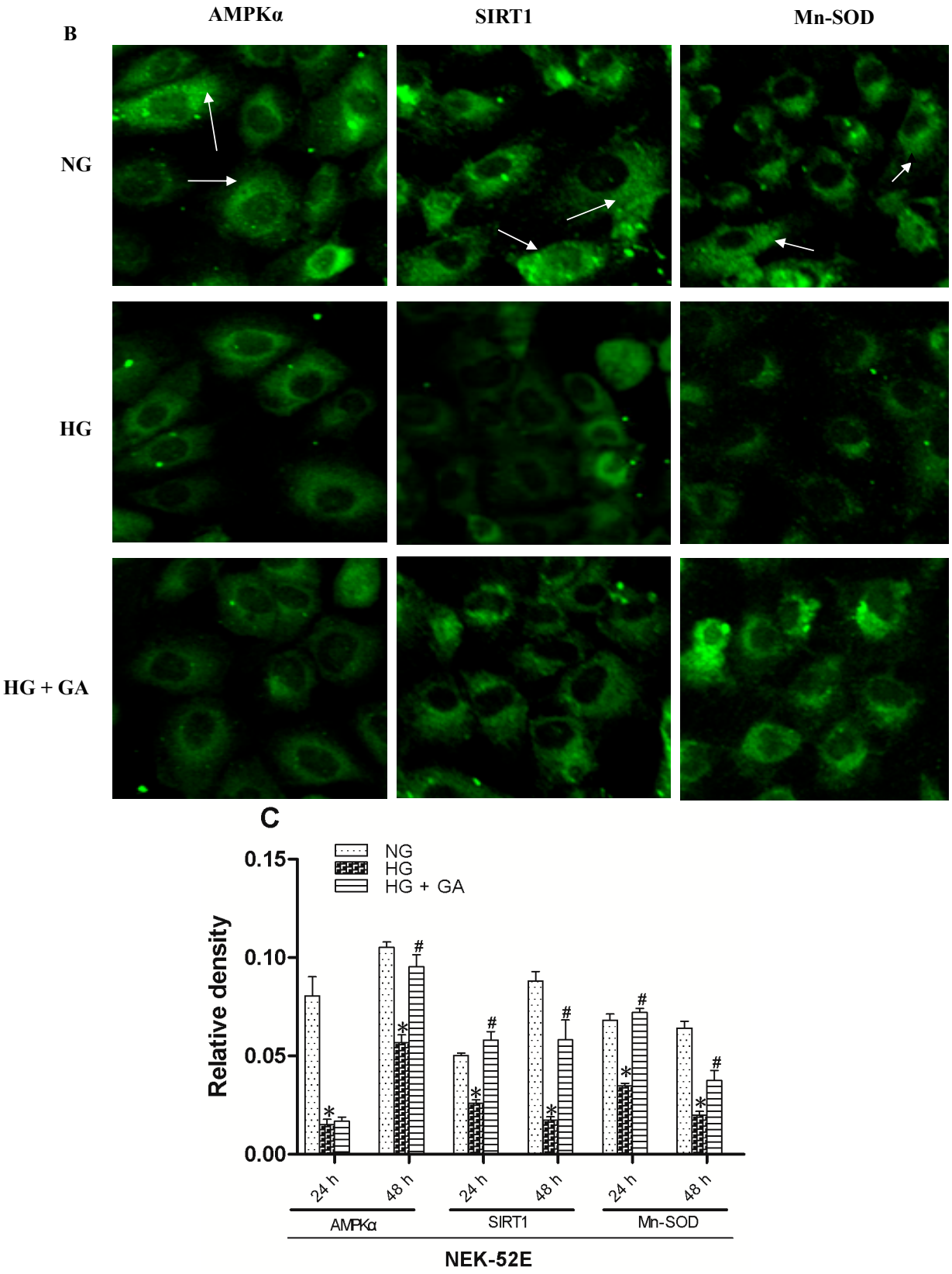


Table 1. Effect of GA on silent information regulator T1 (SIRT1), AMP-activated protein kinase α (AMPKα), manganese-superoxide dismutase (Mn-SOD) and transforming growth factor-β (TGF-β1) proteins expression in NRK-52E cells, as detected by immunohistochemistry.

Group (n = 6)	AMPKα Expression	SIRT1 Expression	Mn-SOD Expression	TGF-β1 Expression
Normal group (24 h)	0.24 ± 0.018	0.21 ± 0.014	0.27 ± 0.018	0.17 ± 0.006
High glucose group (24 h)	0.20 ± 0.027 *	0.18 ± 0.015 *	0.19 ± 0.031 *	0.28 ± 0.019 **
Experimental group (24 h)	0.22 ± 0.025	0.21 ± 0.017 #	0.24 ± 0.028 #	0.18 ± 0.017 ##
Normal group (48 h)	0.29 ± 0.010	0.19 ± 0.017	0.41 ± 0.048	0.23 ± 0.005
High glucose group (48 h)	0.23 ± 0.007 **	0.13 ± 0.006 *	0.22 ± 0.029 *	0.31 ± 0.058 **
Experimental group (48 h)	0.25 ± 0.006 #	0.18 ± 0.005 #	0.28 ± 0.024 #	0.26 ± 0.037 #

* $p < 0.05$ *vs.* NG; ** $p < 0.01$ *vs.* NG; # $p < 0.05$ *vs.* HG; ## $p < 0.01$ *vs.* HG.

2.5. Effect of GA on SIRT1, AMPKα, Mn-SOD and TGF-β1 Proteins Expression, as Detected with Western Blotting

In our experiment, there was a decrease in the expression of AMPKα, SIRT1 and Mn-SOD, and an increase in the expression of the TGF-β1 after treatment of cells with HG. However, the treatment with GA antagonized all the above effects induced by HG (Figure 6A,B).

Figure 6. Western blotting analyses. NRK-52E cells were grouped and treated as in Figure 1 for 24 and 48 h, followed by Western blotting to evaluate expression of proteins as indicated, with Actin as an equal loading control. (**A**) Western blot detect purpose proteins treated with 24 and 48 h pictures; (**B**) Relative levels of protein expression. The intensity of the bands on Western blots were quantified, normalized to Actin, and graphed. * $p < 0.05$ *vs.* NG; # $p < 0.05$ *vs.* HG.

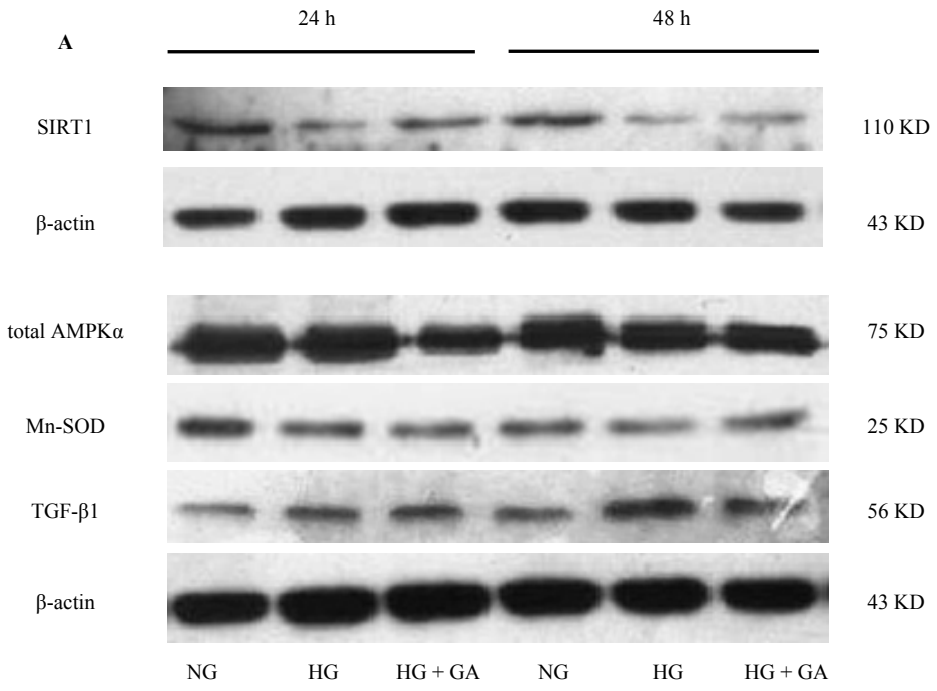
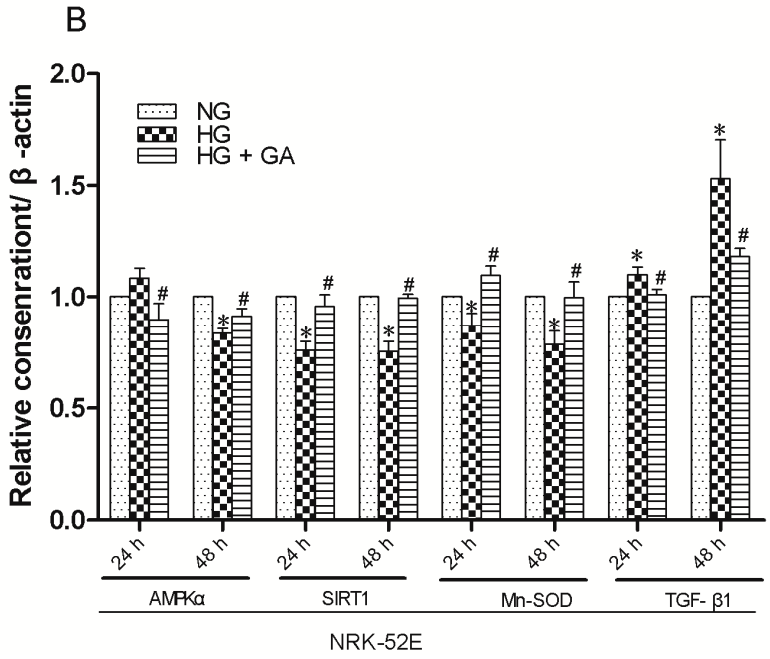


Figure 6. *Cont.*



2.6. Effects of GA on Activities of Antioxidant Enzymes and Oxidative Stress Markers

The activity of SOD and concentration of MDA were lower, whereas concentration of MDA was higher in the HG group than in the control group ($p < 0.05$), suggesting that the cells were suffered from oxidative stress (Table 2). Treatment with GA significantly decreased the concentrations of MDA and significantly increased SOD activity ($p < 0.05$). These results indicate that GA ameliorates oxidative stress in high glucose cells.

Table 2. Effect of GA on model driven architecture (MDA) and SOD in NRK-52E cells.

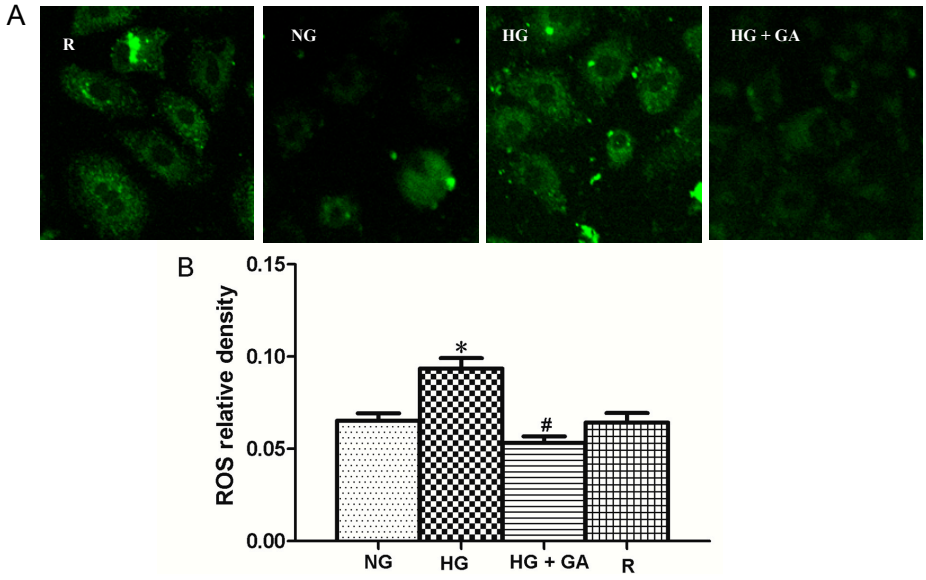
Group (n = 3)	MDA (μmol/L)	SOD (U/mL)
Normal group	7.57 ± 0.680	23.60 ± 0.538
High glucose group	14.66 ± 0.480 **	16.22 ± 0.315 **
Experimental group	9.77 ± 0.468 $^{\#\#}$	19.68 ± 0.952 $^{\#}$

** $p < 0.01$ *vs.* NG; $^{\#}$ $p < 0.05$ *vs.* HG; $^{\#\#}$ $p < 0.01$ *vs.* HG.

2.7. Effect of High Glucose and GA on ROS (Reactive Oxygen Species) Production

We observed that the ROS production was augmented in NRK-52E cells induced with high glucose, peaking after 24 h of treatment (Figure 7A). With the addition of GA, it was decreased. These data indicate that after giving GA, there appears either a decrease in ROS generation or an increase in endogenous ROS scavenging/antioxidant capacity.

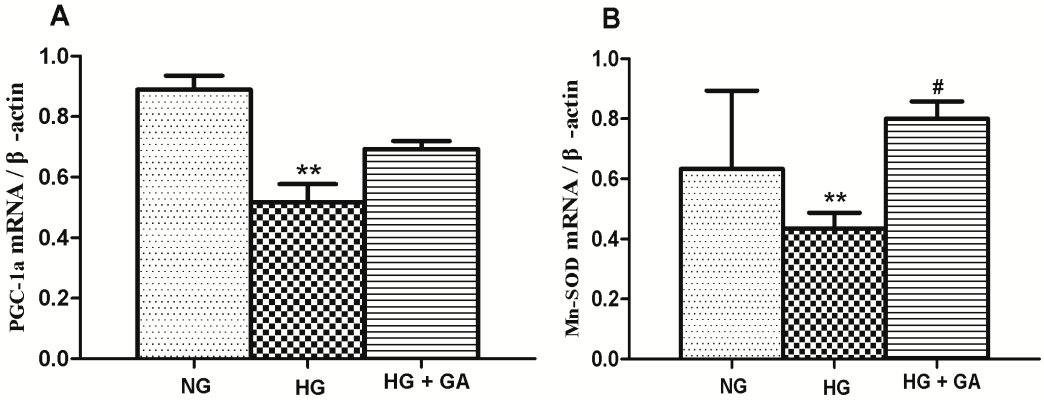
Figure 7. High glucose induces ROS (reactive oxygen species). Cells were grouped and treated for 24 h as indicated, followed by DCFH-DA (2',7'-dichlorofluorescin diacetate) (probe) or rosup (positive control) treatment for 30 min. Cell were then analyzed, using confocal microscopy. R represents rosup. (**A**) confocal microscopy images; (**B**) Histograms. * $p < 0.05$ *vs.* NG; # $p < 0.05$ *vs.* HG.



2.8. Effect of GA on the Expression of Mn-SOD and PGC-lα (PPARγ Co-Activator 1α) mRNA

The effects of GA on Mn-SOD and PGC-lα mRNA in NRK-52E cells have been examined. Exposure of NRK-52E decreased the expression of Mn-SOD and PGC-lα mRNA level in the HG group compared with NG group. The expression of Mn-SOD increased in GA group ($p < 0.05$), but PGC-lα mRNA level did not increase in GA group (Figure 8A,B) ($p > 0.05$).

Figure 8. Real-time RT-qPCR analyses. Effect of GA on Mn-SOD (**A**) and PGC-1α (PPARγ co-activator 1α) (**B**) mRNA expression was measured by real-time fluorescent quantitative RT-qPCR. Values were normalized to mouse housekeeping gene β-actin and then normalized to sham samples. The mRNA levels of Mn-SOD and PGC-1α in the HG group were significantly lower than those in the NG group (** $p < 0.01$). The mRNA levels of Mn-SOD began to return to higher levels in the GA group (# $p < 0.01$). But no change was observed in PGC-lα mRNA level for GA group compared with HG group.



2.9. Discussion

Previous studies have shown that high glucose concentrations promote proliferation of mesangial cells, renal tubular epithelial cells, and vascular smooth muscle cells under the diabetic state [9]. In this study, 30 mmol/L high glucose was used as the stimulator to induce NRK-52E cell proliferation. Rat renal tubular epithelial cells induced by high glucose as an *in vitro* model have been used in studies of early-stage diabetic nephropathy. Clinical trials have shown that high glucose is the principal cause of renal damage in both type 1and type 2 diabetes [10]. MTT is a method to detect cell proliferation activity and was used in this study to detect the NRK-52E cell proliferation. The results shows that high glucose significantly increases NRK-52E cell proliferation after intervention for 24 and 48 h. This demonstrated that NRK-52E cells can be used as *in vitro* model of diabetic nephropathy after injury through induction with 30 mmol/L high glucose. It was also shown that GA can inhibit the cell proliferation induced by HG at the concentration of 100 μmol/L.

Reponses of mesangial cells to injuring stimuli include proliferation, hypertrophy and apoptosis. The degree of these injuries depends on the progress of the cell cycle. In cell cycle progression, traversing the G1-S phase boundary is coupled to DNA synthesis. In vitro, high glucose can promote mesangial cells from G1 to S phase, reducing the proportion of cells in G1 phase, and increasing the proportion of cells in S phase. The high glucose also causes DNA synthesis and promotes cell proliferation [11]. It was shown that GA can inhibit DNA synthesis, resulting in arrest of the cell-cycle transition from G1 to S phase. Compared with NG group, high glucose significantly increased the NRK-52E cells population at S-phase, whereas 100 μmol/L GA blocked the G1-S phase transition after the cells were treated for 48 h. GA may be involved in inhibiting cell proliferation.

These results suggest that GA inhibits high glucose-induced mesangial cell proliferation to execute its protective effect on NRK-52E cell injury.

Although the mechanisms of proliferation of NRK-52E cells induced by high glucose are still poorly defined, mechanism of ROS is well documented in both diabetes and model systems. In addition, high glucose has been frequently shown to augment cell proliferation and DNA synthesis, and it has been suggested as the main contributor to DN in affected subjects [12]. The importance of elevated ROS level in the pathogenesis of the diabetes-related microvascular complications has been well documented [12,13]. High glucose-induced increase of ROS is an important aspect of oxidative stress. The results shows that ROS increased in HG group compared with the NG group. However, it was decreased in GA group compared with the HG group.

The cellular levels of ROS and the presence of oxidative stress are determined not only by rates of ROS generation, but also by their neutralization and degradation using endogenous antioxidants. Oxidative stress leads to lipid peroxidation and thereby formation of the harmful product such as MDA [14]. Accordingly, it induces DNA oxidative damage via generation of 8-OHdG (8-hydroxy-2-deoxy Guanosine) [15]. Meanwhile, the activity of antioxidant defense enzymes decreases [16], which leads to cell damage. The reduced activity changes the oxidative stress markers: decreasing SOD levels and increasing MDA. It was reported that an anti-oxidative role of GA in the amelioration of carbon tetrachloride-induced liver injury [17].

The peroxisome PGC-1α is a small family of transcriptional coactivators, which play a critical role in the control of glucose, lipid, and energy metabolism [18]. The physiological significance of PGC-1α in

mitochondrial energy metabolism has been well demonstrated [19,20]. Studies have shown that high glucose-induced renal cortical injury and oxidative stress is related to down-regulation of PGC-1α. When PGC-1α stimulates mitochondrial biogenesis, SOD generation increases [21]. Therefore, when PGC-1α down-regulates, oxidation and antioxidant system become imbalanced and oxidative stress occurs. In this experiment, we used RT-qPCR method to detect the PGC-1α mRNA. The result showed that compared with NG group, PGC-lα mRNA decreased in HG group. There was no different in GA group compared with HG group.

These results showed that high glucose can promote ROS generation, decrease SOD and increase MDA in NRK-52E cells. It was further confirmed that high glucose can induce ROS production, promote lipid peroxidation, and decrease antioxidant capacity. In GA group, however, it was observed that GA had an antioxidant role, ROS, causing MDA to decrease and SOD to increase. We also used RT-qPCR method to detect the Mn-SOD mRNA. The result showed that Mn-SOD mRNA increase in GA group. These results suggest that GA can reduce the oxidative stress, improve antioxidant system to protect cells from damage. However, the protective effect of glycyrrhizin on high glucose-induced NRK-52E cells' oxidative stress injury may not be regulated by PGC-1α after treatment for 24 h.

Since AMPK is a cellular energy sensor, its activity is highly linked to the change in the intracellular AMP/ATP ratio. The role of AMPK in carbohydrate and protein metabolism, cell cycle regulation, and in mitochondrial biogenesis has been described in literature. AMPK also regulates glucose homeostasis. Insulin deficiency has been proposed as one of the factors causing hypothalamic. AMPK activation and the subsequent increase in food intake have been observed in streptozotocin-induced diabetic rats [22]. Renal AMPK activity was reduced after a high-fat diet (HFD) for 16 weeks [23]. However, its role in mediating renal inflammation was rarely evaluated. Adiponectin and rosiglitazone can activates AMPK through improving insulin sensitivity. We used immunohistochemistry, immunofluorescence and western blot experiments to detect AMPK protein expression. The results showed that AMPKα has a wealth expression in the NRK-52E cells. AMPKα protein expression decreased in high glucose group. GA could make AMPKα protein expression increased after treatment for 48 h. It also showed that AMPK was localized in the cytoplasm.

Sirt1, a NAD-dependent protein deacetylase, is reported to regulate intracellular metabolism and attenuate ROS-induced apoptosis, leading to longevity and acute stress resistance. Both AMPK and SIRT1 have emerged as interesting targets as they are heavily involved in catabolic metabolism, mitochondrial activation, angiogenesis and enhanced cell survival [24–27]. It is well known that the effects of resveratrol are mediated by both SIRT1 and AMPK [28]. However, the direct relationship between kidney-specific Sirt1 and renal tissue survival *in vivo* has not been elucidated. In this study, immunohistochemistry, immunofluorescence and western blot experiment showed that high glucose can reduce the expression of SIRT1, which may be attentuated after giving GA.

TGF-β1 is a key intermediary substance in DN. TGF-β1 can form an autocrine activation of proliferation ring in an autocrine manner to prevent glomerular mesangial cells, epithelial, endothelial cells and proximal tubule cell from proliferation, differentiation and increasing cell diameter. In early diabetic, glomerular and tubular hypertrophy plays an important role. The experimental showed that TGF-β1 protein expression was significantly increased in HG group after 24 and 48 h, which may be reduced after giving GA. Morphology shows that cells were flat in HG group compared with NG group. In the electron microscope after 48 h treatment, stimulation showed that NRK-52E cell's volume

increased comparing to NG group. This suggests that cells hypertrophy in HG group relates to high expression of TGF-β1.

These results suggest that GA protects NRK-52E cells from damage through regulating the expression of AMPK, SIRT1, Mn-SOD and TGF-β1. NRK-52E cells hypertrophy induced by high glucose correlates to high expression of TGF-β1.

3. Experimental Section

3.1. Materials

Cell culture reagents, trypsin EDTA (Calcium disodium), fetal bovine serum (FBS) were obtained from Hyclone laboratories Inc. (South Logan, UT, USA). Flasks, vials, 24 Well cell culture cluster, 96 well cell culture cluster and straws were purchased from Corning Incorporated (Corning, NY, USA). Glycyrrhizin was come from Tokyo Chemical Industry Co. (TCI, Tokyo, Japan). D-(+)-glucose powder was purchased from Sigma (St. Louis, MI, USA). NRK-52E cells were obtained from Chinese Center for Type Culture Collection (Shanghai, China). MTT (5 mg/mL) and cell cycle detection kits were purchased from Keygen Biotech (Nanjing, China). Reaction Oxygen Species Assay Kit was purchased from Beyotime (Shanghai, China). SOD and MDA kits were obrained from Nanjing Jiancheng (Nanjing, China). The antibodies recognizing AMPKα, SIRT1 and Mn-SOD were purchased from Santa Cruz (TX, USA). Anti-β-actin was purchased from Bios (Beijing, China). Trizol was purchased from Invitrogen (Grand Island, NY, USA). Other commercial kits of the Transcript RT/RI Enzyme Mix and the TransStart Top Green qPCR SuperMix were obtained from TransGen Biotech (Beijing, China). All other reagents were from commercial sources and of standard biochemical quality.

3.2. Cell Culture

The cells were cultured in DMEM (Dulbecco's modi-fied Eagle's medium) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in an atmosphere containing 5% CO_2. After preincubation in DMEM supplemented with 0.5% fetal calf serum for 24 h, cells were then treated in three different groups: normal glucose group (NG, 5.6 mM glucose), high glucose group (HG, 30 mM glucose), and high glucose and glycyrrhizic acid group. Cells were incubated for another 24 or 48 h before analyses.

3.3. Cell Proliferation Assay

MTT assay was used to measure cell proliferation. After 24 and 48 h incubation with different compounds as described above, 50 μL MTT was added and cells were cultured for additional 4 h. Subsequently, cells were lysed using dimethylsulfoxide. When the formanzan crystals were completely dissolved, the density (OD) was measured at 490 nm, using a Spectrophot-ometer Microplate Reader come from BioTek (Winooski, VT, USA).

3.4. Flow Cytometry

After 24 or 48 h treatment with different compounds, cells were harvested by trypsinization without EDTA. They were then centrifuged (with 1000× *g*, 10 min, 4 °C) and washed twice with phosphate buffered saline (PBS). Then they were fixed in methanol at 4 °C overnight. Following two washes with PBS, the fixed cells were incubated in RNase at 37 °C for 30 min, followed by a DNA staining with propidium iodide at 4 °C for 30 min in the dark. Then each sample was analyzed using a Flow Cytometer (BD FACSCalibur, Biocompare, San Diego, CA, USA) and the proportion of cells within the G1 and S phases of the cell cycle were determined.

3.5. Histological Studies

After cells were intervened 24 h, the cells were fixed in 95% ethanol. Then stained with hematoxylin-eosin. The stained sections were examined and photographed with a light microscope (BX61, Olympus, Tokyo, Japan).

3.6. Electron Microscopy

After cells were intervened 48 h, they were fixed with 4 °C, then washing with 0.1 mol cacodylate buffer. Experimental procedure includes before fixation, after fixation, dehydration, embedding, slicing and uranyl acetate dye staining. TEM (transmission electron microscopic) comes from H7650 (Hitachi, Shiga, Japan).

3.7. Immunohistochemistry Assay

Cells were incubated with antibodies specific for AMPKα, SIRT1, Mn-SOD (1:200) and TGF-β1 (1:100, BOSTER, Wuhan China) overnight, followed by secondary antibody incubation in IHC Detection Reagent (ZSGB-BIO, Beijing, China) in 37 °C for 45 min. DAB (ZSGB-BIO) was used to stain cytoplasm. Then hematoxylin was used to stain nucleus. Cells were then examined and photographed with light microscope (BX61). Ipwin32 software was used to quantify optical density.

3.8. Immunofluorescence Assay

The cells were cultured on glass coverslips and were treated for 24 and 48 h after growth arrest. Cells were fixed in 4.0% formaldehyde, then blocked with goat serum. Immunofluorescence staining was performed by incubating the fixed cells with anti-AMPKα, SIRT1 and Mn-SOD antibodies (1:200), followed by incubation with FITC (fluorescein isothiocyanate)-conjugated secondary antibody (1:40, Protein Tech Group, Chicago, IL, USA). Cells were viewed with an Olympus FV1000 Laser Scanning Confocal Microscope (Olympus, Center Valley, PA, America). Ipwin32 software (Media Cybernetics Inc., Bethesda, MD, USA) was used to quantify fluorescence intensity.

3.9. Western Blotting

After being treated with different compounds for 24, 48 h, cells were harvested and washed with ice-cold phosphate buffer. Protein was obtained using whole-cell extraction kit (Keygen biotech,

Beijing, China). Protein concentrations were determined using the BCA method (Keygen biotech). Equal amounts of protein were loaded, separated by SDS-PAGE (SDS-polyacrylamide gel) and transferred to nitrocellulose membranes. After being blocked with 5% skimmed milk in Tris-phosphate buffered saline (TPBS) at room temperature, the membranes were incubated overnight at 4 °C with primary antibodies for AMPKα, SIRT1, Mn-SOD (1:300), TGF-β1 (1:100), and Rabbit An-β-Actin (1:1000). After being incubated with the respective second antibody, immune complexes were detected using ECL (chemiluminescent agent) (Amersham Life Science, Arlington Heights, IL, USA) Western blotting reagents. Immunoreactive bands were quantified using the Bio-RAD Gel Imaging System (Bio-RAD, Berkeley, CA, USA). Values were corrected with the internal control (β-actin).

3.10. UV Spectrophotometer

After cells were cultured for 24 h, UV spectrophotomete (VIS-7220N, Beijing, China) was used to detect OD of SOD and MDA between different groups.

3.11. ROS Detection

The cells were cultured on glass coverslips and incubated in the dark with 10 μM/L of DCF (dichlorofluorescein) or Rosup for 30 min at 37 °C. Intracellular ROS production was assessed with an Olympus FluoView 1000 Laser Scanning Confocal Microscope (OLYMPUS) (using ex/em λ = 488 nm/515 nm for DCFH-DN).

3.12. Quantitative Real Time Polymerase Chain Reaction PCR Assay

RNA was extracted using the Trizol reagent, reversely transcribed with an RT kit using oligo (dT) 18 primer (0.5 μg/μL) in a total volume of 20 μL according to Manufacturer's protocol. Real-time PCR was performed and analyzed on a Fluorescent PCR instrument (IQ-5) using cDNA and SYBR Green PCR Master Mix. The primers used were: Mn-SOD forward: 5'-AAGGAGCAAGGTCGCTTACAGA-3', Mn-SOD reverse: 5'-CAAATGGCTTTCAGATAGTCAGGTC-3', PGC-1α forward: 5'-AATCAAGC CACTACAGACACCGC-3', PGC-1α reverse: 5'-CTTTCGTGCTCATTGGCTTCAT-3', The relative amounts of mRNA were determined by the $2^{-\Delta\Delta Ct}$ calculations.

3.13. Statistical Analysis

All quantitative data are expressed as mean ± SD. The differences between two experimental conditions were analyzed using the Student's *t* tests and/or one-way analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant.

4. Conclusions

We concluded that the GA treatment resulted in protective effects on NEK-52E cell injuries induced by high glucose. The protection is obtained by inhibiting the high-glucose induced up-regulation of oxidation factor ROS and MDA, and down-regulation of SOD. However, the protective effect of glycyrrhizin on high glucose-induced NRK-52E cells' oxidative stress injury may not be regulated by PGC-1α. In addition, GA may increase AMPK, SIRT1 and Mn-SOD proteins expression and decreased

expression of TGF-β1 induced by HG. NRK-52E cells hypertrophy induced by high glucose may relate to high expression of TGF-β1. At the cell level, GA may be a potential therapeutic agent for the early stage of DN.

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Author Contributions

Shaozhang Hou, experimental designed and involved in specific experiments; Fangfang Zheng, involved in specific experiments and writing of the paper; Yuan Li, experiments carry out and data analysis; Ling Gao and Jianzhong Zhang experimental design and guidance.

Conflicts of Interests

The authors declare no conflict of interest.

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Glycyrrhizin, silymarin, and ursodeoxycholic acid regulate a common hepatoprotective pathway in HepG2 cells

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ABSTRACT

Background: Glycyrrhizin, silymarin, and ursodeoxycholic acid are widely used hepatoprotectants for the treatment of liver disorders, such as hepatitis C virus infection, primary biliary cirrhosis, and hepatocellular carcinoma.
Purpose: The gene expression profiles of HepG2 cells responsive to glycyrrhizin, silymarin, and ursodeoxycholic acid were analyzed in this study.
Methods: HepG2 cells were treated with 25 μM hepatoprotectants for 24 h. Gene expression profiles of hepatoprotectants-treated cells were analyzed by oligonucleotide microarray in triplicates. Nuclear factor-κB (NF-κB) activities were assessed by luciferase assay.
Results: Among a total of 30,968 genes, 252 genes were commonly regulated by glycyrrhizin, silymarin, and ursodeoxycholic acid. These compounds affected the expression of genes relevant various biological pathways, such as neurotransmission, and glucose and lipid metabolism. Genes involved in hepatocarcinogenesis, apoptosis, and anti-oxidative pathways were differentially regulated by all compounds. Moreover, interaction networks showed that NF-κB might play a central role in the regulation of gene expression. Further analysis revealed that these hepatoprotectants inhibited NF-κB activities in a dose-dependent manner.
Conclusion: Our data suggested that glycyrrhizin, silymarin, and ursodeoxycholic acid regulated the expression of genes relevant to apoptosis and oxidative stress in HepG2 cells. Moreover, the regulation by these hepatoprotectants might be relevant to the suppression of NF-κB activities.

Introduction

Liver cancer is the second most common cause of cancer death worldwide, causing about 746,000 deaths in 2012. The prognosis of liver cancer is very poor and the estimated incidence of new cases is 782,000 in the less developed regions in 2012 (Ferlay et al. 2015). Several studies illustrate that constitutive nuclear factor-κB (NF-κB) activity plays a central role in the hepatic neoplastic progression through the upregulation of anti-apoptotic genes (Kucharczak et al., 2003). Moreover, the inhibition of NF-κB activation in hepatocytes retards and reduces the development of hepatocellular carcinoma in mice (DiDonato et al. 2012). Therefore, the inhibition of NF-κB activation might be an effective strategy to treat liver cancers.

Glycyrrhizin, the triterpenoid saponin from *Glycyrrhiza glabra* L. roots (licorice), consists of one molecule of glycyrrhetinic acid and two molecules of glucuronic acid. Glycyrrhizin exhibits various pharmacological effects, such as anti-inflammatory and protective effects in liver (Li et al. 2014). Therefore, glycyrrhizin analogs, such

Abbreviations: NF-κB, nuclear factor-κB; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; sva, surrogate variable analysis; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; qPCR, quantitative real-time polymerase chain reaction; RQ, relative quantitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC9, histone deacetylase 9; MCM3AP, minichromosome maintenance deficient 3 associated protein; Cerebral, cell region-based rendering and layout; RLU, relative luciferase unit; IL, interleukin; FGF10, fibroblast growth factor 10; COL2A1, collagen type 2 alpha 1; SPIB, Spi-B transcription factor; PRKAB1, protein kinase AMP-activated beta 1; ETHE1, ethylmalonic encephalopathy 1; BID, BH3 interacting domain death agonist; SOD2, superoxide dismutase 2; MAP3K7, mitogen-activated protein kinase kinase kinase 7; tBID, truncated BID; TNF, tumor necrosis factor; ROS, reactive oxygen species.

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as magnesium isoglycyrrhizinate and stronger neo-minophagen C, are effective and safe for the treatment of patients with chronic liver disease and liver dysfunction (Mori et al. 1990; Mao et al. 2009).

Silymarin is a flavonolignan complex from *Silybum marianum* (L.) Gaertn. fruits. Silymarin comprises a number of flavonolignans, including silibinin (silybin A and silybin B), isosilybin A and B, silychristin A and B, silydianin, and other phenolic compounds (Wu et al. 2009). Silymarin exhibits anti-inflammatory and immunomodulatory effects and thus promotes the health of livers (Polyak et al. 2013). In addition, silymarin-type drugs like legalon have been used for the treatment of acute hepatitis and nonalcoholic fatty liver disease in patients (El-Kamary et al. 2009; Loguercio et al. 2012).

Ursodeoxycholic acid, a hydrophilic stereoisomer of chenodeoxycholic acid, is a major component of Chinese black bear's bile (Ohtsuki et al., 1992). Ursodeoxycholic acid is used to treat chronic cholestatic liver diseases, such as primary biliary cirrhosis and primary sclerosing cholangitis (Chapman 2009; Lindor et al. 2009). Moreover, some evidences indicate that ursodeoxycholic acid decreases the levels of alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyl transpeptidase in patients with chronic hepatitis C and protects livers from against methotrexate-induced toxicity (Omata et al. 2007; Uraz et al. 2008).

Few reports have evaluated the genomic alterations elicited by glycyrrhizin, silymarin, and ursodeoxycholic acid. For examples, *Glycyrrhiza glabra* root extract induces the proliferation of MCF-7 cells by activating extracellular signal-regulated kinases 1/2 and Akt pathways (Dong et al., 2007). Treating hepatocytes with ursodeoxycholic acid shows that ursodeoxycholic acid affects the expression of genes directly involved in cell cycle and apoptotic events, and the E2F-1/p53/apoptotic protease activating factor-1 pathway seems to be the target of ursodeoxycholic acid (Castro et al. 2005). In this study, we treated hepatocytes with non-cytotoxic concentrations of glycyrrhizin, silymarin, and ursodeoxycholic acid, and analyzed the gene expression profiles by microarray. The gene expression profiles were further compared to evaluate the different and the common pathways regulated by these compounds.

Materials and methods

Cell culture

The human hepatoma cell line (HepG2) was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). Recombinant HepG2/NF-κB cell, which carried the NF-κB-driven luciferase genes, was constructed as described previously (Hsiang et al. 2009). HepG2 cells and HepG2/NF-κB cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), 100 μg/ml streptomycin, and 100 unit/ml penicillin in a humidified incubator at 37 °C with 5% CO_2.

Chemicals

Glycyrrhizin (purity $\geq$ 95%), silymarin, and ursodeoxycholic acid (purity $\geq$ 99%) were purchased from Sigma (St. Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 200 mM, and stored at −30 °C. Silymarin (product number 254924) is a mixture of toxifolin (4%), silichristin (27.9%), silidianin (2.9%), silybin A (19.3%), silybin B (31.3%), isosilybin A (8.2%), and isosilybin B (2.3%). MG-132, a NF-κB inhibitor, was purchased from Santa Cruz (Dallas, TX, USA) and dissolved in DMSO to a final concentration of 50 mM. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS) (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2).

Total RNA isolation

HepG2 cells (2×10^6 cells, passage number 38) were seeded in a 25-cm^2 flask and incubated at 37 °C for 24 h. Cells were then treated with 5 ml of culture medium containing 0.125‰ DMSO (solvent control) or μM hepatoprotectants, and incubated at 37 °C for another 24 h. Total RNA was extracted from cells treated with or without compounds by RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA was quantified using the spectrophotometer (Beckman Coulter, Fullerton, CA, USA) and further evaluated using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA sample with an RNA integrity number greater than 8.0 was accepted for microarray analysis.

Microarray analysis

Microarray analysis was performed as described previously (Lo et al. 2013; Ho et al. 2014). Briefly, fluorescent RNA targets were prepared from 5 μg of total RNA samples using the MessageAmp™ aRNA kit (Ambion, Austin, TX, USA) and Cy5 dye (Amersham Pharmacia, Piscataway, NJ, USA). Fluorescent targets were hybridized to the Human Whole Genome OneArray™ (Phalanx Biotech Group, Hsinchu, Taiwan). Number of replicates was three. After an overnight hybridization at 50 °C, non-specific targets were washed away and the array was scanned by an Axon 4000 scanner (Molecular Devices, Sunnyvale, CA, USA). Spots with a signal-to-noise ratio $>$ 0 or control probes were selected and normalized by the R program of the limma package (Smyth and Speed 2003). We used surrogate variable analysis (sva) to capture the heterogeneity of expression caused by any variation and to improve the accuracy and reproducibility in analyzing gene expression levels (Leek and Storey 2007). Normalized data were tested by a standard paired *t*-test. The *p*-values were then adjusted for a false discovery rate (FDR) (Benjamini and Hochberg 1995). A value of FDR $<$ 0.5 was considered statistically significant. The fold changes of genes were calculated by dividing the normalized signal intensities of genes in compound-treated cells by those in solvent-treated cells. Genes with fold changes $\geq$ 2.0 or $\leq$ −2.0 and FDR values $<$0.5 were selected as differentially expressed genes and further analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (www.genome.ad.jp/kegg/). We used the Web-based gene set analysis toolkit (bioinfo.vanderbilt.edu/webgestalt/) to test the enriched pathways. Microarray data are minimum information about microarray experiments compliant, and raw data have been deposited in the Gene Expression Omnibus (accession number: GSE67504).

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was reverse-transcribed for 2 h at 37 °C using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed by mixing cDNA, 2 × Power SYBR Green PCR Master kit and 200 nM of forward and reverse primers. The reaction condition was followed: 10 min at 95 °C; 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicates. Relative quantitation (RQ) was calculated using the comparative C_T method ($\Delta\Delta C_T$) which determines the change in expression of a nucleic acid sequence in a test sample (treated group) relative to the same sequence in a calibrator sample (mock group) (Livak and Schmittgen 2001). The ΔC_T value is determined by subtracting the average C_T value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene from the average C_T value of target gene. The $\Delta\Delta C_T$ value is determined by subtracting the ΔC_T value of mock group from the ΔC_T value of treated group. RQ is calculated as $2^{-\Delta\Delta CT}$. Fold changes were further presented as RQ if the RQ value was $\geq$1, or as −1/RQ if the RQ value is $<$1. The primer ser for each gene was followed: histone

deacetylase 9 (HDAC9) forward, 5′-GCAAGAGGAGACAGAGACCG-3′; HDAC9 reverse, 5′-ACTTGGCACTTCACAAGGCT-3′; minichromosome maintenance deficient 3 associated protein (MCM3AP) forward, 5′-CGCTTCCTCTGGTGGTTCTT-3′; MCM3AP reverse, 5′-CTGCACTGCTTGCAAAACCT-3′; GAPDH forward, 5′-TCACCCACACTGTGCCCATCTATGA-3′; GAPDH reverse, 5′-GAGGAAGAGGATGCGGCAGTGG-3′.

Gene interaction network analysis

We constructed three interaction networks of genes with fold changes ≥ 2.0 or ≤ −2.0 in each treatment group using Genomatix Applications software. Then, we merged genes and their interactions in either network into a union network and finally classified this network by GOlorize, a cytoscape plug-in (Garcia et al. 2007). GOlorize first highlighted the nodes that belonged to the same class using color-coding and then constructed an enhanced visualization of the network using a class-directed layout algorithm. Finally, we used Cell Region-Based Rendering and Layout (Cerebral), a cytoscape plug-in, to generate a pathway-like representation of a network. Cerebral uses subcellular localization annotation to create a layered view of a cell, placing nodes in the region corresponding to the appropriate localization (Barsky et al. 2007).

Cell viability assay

HepG2 cells (5×10^6 cells) were cultivated in a 96-well culture plate. After a 24-h incubation at 37 °C, various amounts of glycyrrhizin, silymarin, or ursodeoxycholic acid were added to confluent cell monolayer and incubated for another 24 h. MG-132 (μM), a NF-κB inhibitor, was added to HepG2/NF-κB cells to evaluate the cytotoxicity. Cell viability was monitored by the MTT colorimetric assay as described previously (Cheng et al. 2009). Cell viability (%) was calculated by the equation: (OD of compound-treated cells/OD of solvent-treated cells) × 100.

Luciferase assay

HepG2/NF-κB cells (5×10^6 cells) were cultured in a 96-well culture plate at 37 °C for 24 h, washed with DMEM, and treated with 25 μM of MG-132 or various amounts of glycyrrhizin, silymarin, or ursodeoxycholic acid for another 24 h. Cells were then washed with ice-cold PBS, lysed with 30 μl Triton lysis buffer (50 mM Tris–HCl, 1% Triton X-100, 1 mM dithiothreitol, pH 7.8), and collected with a cell scraper. Luciferase activity was measured as described previously (Cheng et al. 2009). Relative luciferase activity was calculated by dividing the relative luciferase unit (RLU) of compound-treated cells by the RLU of solvent-treated cells.

Statistical analysis

Data were presented as mean ± standard error. Data were analyzed by one-way ANOVA and post hoc Bonferroni test using SPSS Statistics version 20 (IBM, Armonk, NY, USA). A p value less than 0.05 was considered as statistically significant.

Results

Microarray analysis of hepatoprotectant-regulated gene expression

In this study, we chose HepG2 cell, hepatocytes derived from hepatocellular carcinoma, as a cell model to study the gene expression profiles of hepatoprotectants. Cytotoxic studies were first performed. Fig. 1 shows that glycyrrhizin, silymarin, and ursodeoxycholic acid displayed no visible cytotoxic effects at a broad range of concentration (0.5–500 μM). Previous studies indicated that silymarin at 25 μM achieves the highest hepatoprotective effect in FL83B mouse liver cells and at a concentration exceeding 25 μM abruptly increases cell damage in rat hepatocytes (Sainz-Pardo et al. 1994; Lo et al. 2014). In addition, up to 25 μM of glycyrrhizin dose not induce cell death in human epithelial ovarian carcinoma cells (Lee et al. 2010). Therefore, we treated HepG2 cells with 25 μM compounds to analyze novel or common biological pathways regulated by glycyrrhizin, silymarin, and ursodeoxycholic acid in this study.

Microarray data were analyzed by limma and sva packages to examine the differentially expressed genes in cells treated with various hepatoprotectants. We selected genes with fold changes ≥ 2.0 or ≤ −2.0 and FDR values <0.5 as differentially expressed genes for further analysis. Among a total of 30,968 genes, glycyrrhizin, silymarin, and ursodeoxycholic acid downregulated the expression of 777, 684, and 797 genes, respectively. In addition, the expression of 497, 604, and 710 genes was upregulated by glycyrrhizin, silymarin, and ursodeoxycholic acid, respectively. A Venn diagram was used to classify genes those were specific or common in the comparisons. Fig. 1 shows that glycyrrhizin and silymarin affected 145 genes, silymarin and ursodeoxycholic acid affected 196 genes, and glycyrrhizin and ursodeoxycholic acid affected 227 genes. Furthermore, a total of 252 genes was commonly affected by glycyrrhizin, silymarin, and ursodeoxycholic acid.

KEGG pathway analysis of differentially expressed genes altered by three hepatoprotectants

We further analyzed the enriched pathways altered by three hepatoprotectants. Table 1 shows that various KEGG pathways were significantly regulated by glycyrrhizin, silymarin, and ursodeoxycholic acid. No common KEGG pathway was affected by these hepatoprotectants. Glycyrrhizin regulated calcium signaling and T cell receptor signaling pathways, silymarin affected the insulin signaling pathway, and ursodeoxycholic acid affected neuroactive ligand–receptor interaction, tight junction, long-term depression, and extracellular matrix–receptor interaction. Expression levels of genes with p values <0.05 in these pathways are displayed in Table 2.

Interaction network of hepatoprotectant-regulated genes

The relationship between differentially expressed genes affected by each compound was further analyzed by Genomatix Applications software. Genes were correlated based on a review of published data, the Genomatix Knowledge Base, and promoter DNA sequence analysis. Three interaction networks, corresponding to three hepatoprotectants-regulated genes, were constructed. Then, we merged three gene interaction networks using Cytoscape. As shown in Fig. 2, NF-κB was the node in the gene interaction network. A total of 127 genes was affected by all three compounds and associated with NF-κB in this network. The expression profiles of 127 NF-κB-connected genes were then clustered by hierarchical clustering analysis. Fig. 3 shows that genes upregulated by one compound had tendencies of upregulation by other compounds, and vice versa. Among 127 genes, 12 genes with fold changes ≥ 2.0 or ≤ −2.0 were commonly regulated by glycyrrhizin, silymarin, and ursodeoxycholic acid. The gene expression levels of these genes are shown in Table 3. Furthermore, we used Cerebral to generate pathway-like representations of networks for glycyrrhizin, silymarin, and ursodeoxycholic acid treatments. As shown in Fig. 4, these hepatoprotectants affected NF-κB via regulating the same or distinct genes. For example, in the extracellular space, glycyrrhizin modulated NF-κB activity by regulating interleukin 4 (IL4), IL23A, and fibroblast growth factor 10 (FGF10) genes (Fig. 4A), silymarin modulated NF-κB by regulating IL6, IL9, and collagen type 2 alpha 1 (COL2A1) genes (Fig. 4B), and ursodeoxycholic acid affected NF-κB by regulating IL4, IL11, and FGF10 genes (Fig. 4C). In addition, all compounds affected NF-κB

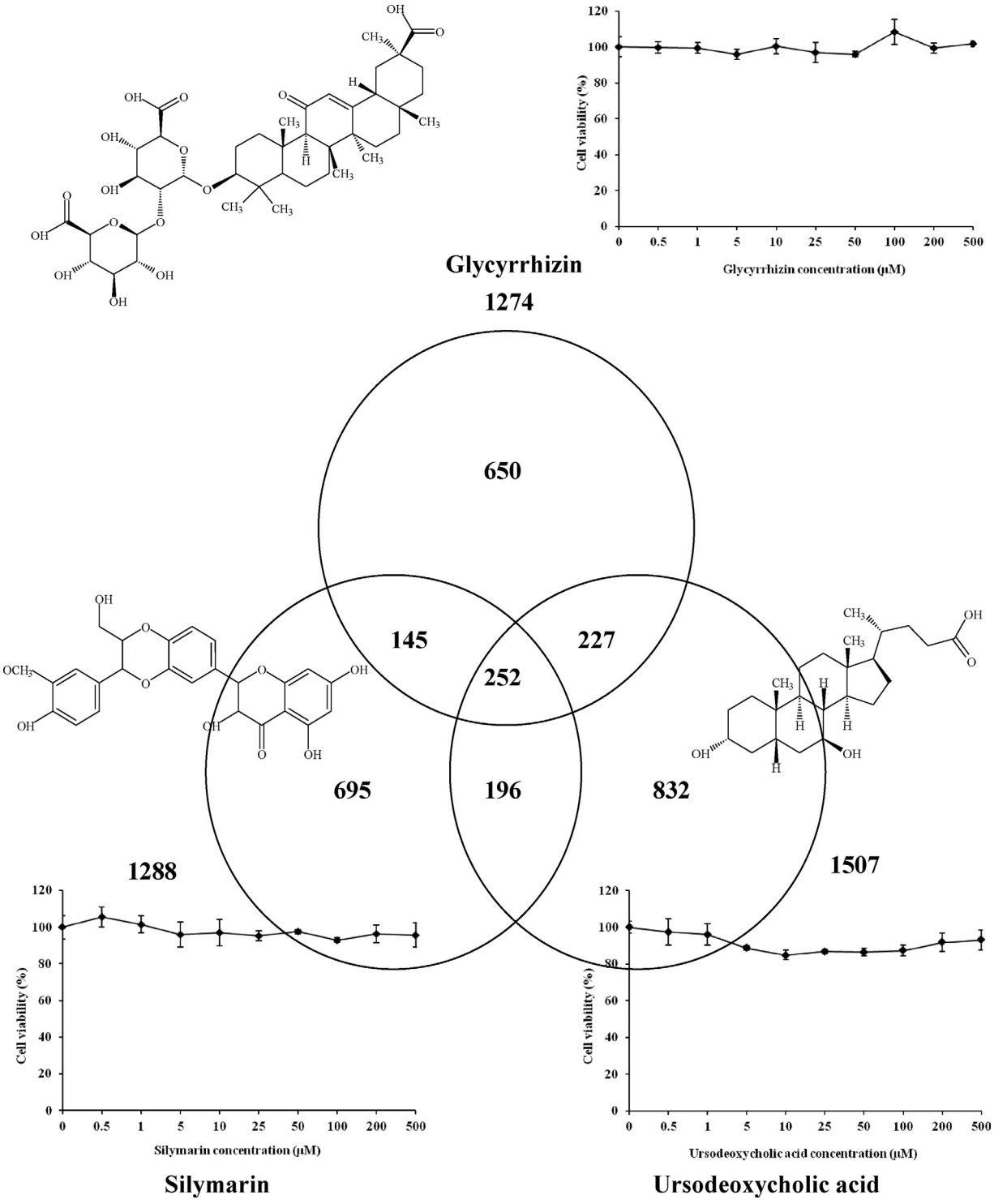

Fig. 1. Venn diagram of differentially expressed genes in HepG2 cells treated with 25 μM glycyrrhizin, silymarin, or ursodeoxycholic acid. The number of genes differentially regulated by glycyrrhizin, silymarin, or ursodeoxycholic acid is shown. Line graphs represent the cell viability of glycyrrhizin-, silymarin-, or ursodeoxycholic acid-treated cells. Values are mean ± standard error of three independent assays.

by regulating HDAC9, MCM3AP, Wilms' tumor 1 (WT1), and Spi-B transcription factor (SPIB) genes in the nucleus.

We further verified microarray data by qPCR. We quantified the expression levels of HDAC9 and MCM3AP genes because these genes are associated with hepatocarcinogenesis and are directly interacted with NF-κB (Wang et al. 2010; Ding et al. 2013). In comparison with mock, the expression of HDAC9 genes was downregulated by glycyrrhizin, silymarin, and ursodeoxycholic acid, with fold changes of −2.08, −11.11, and −3.23, respectively (Table 4). The expression levels of MCM3AP genes were also decreased by glycyrrhizin, silymarin, and ursodeoxycholic acid, with fold changes of −2.56, −1.75, and −2.17, respectively. These findings indicated that the expression of HDAC9 and MCM3AP genes was downregulated by these hepatoprotectants, which were consistent with the findings of microarray data.

Table 1
Classification of genes altered by glycyrrhizin, silymarin, or ursodeoxycholic acid in HepG2 cells by KEGG pathways.

Compound	Pathway	Observed (total)	*p*-value
Glycyrrhizin	Calcium signaling pathway	13 (176)	0.00098
	T cell receptor signaling pathway	8 (93)	0.00416
Silymarin	Insulin signaling pathway	10 (135)	0.00392
Ursodeoxycholic acid	Neuroactive ligand–receptor interaction	22 (254)	0.00037
	Tight junction	10 (136)	0.00496
	Long-term depression	8 (76)	0.00429
	ECM-receptor interaction	8 (87)	0.00679

Table 2
Expression levels of genes which belong to glycyrrhizin-, silymarin-, or ursodeoxycholic acid-altered KEGG pathways in HepG2 cells.

Compound	Gene name	Gene description	Change (folds)	*p*-value
Glycyrrhizin	Calcium signaling pathway			
	ADRA1A	Adrenoceptor alpha 1A	−4.00 ± 1.36	0.0264
	SLC25A4	Solute carrier family 25, member 4	−2.23 ± 0.54	0.0456
	CACNA1E	Calcium channel, voltage-dependent, alpha 1E subunit	2.87 ± 0.72	0.0248
	GRIN2A	Glutamate receptor, ionotropic, *N*-methyl D-aspartate 2A	2.64 ± 0.44	0.0101
	P2RX4	Purinergic receptor P2X, ligand-gated ion channel 4	−3.47 ± 1.25	0.0410
	PPID	Peptidylprolyl isomerase D	−2.22 ± 0.34	0.0144
	T cell receptor signaling pathway			
	CD3Z	CD3Z antigen, zeta polypeptide	−2.17 ± 0.48	0.0384
	IL4	Interleukin 4	2.10 ± 0.36	0.0229
	NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	−2.43 ± 0.20	0.0017
	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1	−2.36 ± 0.40	0.0151
Silymarin	Insulin signaling pathway			
	PDE3A	Phosphodiesterase 3A, cGMP-inhibited	−2.45 ± 0.34	0.0076
	PRKAB1	Protein kinase, AMP-activated, beta 1 non-catalytic subunit	−4.91 ± 0.56	0.0008
	RPS6	Ribosomal protein S6	2.33 ± 0.45	0.0225
	PRKAG3	Protein kinase, AMP-activated, gamma 3 non-catalytic subunit	−2.22 ± 0.32	0.0112
Ursodeoxycholic acid	Neuroactive ligand–receptor interaction			
	GLRA1	Glycine receptor, alpha 1	3.72 ± 0.75	0.0230
	LEPR	Leptin receptor	2.19 ± 0.30	0.0284
	Long-term depression			
	PPP2CB	Protein phosphatase 2, catalytic subunit, beta isoform	−2.75 ± 0.34	0.0147

Table 3
Expression levels of 12 common NF-κB-connected genes in HepG2 cells responsive to glycyrrhizin, silymarin, and ursodeoxycholic acid treatments.

Gene name	Gene description	Glycyrrhizin		Silymarin		Ursodeoxycholic acid	
		Change (folds)	FDR	Change (folds)	FDR	Change (folds)	FDR
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	−5.98 ± 3.61	0.06	−5.71 ± 3.21	0.05	−4.59 ± 1.83	0.06
HDAC9	Histone deacetylase 9	−3.93 ± 1.24	0.02	−4.92 ± 3.68	0.12	−3.81 ± 0.65	0.02
CFTR	Cystic fibrosis transmembrane conductance regulator	−3.64 ± 1.06	0.02	−2.43 ± 0.91	0.10	−7.41 ± 7.75	0.20
ETHE1	Ethylmalonic encephalopathy 1	−3.07 ± 0.83	0.03	−2.73 ± 0.87	0.05	−3.11 ± 1.47	0.14
MCM3AP	Minichromosome maintenance deficient 3 associated protein	−2.92 ± 1.56	0.14	−2.17 ± 1.02	0.20	−2.74 ± 1.85	0.27
SPIB	Spi-B transcription factor	−2.78 ± 0.88	0.05	−3.34 ± 2.65	0.23	−2.33 ± 0.58	0.08
WT1	Wilms' tumor 1	−2.43 ± 1.39	0.22	−2.09 ± 1.44	0.36	−2.10 ± 1.01	0.26
BID	BH3 interacting domain death agonist	−2.20 ± 1.48	0.33	−2.58 ± 2.52	0.40	−7.37 ± 5.26	0.11
C1QTNF3	C1q and tumor necrosis factor related protein 3	2.02 ± 0.75	0.15	2.74 ± 2.29	0.31	2.76 ± 1.86	0.27
SOD2	Superoxide dismutase 2	2.43 ± 1.27	0.19	3.01 ± 2.22	0.23	3.02 ± 2.46	0.31
SFRS1	Splicing factor, arginine/serine-rich 1	2.97 ± 2.36	0.26	2.38 ± 2.59	0.48	3.50 ± 2.68	0.24
UBE2N	Ubiquitin-conjugating enzyme E2N	4.24 ± 1.79	0.04	2.55 ± 1.23	0.15	4.71 ± 0.98	0.02

Effects of hepatoprotectants on NF-κB activities in HepG2 cells

Because NF-κB may play a central role in the glycyrrhizin-, silymarin- and ursodeoxycholic acid-regulated gene network, we wondered whether these compounds were able to affect NF-κB activities. HepG2/NF-κB cells were then treated with various amounts of glycyrrhizin, silymarin, or ursodeoxycholic acid, and the luciferase activity was determined 24 h later. As shown in Fig. 5, MG-132, a well-known proteasome inhibitor that inhibits NF-κB activity by inhibiting IκB degradation, suppressed significantly the NF-κB activity in HepG2 cells. Glycyrrhizin, silymarin, and ursodeoxycholic acid suppressed NF-κB activities in a dose-dependent manner. The EC_{50} value of silymarin on the inhibition of NF-κB activity was 64 ± 9.17 μM, while EC_{50} values of glycyrrhizin and ursodeoxycholic acid were >500 μM. No visible cytotoxic effects were observed. These findings indicate that glycyrrhizin, silymarin, and ursodeoxycholic acid inhibited NF-κB activities in HepG2 cells.

Discussion

In this study, we investigated the gene expression profiles of HepG2 cells in response to glycyrrhizin, silymarin, and ursodeoxycholic acid treatments. Differentially expressed genes were categorized according to KEGG pathways, and we found that hepatoprotectant-regulated genes were associated with various biological pathways. For example, adrenoceptor alpha 1A, glutamate receptor ionotropic *N*-methyl D-aspartate 2A, and purinergic receptor P2X ligand-gated ion channel 4 in calcium signaling pathway,

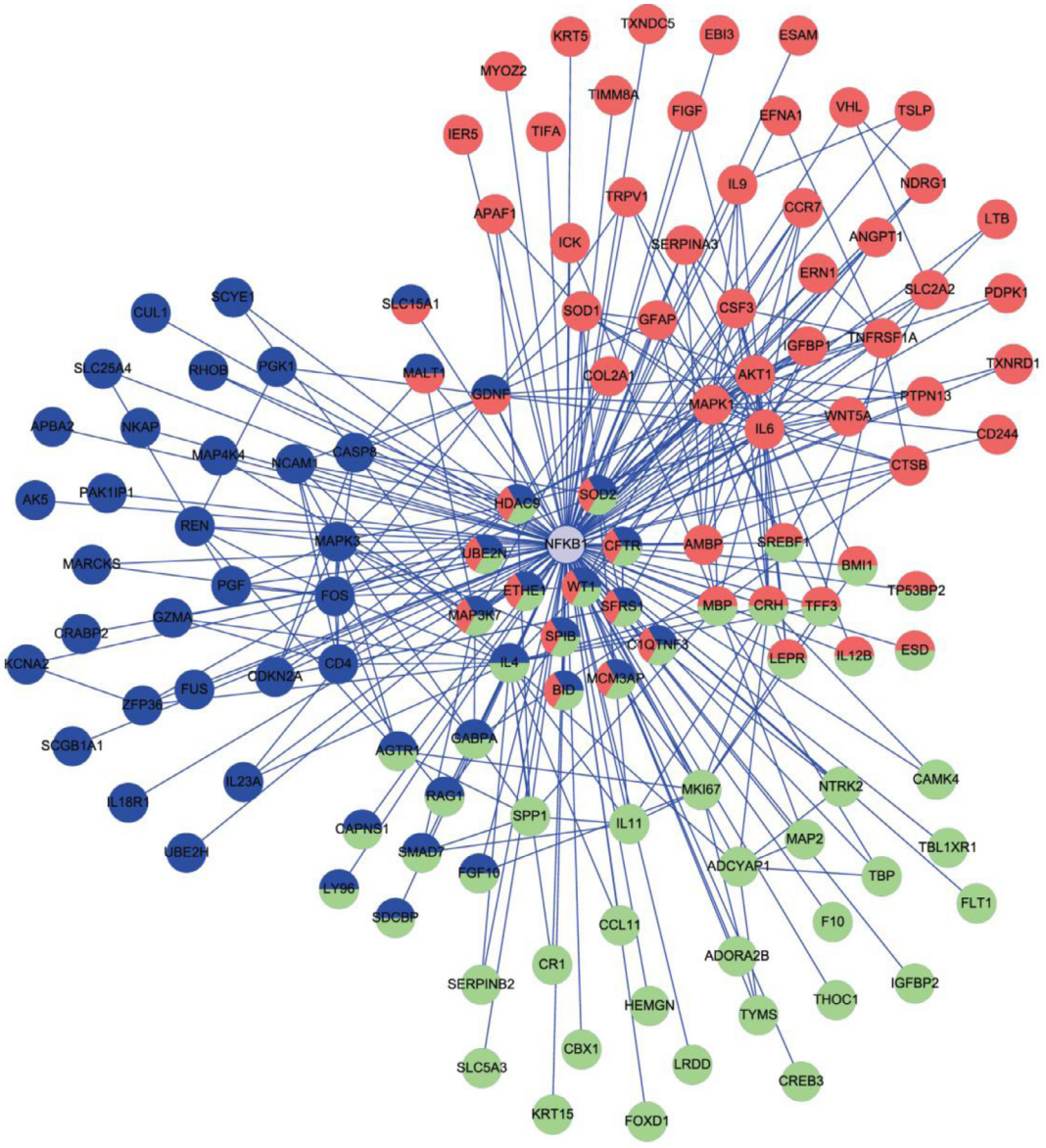

Fig. 2. Network analysis of glycyrrhizin-, silymarin-, and ursodeoxycholic acid-regulated genes in HepG2 cells. Differentially expressed genes, which were affected by either compound, were analyzed by BiblioSphere Pathway Edition software. Then, the network was visualized and classified by cytoscape software. Glycyrrhizin-, silymarin-, and ursodeoxycholic acid-regulated genes are colored in blue, red, and green, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Expression levels of HDAC9 and MCM3AP genes in glycyrrhizin-, silymarin-, and ursodeoxycholic acid-treated HepG2 cells by qPCR.

Gene	Sample	Avg C_T	Average C_T of GAPDH	ΔC_T[a]	$\Delta\Delta C_T$[b]	Change (folds)[c]
HDAC9	Glycyrrhizin	26.47 ± 0.06	30.04 ± 0.01	−3.41 ± 0.17	1.05 ± 0.17	−2.08
	Silymarin	37.07 ± 1.07	30.13 ± 0.03	6.94 ± 1.07	3.48 ± 1.07	−11.11
	Ursodeoxycholic acid	27.37 ± 0.13	30.13 ± 0.03	−2.76 ± 0.13	1.70 ± 0.13	−3.23
MCM3AP	Glycyrrhizin	33.89 ± 1.06	30.04 ± 0.01	4.00 ± 1.07	1.36 ± 1.07	−2.56
	Silymarin	33.52 ± 1.19	30.07 ± 0.01	3.44 ± 1.19	0.81 ± 1.19	−1.75
	Ursodeoxycholic acid	33.90 ± 1.06	30.13 ± 0.03	3.77 ± 1.06	1.13 ± 1.06	−2.17

[a] The ΔC_T value is determined by subtracting the average C_T value of GAPDH gene from the average C_T value of target gene. The standard deviation of the difference is calculated from the standard deviations of the target gene and GAPDH gene.

[b] The $\Delta\Delta C_T$ value is determined by subtracting the ΔC_T value of mock group from the ΔC_T value of treated group. This is a subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta$CT is the same as the standard deviation of ΔC_T value.

[c] RQ is calculated as $2^{-\Delta\Delta CT}$. Fold changes were presented as RQ if the RQ value was ≥1, and as −1/RQ if the RQ value is <1.

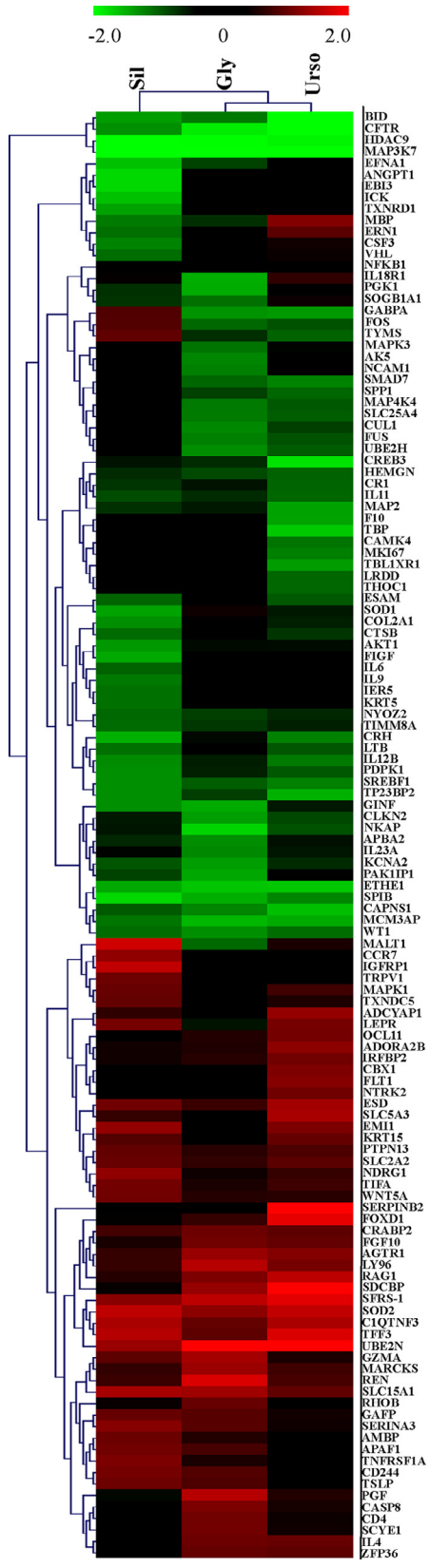

Fig. 3. Hierarchical clustering analysis of 127 NF-κB-connected genes affected by either glycyrrhizin (Gly), silymarin (Sil), or ursodeoxycholic acid (Urso). Normalized $\log_2$ expression values are color-coded according to the legend on the top. Increased levels are colored red and decreased levels are colored green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and glycine receptor alpha 1 and leptin receptor in neuroactive ligand–receptor interaction mediate actions in the nervous system through the binding of different neurotransmitters (Betz et al. 1999; Koshimizu et al. 2003; Vial et al. 2004; Ryan et al. 2008). Neuroblastoma RAS viral oncogene homolog and phosphoinositide-3-kinase regulatory subunit 1 in T cell receptor signaling pathway are also involved in insulin signaling pathway (Saltiel and Kahn 2001). Protein kinase AMP-activated beta 1 (PRKAB1) and PRKAG3 are known to be associated with lipogenesis (Bailey 2007). Based on these results, we suggested that these hepatoprotectants might regulate pathways involved in neurotransmission, and glucose and lipid metabolism in liver.

We further investigated differentially expressed genes by gene interaction network analysis. We found that NF-κB seemed to be in the central part of the network and 127 genes were connected directly to NF-κB. Furthermore, we found that 12 NF-κB-connected genes were differentially expressed in all three compound treatments. Among them, HDAC9, MCM3AP, SPIB, WT1, ethylmalonic encephalopathy 1 (ETHE1), BH3 interacting domain death agonist (BID), superoxide dismutase 2 (SOD2), and mitogen-activated protein kinase kinase kinase 7 (MAP3K7) genes are known to be involved in hepatocarcinogenesis, apoptosis, and anti-oxidative pathways, and their expressions are directly regulated by NF-κB. For examples, HDAC9 is a transcriptional regulator of the histone deacetylase family, which is regulated by Sp1 and NF-κB (Ma et al. 2011). Recent study indicated that HDAC9 promotes hepatocellular carcinoma progression by inhibiting p53 transcriptional activity (Ding et al. 2013). MCM3AP is an acetyltransferase that acetylases MCM3, and plays a role in DNA replication (Takei et al. 2001). It is also a novel hepatitis B virus integration site, and upregulation of MCM3AP promotes the hepatocarcinogenesis by affecting flanking sequences (Wang et al. 2010). SPIB is a transcription factor that binds to a purine-rich sequence of promoters (Bonadies et al. 2010). Using a random-walk-based community detection algorithm, Petrochilos et al. (2013) have identified that SPIB is functionally related to cancer and shows a promise as a therapeutic target. WT1 encodes a transcription factor involved in cell growth and development. The presence of NF-κB-responsive elements in the promoter of WT1 gene suggests that the transcription of WT1 gene is regulated by NF-κB pathway (Dehbi et al. 1998). WT1 is expressed in a substantial proportion of hepatocellular carcinoma contributing to tumor progression and resistance to chemotherapy, suggesting that WT1 may be an important target for liver cancer treatment (Perugorria et al. 2009). ETHE1 gene accelerates the export of NF-κB from nucleus and inhibits p53-dependent apoptosis, thus contributing to hepatocarcinogenesis (Higashitsuji et al. 2002). In our study, all three compounds reduced the expressions of HDAC9, MCM3AP, SPIB, WT1, and ETHE1 genes at non-toxic concentrations in hepatocytes, suggesting the anticancer potentials of these compounds.

In addition to ETHE1, BID gene product is a specific proximal substrate of caspase-8 in the Fas apoptotic signaling pathway (Li et al. 1998). While full-length BID is localized in cytosol, truncated BID (tBID) translocates to mitochondria and thus transduces apoptotic signals from cytoplasmic membrane to mitochondria. The SOD2 gene encodes an intramitochondrial free radical scavenging enzyme, the first line of defense against superoxide produced by oxidative phosphorylation. SOD2 maintains the integrity of mitochondrial enzymes that are inactivated by superoxide (Li et al. 1995). SOD2 and tBID are known to be associated with apoptosis via a mitochondrial pathway (Yin 2000; Pardo et al. 2006). The ability of glycyrrhizin, silymarin, and ursodeoxycholic acid to reduce BID or induce SOD2 may protect HepG2 cells from damage by suppressing apoptosis via the mitochondrial pathway.

MAP3K7 is linked to IL1 receptor and tumor necrosis factor (TNF) receptor signalings (Sato et al. 2005). MAP3K7 is activated by

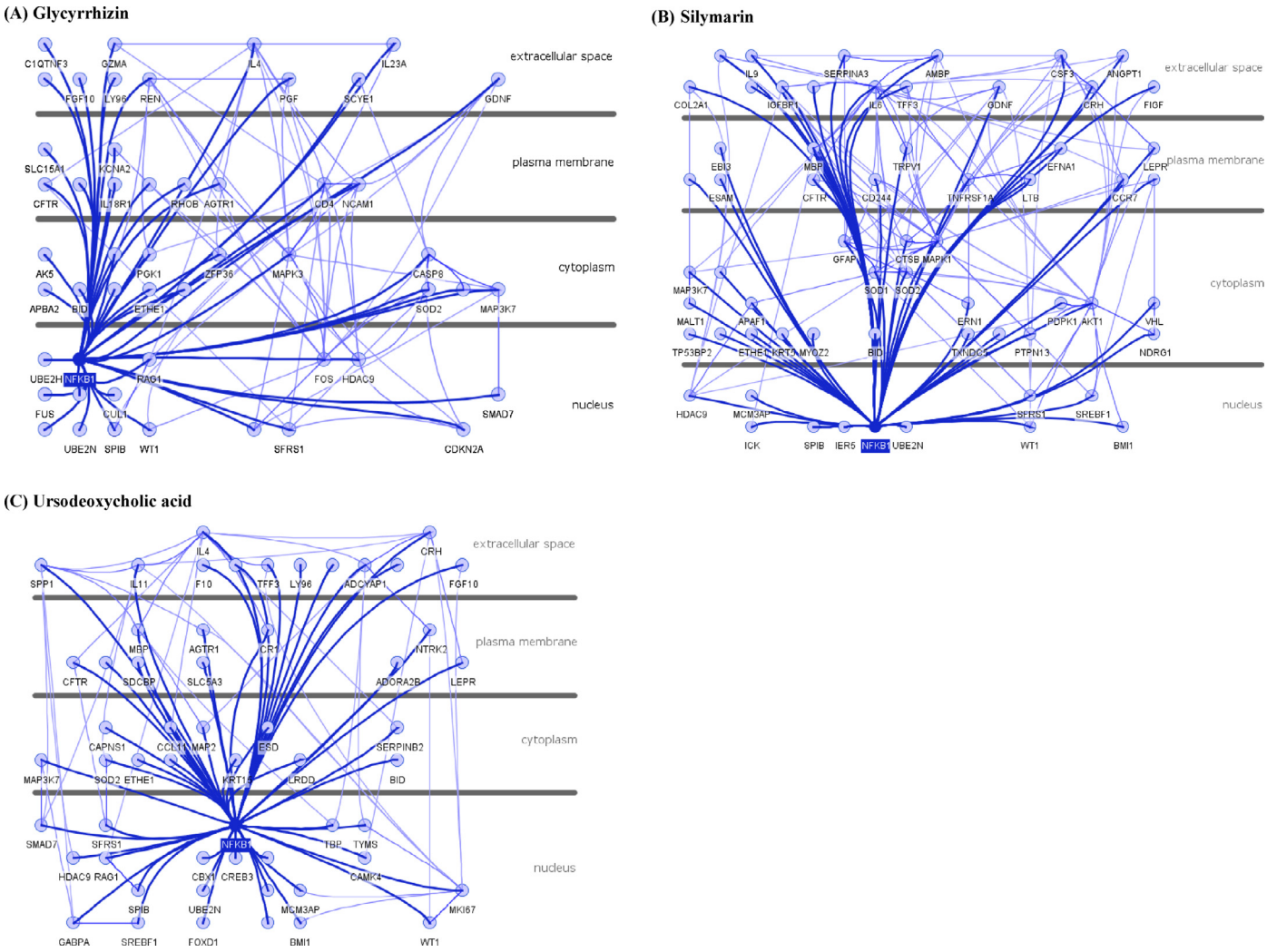

Fig. 4. Cerebral layouts generated by cytoscape plug-in illustrate the pathway-like representations of networks for glycyrrhizin (A), silymarin (B), and ursodeoxycholic acid (C) treatments in HepG2 cells.

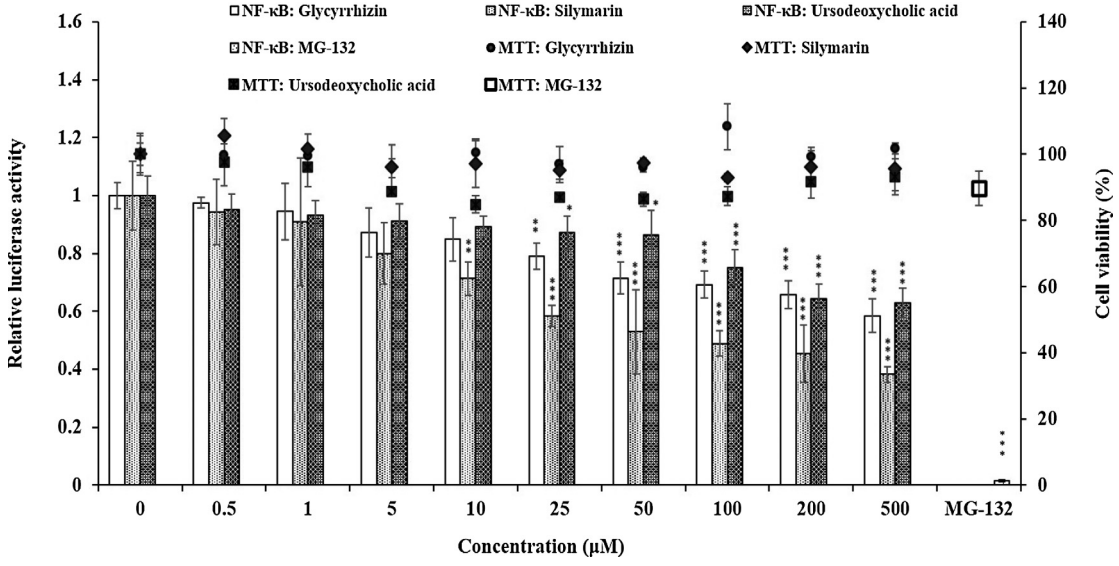

Fig. 5. Inhibitory effects of glycyrrhizin, silymarin, and ursodeoxycholic acid on NF-κB activities in HepG2 cells. HepG2/ NF-κB cells were seeded into 96-well plates and treated with 25 μM of MG-132 or various amounts of glycyrrhizin, silymarin and ursodeoxycholic acid. Luciferase activity and cell viability were determined at 24 h. The bars represent the relative luciferase activity, which is presented as comparison with the RLU relative to solvent-treated cells. The spots represent the cell viability during treatment. Values are mean ± standard error of three independent assays. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with mock.

chemical and physical stresses and regulates stress-induced activation of activator protein-1 and NF-κB (Ishitani et al. 2003; Takaesu et al. 2003; Shim et al. 2005). Hypoxia-activated MAP3K7 results in the activation of c-Jun-dependent transcription in response to oxidative stress (Blanco et al. 2007). In addition, BID is also able to induce the generation of reactive oxygen species (ROS) following death receptor activation (Ding et al. 2004). However, SOD2 plays a critical cytoprotective role against oxidative stress (Delhalle et al. 2002). In this study, the up-regulation of SOD2 and the down-regulation of BID and TAK1 by glycyrrhizin, silymarin, and ursodeoxycholic acid suggested the anti-oxidative properties of these compounds.

Hepatocytes exposed to BID siRNA are resistant to Fas and TNF-α-induced cell death (Yin et al. 1999; Guicciardi et al. 2005). Treatment of lymph-proliferative mice with BID antisense also ameliorates liver injury following bile duct ligation (Higuchi et al. 2001). Additionally, overexpression of SOD2 is effective against mitochondrial oxidative stress in hepatocytes and is protective against alcohol-induced liver injury in an enteral feeding rat model (Wheeler et al. 2001). The up-regulation of SOD2 in hepatocytes is associated with a reduction of ROS-induced injury after ischemia-reperfusion (Chen et al. 2006). Dominant-negative MAP3K7 induces G0 exit in the quiescent liver and accelerates hepatic cell cycle progression during regeneration (Bradham et al. 2001). Moreover, the inhibition of MAP3K7/c-Jun N-terminal kinase decreases the proliferation of hepatic stellate cells, a critical effector in hepatic fibrogenesis (Schnabl et al. 2001). Therefore, the differential expression of BID, SOD2, and TAK1 genes by glycyrrhizin, silymarin, and ursodeoxycholic acid suggested the hepatoprotective effects in livers.

NF-κB signaling pathways have a central function in the regulation of inflammation–fibrosis–cancer axis. NF-κB activation is crucially involved both in the fibrogenesis and in the initiation and promotion of liver cancers (Sun and Karin 2008; Luedde and Schwabe 2011). However, the dual role of NF-κB activation in hepatocarcinogenesis indicates that activation of NF-κB not only displays beneficial effects but also suppresses the viability of hepatocytes. For examples, in the early stages of tumorigenesis, NF-κB activation prevents the death of hepatocytes and thus avoids the release of proinflammatory cytokines by necrotic hepatocytes. However, in the late stages of tumorigenesis, NF-κB activation promotes the survival of transformed hepatocytes and elicits the malignancy of liver cancers (Sun and Karin 2008; Luedde and Schwabe 2011). Glycyrrhizin, silymarin, and ursodeoxycholic acid inhibited NF-κB activities in HepG2 cells, a human hepatoblastoma cell line, suggesting that glycyrrhizin, silymarin, and ursodeoxycholic acid might exhibit hepatoprotective effects by altering the survival of hepatoblastoma cells. Moreover, this finding was also consistent with previous reports that indicate glycyrrhizin, silymarin, and ursodeoxycholic acid play important roles in the treatment of hepatocellular carcinoma by reducing tumor cell proliferation or reducing the angiogenesis of liver (Kuiper et al. 2010; Li et al. 2014; Mastron et al. 2015).

In conclusions, this study demonstrated that different hepatoprotectants regulated different biological pathways. However, these hepatoprotectants also regulated the expression of common genes through a central molecule, NF-κB. Moreover, non-toxic dosages of hepatoprotectants inhibited NF-κB activities. Because the genes regulated by hepatoprotectants are relevant to antioxidant or anti-apoptotic pathways, we proposed that these compounds exerted their hepatoprotective effects by regulating apoptosis and oxidative stress in hepatocarcinoma cells.

Conflict of interest

There was no conflict of interest.

Acknowledgments

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Synergistic effect of cadmium chloride and acetaldehyde on cytotoxicity and its prevention by quercetin and glycyrrhizin

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Abstract

Cadmium chloride at concentrations of 10–50 mM and acetaldehyde (AA) at 1–5 mM showed synergistic toxic effects on V79 cells in vitro. Furthermore, synergistic effects of these chemicals were also observed in mutagenicities of the *Hprt* gene within certain dose ranges (cadmium chloride 5–10 mM, and AA 1–2.5 mM). Moreover, lipid peroxide formation, malondialdehyde (MDA) formation, detected by 2-thiobarbituric acid (TBA) reaction and the mitochondrial membrane potentials detected by rhodamine 123 uptake were significantly increased with the combined effect of cadmium and AA in V79. Thus, the cytotoxicity and genotoxicity displayed by combination of these chemicals can be considered to be associated with oxidative stress. Further, these effects were efficiently reduced by quercetin and less efficiently with glycyrrhizin.

Keywords: Quercetin; Glycyrrhizin; Cadmium; *Hprt*; Genotoxicity

1. Introduction

Cadmium, one of the most potent hazard inorganic substances in our environment, has been found to have severe toxic and genotoxic effect in animal both in vivo and in vitro system, and even in human as well [1–8]. In addition, human exposure to acetaldehyde (AA) is widespread and occurs regularly. The principal routes of direct exposure to AA are gastrointestinal and inhalation. AA exposure also exists endogenously as a primary metabolite of ethanol oxidation, and thus, alcohol consumption is an additional risk factor in conjunction with exogenous sources of environmental and occupational exposures to AA [9]. AA has been proposed that the mutagenic, carcinogenic and teratogenic effect of ethanol be mediated via this metabolite [10]. Thus, humans are exposed to various environmental hazardous substances, the effect of the toxic chemical in human lymphocytes, such as cytotoxicity and genotoxicity, has currently been concerned [11–15]. Oxidative stress is one of the main causes of cytotoxicity or genotoxicity. Thus, the oxidative stress is considered to be associated with many diseases, for example aging, cardiovascular disease, and cancer [16–18]. An important etiological mechanism of these diseases may be a causal relationship between the presence of oxidants and the generation of lipid hydroperoxides. These hydroperoxides can decompose to alkoxy-($RO^{\bullet}$) and peroxy-($ROO^{\bullet}$) free radicals that can oxidize other cell components,

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resulting in changes in enzyme activity or the generation of mediators (i.e. malondialdehyde (MDA) and reactive oxygen species (ROS)) that can cause a mitochondrial depolarization or further cell damage [19]. Therefore, the formation of MDA or uptake of rhodamine 123 is considered as an index of lipid peroxidation that bring cells injury.

On the other hand, amongst the most common of Asiatic folk medicine glycyrrhizin is acted as an anti-inflammatory agent on neutrophil functions including ROS generation [20]. Thus, glycyrrhizin can be considered as a quenching agent of free radicals and a blocking agent of lipid peroxidation chain reactions. Furthermore, the average daily intake of major flavonols, such as quercetin, myricetin and kaempherol, is in an amount of around 16 mg/day [21]. Quercetin is a highly effective antioxidant that has been shown to inhibit free radical mediated damage of low-density lipoproteins in vitro [22,23]. Moreover, the abilities of quercetin to scavenge free radicals and block lipid peroxidation were tested by Formica et al. against cardiovascular disease [24,25].

In the present study, we report herein that the combined effect of cadmium and AA on cytotoxicity or genotoxicity was studied in V79 cells. In addition we examined effect of these chemicals on the formation of lipid peroxides by detecting by 2-thiobarbituric acid (TBA) reaction, and on the cell injuries due to lipid peroxidations by measuring rhodamine 123 uptake. Further, we examined effects of natural antioxidants, quercetin and an Asiatic folk medicine, glycyrrhizin by co-exposure with these toxic agents on various biological markers for toxicities in V79.

2. Material and methods

2.1. Chemicals

Quercetin, glycyrrhizin, $CdCl_2$, MEM, penicillin–streptomycin, trypsin–EDTA, 6-thioguanine (6-TG), dulbeccols phosphate buffered saline (PBS), EMS, TBA, MTT were purchased from SIGMA company (St. Louis, USA). Chinese hamster lung cells were obtained from Professor Ruey-Hseng Lin, Chung Shan Medical and Dental College at Taichung in Taiwan. AA and solvents (Merck, Darmstadt, Germany) and other reagents or dishes (Nunc, Denmark) were obtained from the indicated suppliers.

2.2. Cell growth

V79 cells from lung fibroblasts of Chinese hamster were grown on a monolayer in using MEM medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 75 cm^2 flask. Under the incubation condition with 5% CO_2 in humidified air at 37°C, the cells have a generation time of about 12 h.

2.3. Microculture tetrazolium (MTT) assay

V79 cells in triplicate (three wells) were plated into a series of 24-well culture plates (1 ml culture/well) at a cell density of 5×10^4 cells/ml. Following a 18 h incubation at 37°C, 5% CO_2, 100% relative humidity, MEM medium was removed and washed with PBS. A volume of 1 ml culture medium containing drug was dispensed within appropriate wells.

According to Alley et al. method with a MTT assay [26], the combined effect of cadmium and AA on the growth of V79 cells was performed. Cell cultures were incubated at 37°C in 5% CO_2 humidified atmosphere for 2–18 h. Cell line growth and growth inhibition were measured at 563 nm with a scanning spectrophotometer (Shimadzu UV-260). Briefly, the V79 cells at a density of 5×10^4 cells/dish were treated with cadmium and/or AA at various concentrations. After a 2–18 h incubation, medium was replaced by the one containing 100 μl MTT (5 mg/ml) with PBS washing in between. The cells were incubated for another 4 h, then the blue crystals that are the metabolized product of MTT were extracted by isopropanol. Absorbence at 563 nm was determined and used for the measurement of the proportion of surviving cells.

2.4. Colony efficiency assay

V79 cells were seeded at 200 cells/flask (25 cm^2). After a 18 h incubation, the medium was removed and washed twice with PBS solution. Cells were exposed to the indicated doses of drug for 4 h. Subsequently, fresh drug-free culture medium was added and after 7 days incubation, the cultures were fixed and stained. The mean colony-forming efficiency was determined

from triplicate flasks and based on colonies each containing at least 50 cells.

2.5. Mutants in the Hprt gene

The mutagenicity of drugs was determined by the replating method [27]. V79 cells were incubated in MEM medium containing hypoxanthinine, aminopterin, and thymidine (HAT). After 3 days, the cells were replated at 1×10^6 cells/75 cm^2 flask and incubated for 18 h. Then the medium were removed and washed twice with PBS. Fresh MEM culture medium, culture medium containing drug was added and incubated for 4 h at 37°C and 5% CO_2 in comparison with treated methanesulfonic acid ethyl ester (EMS) as the positive control. Subsequently, drug culture medium was removed and washed twice with PBS, and then the cells were dissociated by treatment with trypsin–EDTA. A total of 2×10^5 cells were replated each at 9 cm^2 dish in five plates with MEM medium for 3 days. Subcultures at 2×10^5 cells/9 cm^2 dish for 4 days, and 2×10^5 cells were replated again each at 9 cm^2 dish in five plates in medium containing 10 mg/ml 6-TG. Following 7 or 8 days incubation, the 6-TG-resistant mutant colonies formed were fixed, stained, and counted.

On the other experiment, cell survival immediately post mutagen treatment was independently determined after 7 days incubation (200 cells/50 mm dish) in MEM without selective agents. Colonies were counted by mutant frequencies estimated.

2.6. Lipid peroxidation assay

Following Yagi's method, the lipid peroxide level was determined by fluorometric measurement after reaction with TBA [28]. The level is expressed in term of malondialdehe (MDA) as standard.

The scraped cells were resuspended in 50 mM phosphate buffer, and divided into two parts. One part was used for the estimation of lipid peroxidation by MDA–TBA formation. The cells were added and mixed with 3% sodium dodecyl sulfate (SDS), 0.1 N HCl, 10% phosphotungastic acid, and 0.7% TBA, and the reaction mixture was heated at 95°C in dark for 10 min. After cooling, *n*-butanol was added and the mixture was shaken vigorously and centrifuged at 3000 rpm for 10 min. The supernatant was determined with fluorometer (Ex/Em = 515/555 nm) in comparison to 1,1,3,3-tetramethoxypropane standards in an identical buffer. The other part was centrifuged at 10 000*g* for 15 min, the protein free lysate was obtained. Proteins were measured with a commercial kit (Bio-Rad) using bovine serum albumin as the standard.

2.7. Mitochondrial membrane potential

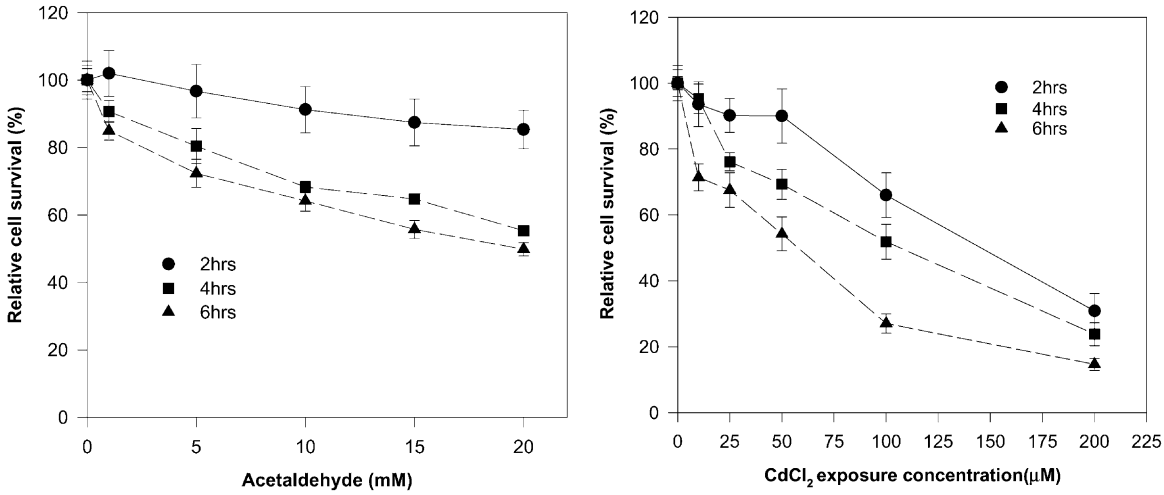
According to Lemasters and co-workers method, changes in mitochondrial membrane potential were evaluated from uptake of rhodamine 123 [29]. V79 cells (1×10^6 cells/25 cm^2 flask) were incubated with 1 μM rhodamine 123 at 37°C. Following 30 min incubation, the various concentration of drug was dispensed within appropriate flasks. After 4 h time, the MEM medium was removed and washed with PBS buffer. The cells (1×10^6) were suspended in PBS containing 0.1% Triton X-100. Standing 10 min, the samples were centrifuged (6000*g*), and rhodamine 123 concentration in the supernatant was determined with a Sequoia–Turner fluorometer at excitation and emission wavelengths of 490 and 515 nm in comparison to rhodamine 123 standards in an identical buffer.

2.8. Statistical analysis

The results are reported as means ± S.D. from individual determinations. Statistical differences were analyzed according to Student's *t*-test; significant differences were established at $P < 0.05$.

3. Results

Effects of cadmium and AA on cell survivals are shown in Fig. 1a and b. Treatment with 10–200 μM cadmium and 1–20 mM AA alone reduced survivals below the control ($P < 0.05$). For the combined effect of cytotoxicity between cadmium and AA, we examined on the co-exposure to V79 cells at the dosage of the concentration 10, 25, 50 μM cadmium and 1, 5 mM AA because both retain cell viability above 50% compared to the control cell, respectively. The results exhibited synergistic cytotoxicity of co-exposure of cadmium and AA on V79 cells for treatment in 4 h



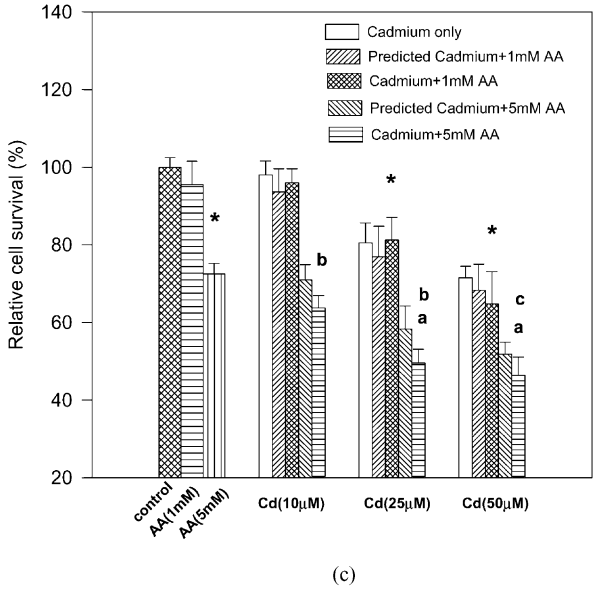


Fig. 1. (a) Effect of AA on survivals of V79 cells. The survivals were determined by MTT assay at 2, 4, and 6 h after addition of AA, in quadruplicate. (b) Effect of cadmium on survivals of V79 cells. The survivals were determined by MTT assay at 2, 4, and 6 h after addition of cadmium, in quadruplicate. (c) Effect of cadmium (Cd) and AA (acetaldehyde) on survivals of V79 cells. The survivals were determined by MTT assay at 4 h after addition of Cd and AA, in quadruplicate. (∗): compared with control ($P < 0.05$); (a): compared with AA ($P < 0.01$); (b, c): compared with Cd (b: $P < 0.01$, c: $P < 0.05$).

interval using MTT assay (Fig. 1c). The cell growth of V79 cells was inhibited by 36, 51, and 56% observed mortality at the concentration 10, 25, 50 μM cadmium and 5 mM AA, respectively.

After our evaluation of the acute toxicity, we focused on the chronic toxicity of co-exposure of cadmium and AA by colony assay. Similarly, we first assessed the cytotoxicities of cadmium and AA on

Table 1
Effect of cadmium ($CdCl_2$) and acetaldehyde (AA) on survivals of V79 cells by colony assay, in triplicate

Dose of $CdCl_2$ (μM)	Relative cell survival (%)			
	V79 cells	V79 cells (add 1 mM AA)	V79 cells (add 2.5 mM AA)	V79 cells (add 5 mM AA)
0	100.0 ± 2.0	96.7 ± 8.9	74.4 ± 2.5**	19.23 ± 2.0**
5	100.0 ± 3.8	96.9 ± 5.4	68.5 ± 6.1[a,b]	–[c]
10	94.9 ± 3.9*	77.4 ± 4.9[a,b]	36.2 ± 2.0[a,b]	–[c]
15	81.8 ± 3.1**	46.4 ± 6.9[a,b]	11.5 ± 3.5[a,b]	–[c]
20	60.0 ± 3.5**	–[c]	–[c]	–[c]

[a] Compared with AA ($P < 0.01$).
[b] Compared with Cd ($P < 0.01$).
[c] No detection.
* $P < 0.05$.
** $P < 0.01$ (compared with control).

V79 cells for treatment in 4 h interval, and subsequently allowed damage cells growing for 7 days to form colony. The results were shown on the Table 1, however, the mean percentage survival ($P < 0.01$) of cells exposed to 5 mM AA was 19% compared with control. Thus, of the substances and concentration we tested on the co-exposure to V79 cell at the concentration 5, 10, 15 μM cadmium and 1, 2.5 mM AA, respectively. The result, based upon a model of independent joint action [30], was shown on the Table 2. Obviously, it showed that the percentage of observed mortality is twice time more than that of predicted mortality except 5 μM cadmium. For example, treatment with both 10, 15 μM cadmium and 1, 2.5 mM AA was exhibited by 23, 54, 64, and 89% observed mortality, respectively. Thus, it indicated that the combined chronic cytotoxicity of cadmium and AA be synergistic effect on V79 cells by colony assay.

We further studied the mutagenic with combination effects of cadmium and AA on V79 cells, and compared the effects of cadmium alone and in combination with AA in a subtoxic or low mutagenic concentration. As shown in Table 3, it observed mutagenesis assay at *Hprt* gene was similar result to cytotoxicities. It showed that mutant at dose 5–15 μM cadmium and 2.5 mM AA was counted by plating efficiency frequency with 34, 7, 3%, respectively, in contrast to positive control EMS (93%). On the other hand, 11, 18, and 14 clones (EMS 173) were determined mutant cells by colony treated 6-TG. The results also assessed that observed mutant in comparison with predicted mutant is synergistic interaction of cadmium and AA on V79 cells by colony assay (Table 4).

Accordingly, Yagi's method [28] lipid peroxidation can be assayed by determining the rate of production of TBA-reactive components, which can be expressed

Table 2
Comparison of observed and predicted mortality induced by AA and cadmium on V79 cells by colony assay[a]

Dose	Observed relative mortality (%)	Predicted relative mortality (%)	Interaction
1 mM AA + 5 μM $CdCl_2$	3.1 ± ND	3.3 ± ND	+
1 mM AA + 10 μM $CdCl_2$	22.6 ± 4.9	8.2 ± ND	>+
1 mM AA + 15 μM $CdCl_2$	53.6 ± 6.9	20.9 ± 9.6	>+
2.5 mM AA + 5 μM $CdCl_2$	31.5 ± 6.1	25.6 ± 4.5	+
2.5 mM AA + 10 μM $CdCl_2$	63.8 ± 2.0	29.4 ± 4.7	>+
2.5 mM AA + 15 μM $CdCl_2$	88.5 ± 3.5	39.1 ± 4.1	>+

[a] Independent joint action model: $P(M) = P(A) + P(B) - P(AB)$. ND: non-detectable response; (+): additive; (>+): synergistic; AA: acetaldehyde; $P < 0.05$.

Table 3
Effect of cadmium and AA on *Hprt* mutant frequencies of V79 cells in V79 cells by colony assay, each dose has been performed in 10 experiments for collecting enough mutant cells[a]

Dose	Plating efficiency (%)	Mutant cells (numbers/10^6 cells)
Control	100.0 ± 3.9	0
5 μM $CdCl_2$	84.4 ± 4.7	0
10 μM $CdCl_2$	46.8 ± 5.4**	4
15 μM $CdCl_2$	30.2 ± 3.9**	2
2.5 mM AA	71.9 ± 5.0*	3
2.5 mM AA + 5 μM $CdCl_2$	34.2 ± 2.1[a]	11
2.5 mM AA + 10 μM $CdCl_2$	7.1 ± 1.0[a]	18
2.5 mM AA + 15 μM $CdCl_2$	2.7 ± 0.6[a]	14
600 μg/ml EMS	93.2 ± 1.6	173

[a] Compared with AA or $CdCl_2$ ($P < 0.01$); AA: acetaldehyde.
* $P < 0.05$ (compared with control).
** $P < 0.01$ (compared with control).

Table 4
Comparison of observed and predicted mutant frequency induced by AA and cadmium[a]

Dose	Observed mutant frequency (1×10^6)	Predicted mutant frequency (1×10^6)	Interaction
2.5 mM AA + 5 μM $CdCl_2$	11	3	>+
2.5 mM AA + 10 μM $CdCl_2$	18	7	>+
2.5 mM AA + 15 μM $CdCl_2$	14	5	>+

[a] $N(AB) = N(A) + N(B) - N(C)$ (independent joint action model): $N(AB)$, number of observed mutant; $N(A) + N(B) - N(C)$, number of predicted mutant; $N(A)$, number of A mutant; $N(B)$, number of B mutant; $N(C)$, number of control mutant; >+, synergistic; AA, acetaldehyde.

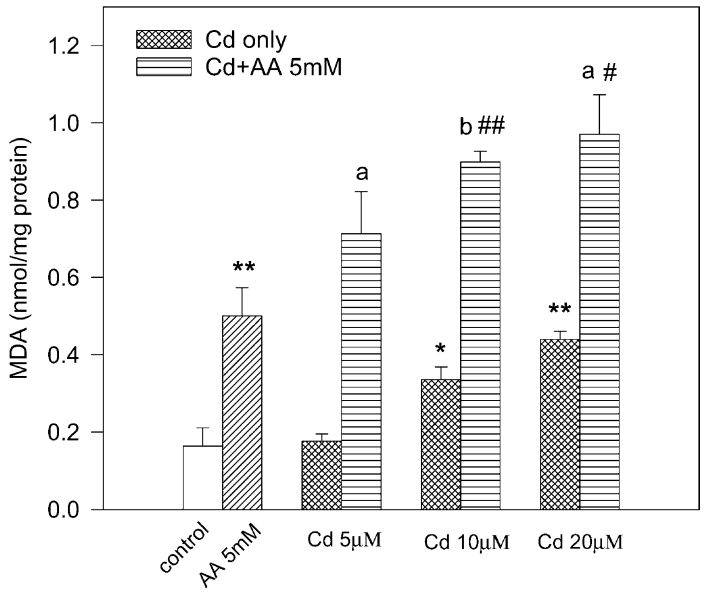


Fig. 2. Effect of cadmium and AA on lipid peroxidation in V79 cells, in triplicate. (∗, ∗∗): compared with control (∗: $P < 0.05$, ∗∗: $P < 0.01$); (#, ##): compared with AA (#: $P < 0.05$, ##: $P < 0.01$); (a, b): compared with Cd (a: $P < 0.05$, b: $P < 0.01$).

Table 5
Comparison of observed and predicted mortality on V79 cells by lipid peroxidation assay[a]

Dose	Observed relative mortality (nmol/mg)	Predicted relative mortality (nmol/mg)	Interaction
5 mM AA + 5 μM $CdCl_2$	0.71 ± 0.17	0.51 ± 0.07	>+
5 mM AA + 10 μM $CdCl_2$	0.90 ± 0.03	0.67 ± 0.08	>+
5 mM AA + 20 μM $CdCl_2$	0.97 ± 0.12	0.78 ± 0.07	>+

[a] $N(AB) = N(A) + N(B) - N(C)$ (independent joint action model): $N(AB)$, amount of observed MDA; $N(A) + N(B) - N(C)$, amount of predicted MDA; $N(A)$, amount of A MDA; $N(B)$, amount of B MDA; $N(C)$, amount of control MDA; >+, synergistic; AA, acetaldehyde.

as MDA equivalents. The results were expressed at the lipid peroxidation on the growth of V79 cells when treatment with 5–20 μM cadmium and 5 mM AA (Fig. 2). For example, cadmium induced the MDA formation in a dose-dependent manner, and the results showed a synergistic increase MDA production by 0.2 nmol/mg protein (Table 5). Furthermore, several groups [31–35] reported that mitochondria are a primary target of toxic injury and that their dysfunction ultimately leads to plasma membrane damage or cell death. For our investigation, mitochondria function was affected from uptake of rhodamine 123 in significant enhancement (200%) in >10 μM cadmium and 5 mM AA treated cells, while a few increment was found in 5 mM cadmium treated (Fig. 3).

As above described, glycyrrhizin and quercetin are acted as good antioxidant agents. To investigate the antioxidant activity of glycyrrhizin and quercetin as anti-carcinogenic agents, we first examined the cytotoxicities of the exposure to 10–500 μM glycyrrhizin or 5–80 μM quercetin alone on V79 cells, both drugs retain cell viability above 80% compared to the control cell by MTT assay. Furthermore, treatment with 10 and 20 μM quercetin was increased by the inhibition of lipid peroxidation in 15 and 20%, respectively (Fig. 4), but not treatment with 50–200 μM glycyrrhizin. Finally, 5–20 μM quercetin and 50–200 μM glycyrrhizin showed significant reduced uptake of rohdamine 123 on V79 cells (Figs. 5 and 6).

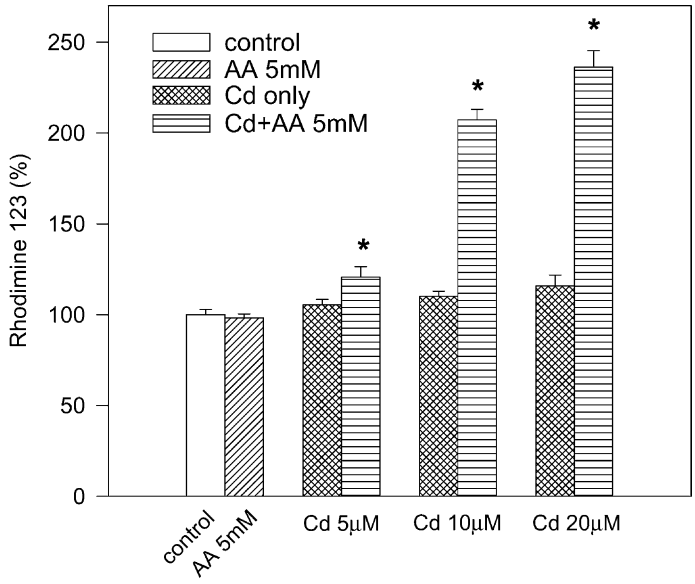


Fig. 3. Effect of cadmium and AA on mitochondrial membrane potential in V79 cells, in triplicate. (∗): compared with Cd or AA (∗: $P < 0.01$).

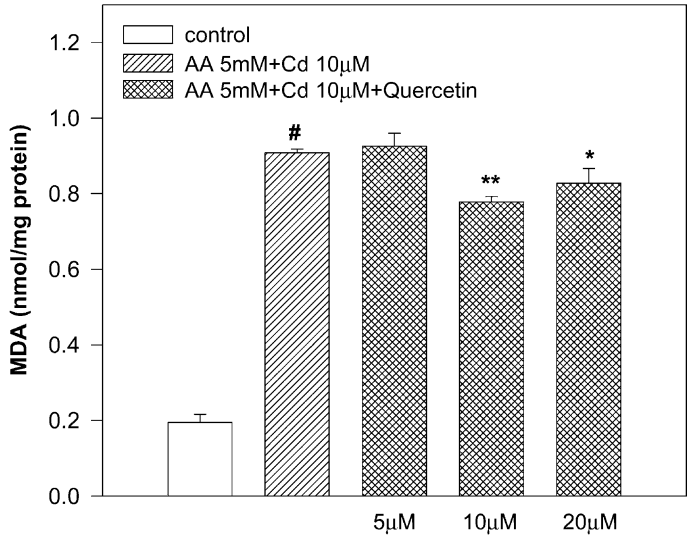


Fig. 4. Effect of quercetin on lipid peroxide levels induced by cadmium and AA in V79 cells, in triplicate. (#): compared with control (#: $P < 0.01$); (∗, ∗∗): compared with AA and Cd (∗: $P < 0.05$, ∗∗: $P < 0.01$).

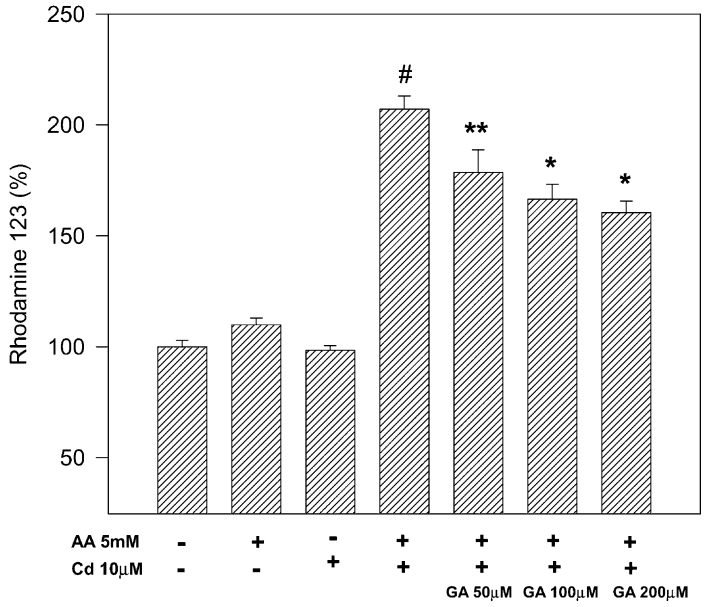


Fig. 5. Effect of glycyrrhizin (GA) on cellular injury detected by rhodamine 123 uptake in V79 cells, in triplicate. (#): compared with control (#: $P < 0.01$); (∗, ∗∗): compared with AA and Cd (∗∗: $P < 0.05$, ∗: $P < 0.01$).

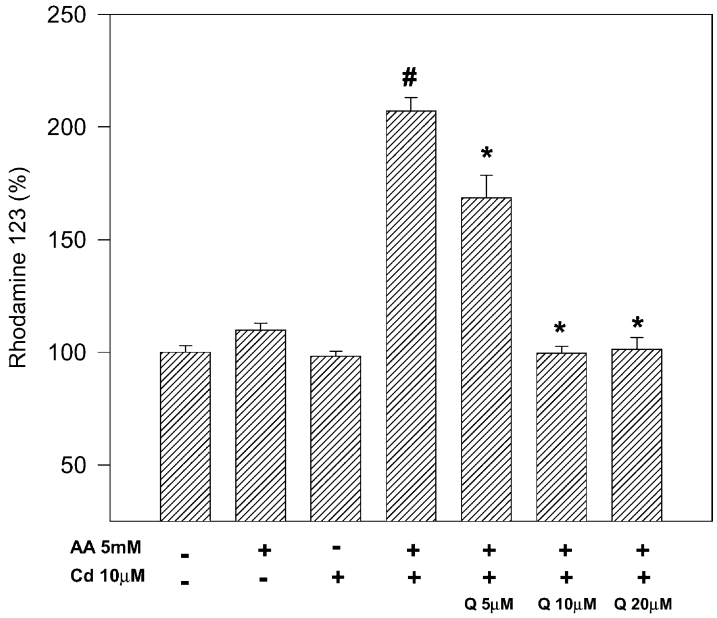


Fig. 6. Effect of quercetin (Q) on cellullar injury detected by rhodamine 123 uptake in V79 cells, in triplicate. (#): compared with control (#: $P < 0.01$); (∗): compared with AA and Cd (∗: $P < 0.01$).

4. Discussion

Recently we have investigated the antioxidant and anti-tumor activity of caffeic acid phenethyl ester (CAPE) analogous, and the result indicated that CAPE be acting as potential chemotherapic agent against oral cancer [36,37]. Similarly, quercetin and glycyrrhizin have drawn our attention because of its antioxidant, anti-tumor, and anti-inflammatory properties. To evaluate them as chemopreventive agents, we started to test the cytotoxicity of cadmium and AA on V79 cells. Under our investigation, the cytotoxicities of cadmium and AA on V79 cells were studied by the MTT or colony assay. According to our studies, both acute and chronic results showed a significant synergistic effect on cytotoxicity of co-exposure of cadmium and AA in V79 cells (Fig. 1). Similarly, at *Hprt* mutant assay, the results have also shown that the combined chronic genotoxicity of cadmium and AA be synergistic effect on V79 cells by colony assay.

After cytotoxicity and genotoxicity studies, we further investigated the formation of MDA is considered an index of lipid peroxidation that causes cell injury. For instance, the cytotoxicity and genotoxicity may be considered to be associated with oxidative stress, the lipid peroxidation and mitochondrial membrane potential assays were tested. Our observations may indicate that a mitochondrial permeability transition in response to co-exposure of cadmium and AA leads to plasma membrane damage or cell death. Although the mechanism of cytotoxicities or mutagenicities on the combined exposure of cadmium and AA is still not understood, it is well known that the protection of cells from oxidative damage can be accomplished through an antioxidant agent. Fortunately, our results assessed that reduced the mortality of V79 cells from oxidative damage by quercetin was two-folds than that of glycyrrhizin. Even though the mechanism of reducing the damage of plasma membrane by glycyrrhizin is still not understood, it should be different pathway from quercetin for protecting V79 cells.

In conclusion, based on the above assay results, combined treatment of cadmium and AA on V79 cells has shown a significant synergistic effect of cytotoxicity and genotoxicity. In addition, the superoxide radical species have been implicated in the increased cytotoxicity or mutagenicity of cadmium and AA on V79 cells by studies of lipid peroxidation formation

and uptake rhodamine 123. Moreover, the ability of quercetin and glycyrrhizin to scavenge free-radicals and block lipid peroxidation raises the possibility that they may act as protective factors against carcinogenesis, and it implies that quercetin is better than glycyrrhizin as chemopreventor against cytotoxicity and genotoxicity on co-exposure of cadmium and AA in V79 cells. Furthermore, quercetin is consistent with structure and activity relationship that the aryl units contain at least one aryl ring required *o*-bis-hydroxylation for significant inhibitory potency of antioxidants. Therefore, further investigation should address how cadmium and AA affect cytological activities in normal cells. Further in vivo assays are currently underway to evaluate the efficacy of quercetin and glycyrrhizin as chemopreventive agents against carcinogenesis.

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The authors are indebted to Professor Ruey-Hseng Lin for the generous gift of V79 cells. For financial support, we thank the National Science Council of the Republic of China (Research Grant NSC 87-2113-M-040-001 and NSC 89-2113-M-018-003).

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A Long-Term Glycyrrhizin Injection Therapy Reduces Hepatocellular Carcinogenesis Rate in Patients with Interferon-Resistant Active Chronic Hepatitis C: A Cohort Study of 1249 Patients

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To elucidate the influence of a glycyrrhizin therapy on hepatocarcinogenesis rate in interferon (IFN)-resistant hepatitis C, we retrospectively analyzed 1249 patients with chronic hepatitis with or without cirrhosis. Among 346 patients with high alanine transaminase value (twice or more of upper limit of normal), 244 patients received intravenous glycyrrhizin injection and 102 patients did not, after judgment of IFN resistance. Crude carcinogenesis rates in the treated and untreated group were 13.3%, 26.0% at the 5th year, and 21.5% and 35.5% at the 10th year, respectively ($P = .0210$). Proportional hazard analysis using time-dependent covariates disclosed that glycyrrhizin treatment significantly decreased the hepatocarcinogenesis rate (hazard ratio 0.49, 95% confidence interval 0.27–0.86, $P = .014$) after adjusting the background features with significant covariates. Glycyrrhizin injection therapy significantly decreased the incidence of hepatocellular carcinoma in patients with IFN-resistant active chronic hepatitis C, whose average aminotransferase value was twice or more of upper limit of normal after interferon.

KEY WORDS: chronic hepatitis; hepatitis C virus; glycyrrhizin; hepatocellular carcinogenesis; cancer prevention.

Until recently, hepatitis C virus (HCV) has been reported to be a causative agent of hepatocellular carcinoma (HCC) aside from hepatitis B virus (1–5). In our cohort studies of Japanese patients with HCV-related cirrhosis (5), the cumulative appearance rates of HCC at the 5, 10, and 15 years were 21.5%, 53.2%, and 75.2%, respectively. The carcinogenesis rate was higher in those patients with cirrhosis caused by HCV than in those with hepatitis B virus-related cirrhosis.

Interferon (IFN) is effective in eliminating HCV in some patients with chronic hepatitis C (6–8) and cirrhosis (9–11), and in reducing hepatocellular carcinogenesis rate through suppression of necro-inflammatory process and reduction of serum alanine transaminase (ALT). Kasahara *et al.* (6) reported that sustained normal ALT value after IFN therapy was significantly associated with a decreased hepatocellular carcinogenesis rate in patients with chronic hepatitis C. Our data (7) also demonstrated an anticarcinogenic activity of IFN in patients who attained normal ALT

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level after the therapy compared with IFN-treated patients without normalization of ALT.

Oka *et al.* (12) reported in a randomized controlled trial that a kind of medicinal herb, *Sho-saiko-to*, significantly decreased hepatic carcinogenesis rate in patients with HBsAg-negative cirrhosis. Tarao *et al.* (13) showed that HCC appearance rate was significantly higher in HCV-related cirrhotic patients with a high ALT value of 80 IU/mL or more than that of those with lower ALT value (<80 IU/mL), and also suggested that treatment of cirrhosis and prevention of HCC should be directed to suppress the necro-inflammation of HCV-related hepatitis. A glycyrrhizin-containing product, Stronger Neo-Minophagen C (SNMC; Minophagen Pharmaceutical Co. Ltd., Tokyo, Japan), is widely used in Japan for suppression of hepatitis activity and for prevention of disease progression in patients with hepatitis B virus and HCV-induced chronic hepatitis. Glycyrrhizin has been reported to suppress hepatic inflammation with an effect to improve the elevated ALT levels and histologic findings of the liver (14–17). We reported its favorable effect on hepatocellular carcinogenesis in those patients with chronic hepatitis C who received glycyrrhizin for more than 10 years (18).

To elucidate whether glycyrrhizin suppress the carcinogenesis rate in patients with IFN-resistant chronic hepatitis C, we retrospectively assessed a cohort of 1249 patients without sustained virologic response (SVR) after IFN therapy.

PATIENTS AND METHODS

Study Population. A total of 1249 consecutive Japanese patients with chronic hepatitis C with or without cirrhosis were examined, who did not show an SVR of HCV-RNA under IFN therapy. Sera of the patients showed positive anti-HCV (second-generation anti-HCV kit, enzyme-linked immunosorbent assay, Dainabot, Tokyo, Japan), positive HCV-RNA (nested PCR), and negative hepatitis B surface antigen (HBsAg; radioimmunoassay, Dainabot). Anti-HCV and HCV-RNA were assayed using stored frozen sera at –80°C. There were 778 men and 471 women aged 18–81 years, with a median age of 53 years in the study. They were diagnosed as having liver cirrhosis by peritoneoscopy, liver biopsy, or both between 1987–2002.

All the patients had a history of receiving once or more times of IFN therapy: 1179 patients underwent IFN monotherapy only and the other 70 patients had received an IFN plus ribavirin combination therapy before the entry of this study. A total of 347 patients showed a normal ALT for at least 6 months after cessation of IFN (biochemical responders), and the other 902 patients abnormal ALT at 6 months after the end of IFN therapy. A retrospective cohort study was performed using these 1249 consecutive patients with chronic hepatitis or cirrhosis who failed to show SVR.

Glycyrrhizin Treatment. Glycyrrhizin therapy was performed using intravenous injection of SNMC. The preparation contains 0.2% (4 mg) glycyrrhizinic acid as the main active constituent, 2% (40 mg) glycine, and 0.1% (2 mg) L-cysteine in 20-mL ampoules.

Of 1249 patients with IFN-resistant chronic liver disease, 453 patients underwent glycyrrhizin injection therapy and the remaining 796 patients did not receive the therapy until the end of observation. The purpose of the introduction of the glycyrrhizin injection therapy was to suppress elevated ALT and to prevent disease progression in all the patients. Of the 453 patients, 129 (28.5%) received a daily dose of 40–60 mL of SNMC (80–120 mg as glycyrrhizin) and 324 (71.5%) received 80–100 mL (160–200 mg as glycyrrhizin). A total of 110 patients received the treatment for less than 2 years and 107 patients continued the therapy for 2–4 years, 132 patients for 4–6 years, and the remaining 104 patients for 6 years or longer. When the treatment was regarded as effective from the viewpoint of ALT levels, treatment was usually continued for a period as long as possible. As a result, a median daily dose of 100 mL of SNMC was administered 3 times a week during a median period of 4.3 years (range, 0.1–14.5 years) in the treated group.

Two (0.44%) of 453 treated patients were withdrawn from the glycyrrhizin injection therapy because of side effects: 1 because of hypertension and 1 from skin rash.

Background and Laboratory Data of Patients With and Without Glycyrrhizin Therapy. Table 1 summarizes the profiles and data of the patients at the time of diagnosis of chronic hepatitis with or without cirrhosis. The male/female ratio was not different between the 2 groups. Median age was older by 2 years in the treated group than in the untreated group ($P < .001$). Results of histologic staging of liver disease were classified according to Desmet *et al.* (19). F1stage hepatitis was found significantly more often in the untreated group than in the glycyrrhizin group ($P < .001$, χ^2 test). Both AST and ALT median levels were significantly higher in the treated group than in the untreated group ($P < .001$). HCV subtype was analyzed by the immunoserologic typing method with a commercial kit (Kokusai Diagnostic Corporation, Kobe, Japan): serologic group 1 indicated genotypes 1a and 1b, and group 2 included 2a and 2b subtypes. The rate of HCV serologic group 1 was significantly higher in the glycyrrhizin group than in the untreated group ($P = .032$).

Follow Up. Follow-up of the patients was made monthly after the judgment of IFN-resistance by monitoring hematologic, biochemical, and virologic data. Imaging diagnosis with ultrasonography (US) and/or computed tomography (CT) was made 3 or more times per year in a majority of patients with cirrhosis and once a year in patients without cirrhosis. Angiographic study was performed only when HCC was strongly suspected on US or CT.

When angiography revealed a characteristic hypervascular nodule suggesting a specific finding for HCC, no histologic examination was made in a majority of these patients. An increasing trend of tumor markers was also taken into account in establishment of the diagnosis of HCC. Microscopic examination through a fine needle biopsy was also performed in patients whose angiogram did not show a typical image of HCC.

The number of cases lost to follow-up was 121 (9.7%): 28 patients (6.2%) in the glycyrrhizin group and 93 (11.7%) in the untreated group. Because the outcomes regarding appearance of HCC were not identified in these patients, they were dealt as censored data in the following statistics (20). Death unrelated to HCC was also classified as withdrawal and regarded as a censored case. The median observation period of the total number of patients was 5.7 years with a range of 0.1–16.1 years. Because

TABLE 1. PATIENT PROFILES AND LABORATORY DATA AT TIME OF JUDGMENT OF IFN RESISTANCE

	Glycyrrhizin Group (n = *453*)	*Untreated Group* (n = *796*)	P
Demographics			
Gender (M/F)	283/170	495/301	.92
Age (y)*	54 (25–81)	52 (18–77)	<.001
Observation period (y)*	8.3 (0.1–16.1)	5.1 (0.1–13.1)	<.001
Liver histology			
F1	146 (32.7%)	502 (64.0%)	<.001
F2	193 (43.3%)	192 (24.5%)	
F3	38 (8.5%)	52 (6.6%)	
F4	69 (15.5%)	38 (4.8%)	
Laboratory data*			
Aspartic transaminase (IU/L)*	81 (19–446)	54 (11–355)	<.001
ALT (IU/L)*	122 (12–630)	83 (10–822)	<.001
HCV serologic group 1 (1a or 1b)	360 (80.2%)	582 (73.7%)	.032
Group 2 (2a or 2b)	73 (16.3%)	165 (20.9%)	
Others	16 (3.6 %)	43 (5.4%)	

*Expressed as median (range).

many patients receiving glycyrrhizin therapy migrated from the untreated group to the treated group, observation period of the untreated group was significantly shorter than that of the treated group (see Table 1). The date of the last follow-up for this study was September 1, 2003.

Statistical Analysis. Nonparametric procedures were employed for the analysis of background characteristics of the patients, including Mann-Whitney U-test and χ^2 method. HCC appearance rates were calculated from the time period between the judgment of IFN ineffectiveness and appearance of HCC in each group, using Kaplan-Meier technique (20). The differences in carcinogenesis curves were tested using the log-rank test. Independent factors associated with the appearance rate of HCC were studied using time-dependent Cox regression analysis (21). An interaction term of IFN treatment and "waiting time" to the therapy was introduced in the analysis as a time-dependent covariate. The independence of treatment factor from "waiting time" was also confirmed by log-minus-log plot of proportional hazard model. Several variables were transformed into categorical data consisting of 2–3 simple ordinal numbers to estimate each hazard ratio. All factors found to be at least marginally associated with liver carcinogenesis ($P < .15$) were tested by the multivariate Cox proportional hazard model. A P-value of less than .05 was considered to be significant. All data analysis was performed using the computer program SPSS version 11 (22).

TABLE 2. INDEPENDENT RISK FACTORS AFFECTING HEPATOCELLULAR CARCINOGENESIS

Factors	*Category*	*Risk Ratio* (95 % *CI*)	P
Fibrotic stage			
	F1	1	
	F2–3	2.94 (1.20–7.21)	.018
	F4 (cirrhosis)	9.21 (3.73–22.8)	<.001
Gender			
	1: Female	1	
	2: Male	2.80 (1.35–5.81)	.006
Glycyrrhizin injection			
	1: No	1	
(SNMC)*	2: Yes	0.49 (0.27–0.86)	.014

Time-dependent Cox proportional hazard analysis. *SNMC, Stronger Neo-Minophagen C (herbal medicine containing glycyrrhizin).

RESULTS

Initial Aminotransferase and Carcinogenesis Rates

Patients with and without glycyrrhizin therapy were classified into 6 categories according to average ALT value during the first year after cessation of IFN therapy: group 1, normal ALT; group 2, <1.5 times of upper limit of normal (ULN); group 3, 1.5–2 times ULN; group 4, 2–3 times ULN; group 5, 3–4 times of ULN; and group 6, >4 times ULN. Hepatocellular carcinogenesis rates were 2.5%, 5.0%, 8.1%, 11.8%, 12.0%, and 12.7% at the end of 5 years and 6.6%, 7.2%, 19.6%, 15.1%, 21.0%, and 39.3% at 10 years, respectively (Figure 1). There was a significant statistical difference among the 6 subgroups (log-rank test, $P < .0001$). The higher the average ALT, the higher the carcinogenesis rate was.

Influence of Glycyrrhizin on Carcinogenesis in Patients With High Aminotransferase

Glycyrrhizin therapy was usually performed in patients with a high ALT value and high hepatitis activity. In this retrospective study, average ALT values were significantly different between the treated and untreated groups: group 1, normal average ALT was found in 38 among patients with glycyrrhizin therapy and in 188 among patients without therapy; in group 2, ALT <1.5 times of ULN was found in 42 and 331; in group 3, 1.5–2 times ULN in 84 and 138; in group 4, 2–3 times ULN in 143 and 92; in group 5, 3–4 times in 53 and 29; and in group 6, ALT

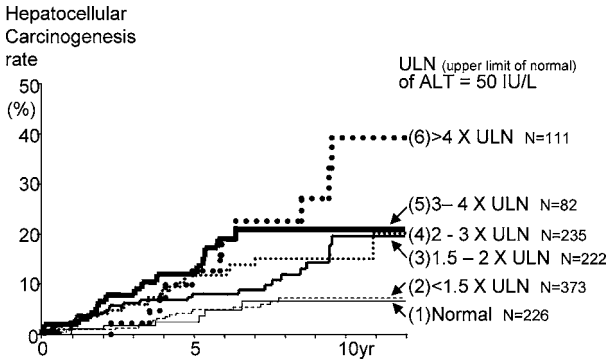

Fig 1. Carcinogenesis rates according to initial ALT values classified into six groups: (1) normal ALT, (2) <1.5 times ULN, (3) 1.5–2 times ULN, (4) 2–3 times ULN, (5) 3–4 times ULN, and (6) >4 times of ULN. The higher the average ALT, the higher the carcinogenesis rate was.

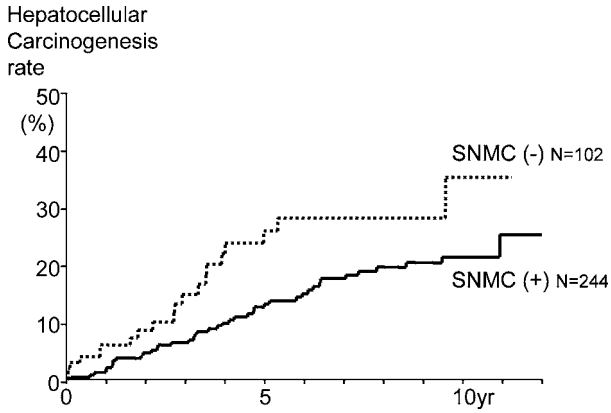

Fig 2. Carcinogenesis rates in patients with high average ALT values of twice or more of ULN, excluding those patients with biochemical responders who continued a normal ALT value at least 6 months just after IFN therapy. The carcinogenesis rate of glycyrrhizin-treated group was significantly lower than that of the untreated group (log-rank test, $P = .0210$).

>4 times ULN in 93 of the glycyrrhizin group and 18 of the untreated group. The rate of a high ALT value of twice or more of ULN in the glycyrrhizin treated group (64.2%, 289/453) was significantly higher than that of the untreated group (16.2%, 129/796).

Of the 418 patients with a high average ALT in both groups, 68 patients showed a normal ALT value for at least 6 months just after IFN therapy (biochemical response). Because biochemical response with normal ALT for a certain period after IFN was likely to affect carcinogenesis rates in those patients, biochemical responders were excluded in the following analyses about the influence of glycyrrhizin on carcinogenesis: after all, 244 patients with glycyrrhizin therapy and the 102 patients without therapy were assessed.

Cumulative hepatocellular carcinogenesis rates were calculated in these 346 patients with a high average ALT values, excluding biochemical responders from both groups. Carcinogenesis rates in the glycyrrhizin group and the untreated group were 6.5% and 13.3% at the end of year 3, 13.3% and 26.0% at the end of year 5, 17.7% and 28.3% at the end of year 7, and 21.5% and 35.5% at year 10, respectively (Figure 2). In the stratified and selected patient group, the carcinogenesis rate of glycyrrhizin-treated group was significantly lower than that of the untreated group (log-rank test, $P = .0210$).

Carcinogenesis Rates According to Hepatitis Staging

Crude carcinogenesis rates were compared between the groups, according to each hepatitis stage. In F1 stage chronic hepatitis, hepatocellular carcinogenesis rates in the glycyrrhizin group ($n = 82$) and the untreated group ($n = 32$) were 1.4% and 4.2% at year 5 and 7.0% and 12.1% at 10 years, respectively (Figure 3A). In F2–3 stage chronic hepatitis, hepatocellular carcinogenesis rates in the glycyrrhizin group ($n = 121$) and the untreated group ($n = 53$) were 14.8% and 28.4% at the end of year 5, and 21.5% and 38.6% at year 10, respectively (Figure 3B). In patients with F4 stage chronic hepatitis (cirrhosis), hepatocellular carcinogenesis rates in the glycyrrhizin group ($n = 38$) and the untreated group ($n = 15$) were 35.2% and 58.0% at the end of year 5, and 57.2% and 58.0% at year 10, respectively (Figure 3C).

In each fibrotic stage of hepatitis, carcinogenesis rates were lower in the glycyrrhizin group than in the untreated group, but statistical significance was not obtained owing to shortage of patient number in these stratified groups.

Aminotransferase Activity Before and After Glycyrrhizin Therapy

ALT values in the patients with glycyrrhizin treatment were serially assessed in those patients who began the therapy after they had shown a high average ALT value (Figure 4). Median value of ALT at the beginning of the glycyrrhizin therapy was 150 IU/L (25th percentile 120, 75th percentile 221), 72 IU/L at month 3, 70 IU/L at month 6, and 64 IU/L (25th percentile 48, 75th percentile 93) at month 12, respectively. ALT value significantly decreased after the initiation of glycyrrhizin injection therapy.

Factors Affecting Carcinogenesis Rates in Active Hepatitis and Cirrhosis

In the selected patients with active hepatitis with an average ALT value of twice ULN or higher, multivariate analysis was performed to explore associating factors with carcinogenesis, using time-dependent Cox proportional hazard model. Time between the judgment of IFN

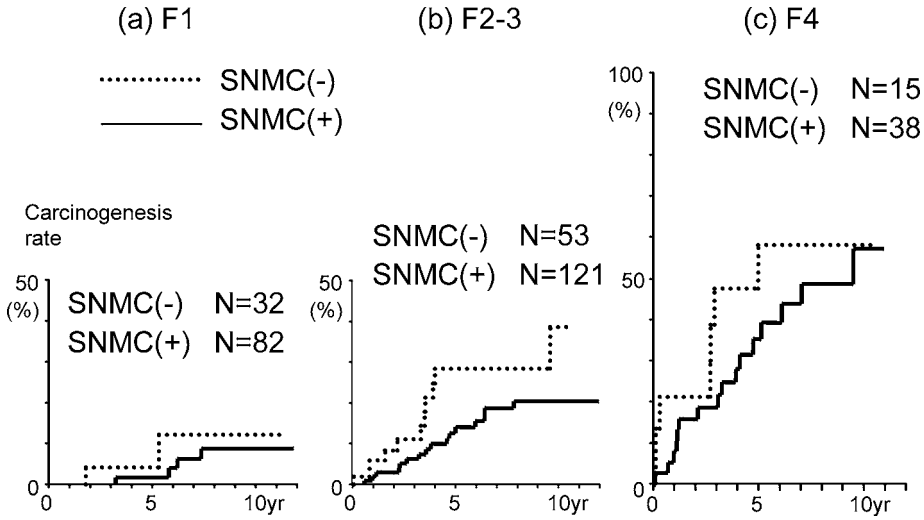

Fig 3. Carcinogenesis rates according to hepatitis staging: (a) F1 stage hepatitis, (b) F2–F3 stage hepatitis, and (c) F4 or cirrhotic stage. In each fibrotic stage of hepatitis, carcinogenesis rates were lower in the glycyrrhizin group than in the untreated group.

ineffectiveness and initiation of glycyrrhizin therapy was set as a time-dependent variable to clarify the significance of glycyrrhizin therapy in the clinical course of HCV-related chronic liver diseases. Patients with biochemical response with a normal ALT value sustained for at least 6 months after IFN therapy were also excluded from the analysis.

In multivariate analysis, following 3 factors influenced the carcinogenesis: fibrotic staging, gender ($P = .006$), and glycyrrhizin therapy ($P = .014$). When a hazard of F1 stage fibrosis for carcinogenesis was set as 1 in the model, hazard ratio of F2–F3 stage fibrosis was calculated as 2.94 ($P = .018$), and that of F4 (cirrhosis) was estimated as 9.21 ($P < .001$). Similarly, the hazard ratio for carcinogenesis of male gender was 2.80, and use of glycyrrhizin independently decreased the carcinogenesis rate in patients with active chronic hepatitis after IFN therapy. Following factors did not affect the HCC appearance rate significantly: age, association of diabetes mellitus, serologic grouping of HCV, HCV-RNA concentration, AST, ALT at the time before IFN therapy, and bilirubin.

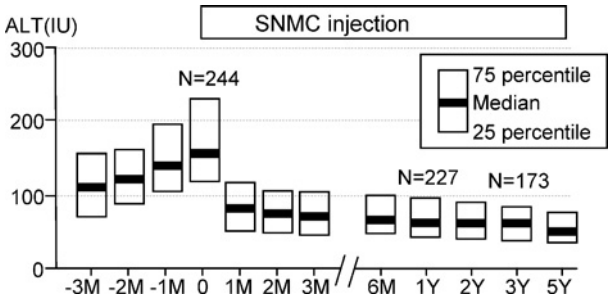

Fig 4. Aminotransferase activity before and after glycyrrhizin therapy. ALT value significantly decreased after the initiation of glycyrrhizin injection therapy.

DISCUSSION

IFN is effective in patients with chronic liver disease caused by HCV, from the viewpoints of anti-inflammatory effect and cancer prevention (6–11). Although the carcinogenesis rate is noticeably reduced when aminotransferase becomes normal with or without HCV-RNA eradication (6–8) after the therapy, the rate of normalization of ALT after IFN therapy is approximately half of patients with high viral load and group 1 HCV-subtype.

This retrospective study was undertaken to evaluate whether long-term glycyrrhizin injection therapy could decrease hepatocellular carcinogenesis rate in patients with IFN-resistant HCV-related chronic hepatitis and cirrhosis. Because it requires at least 5 years to show a statistical difference in carcinogenesis rate from hepatitis or cirrhosis between glycyrrhizin-treated and "untreated" groups, a prospective randomized trial using untreated control patients is difficult from both ethical and medical viewpoints in Japan, where glycyrrhizin injection therapy is covered by standard medical insurance and is already regarded as a usual choice of therapy as a salvaging procedure for IFN-ineffective patients. We, therefore, attempted to carry out this retrospective cohort study to prove an anticarcinogenic activity of glycyrrhizin, with a statistical adjustment using possible covariates explored in multivariate analysis.

Because glycyrrhizin injection therapy was chiefly performed for patients with a high ALT value and because cancer prevention was meaningful in just those patients with a high carcinogenesis risk with high hepatitis activity, we analyzed the role of a long-term glycyrrhizin injection therapy in the patients with a high ALT value. The treated group consisted of significantly more numbers of patients with a high ALT value of twice or more of ULN. When carcinogenesis rates were assessed only in those patients with a high ALT value of twice or more ULN excluding biochemical responders, the rate of the treated group became significantly higher than that of the untreated group ($P = .021$). The cancer preventive effect of glycyrrhizin in IFN-resistant patients was also confirmed by time-dependent Cox proportional analysis that adjusted the background features of the retrospective cohort (hazard ratio = 0.49, $P = .014$). We previously reported a study focused on the anticarcinogenic action of glycyrrhizin for patients with chronic hepatitis C, but the pilot study only demonstrated that 10 years or longer treatment with glycyrrhizin ($n = 84$) could suppress the carcinogenesis rate (18). Current study dealing with a large cohort ($n = 1249$) showed that glycyrrhizin injection therapy significantly decreased carcinogenesis rate irrespective of the length of treatment when comparison was made in a selected patient cohort with high hepatitis activity.

Although a statistically significant difference was not shown for a lack of sufficient patient number in subgroups of chronic hepatitis and cirrhosis, this study also demonstrated that glycyrrhizin was effective not only in chronic hepatitis but also in cirrhosis. Considering that liver cirrhosis generally shows a resistance to IFN treatment, our current study demonstrated encouraging results from the viewpoint of HCC prevention. When IFN therapy was attempted in 7 patients with decompensated cirrhosis by Nevens *et al.* (23), complications sometimes occurred in these patients, including variceal bleeding, aggravation of ascites or encephalopathy, development of pneumonia, and recurrence of spontaneous bacterial peritonitis or gastric ulcer bleeding. Because patients with cirrhosis usually showed lower platelet and leukocyte counts than those with chronic hepatitis and because cirrhotic patients tended to show deterioration with a large dose of IFN, glycyrrhizin therapy proved to be a useful alternative of therapy. Intermittent long-term glycyrrhizin therapy was well tolerated with withdrawal of only 2 patients (0.44%).

Because carcinogenesis is not a single-step event but a complex, multistep process, the exact mechanism of the glycyrrhizin activity in suppression of liver carcinogenesis remains unknown. One of the principal roles of long-term administration of glycyrrhizin in decreasing the carcinogenesis rate is considered to be anti-inflammatory, which blocks the active carcinogenic process of continuous hepatic necro-inflammation and cell damage. In the treated group, median ALT values markedly decreased after initiation of the glycyrrhizin injection, suggesting that pathologic process of hepatocyte necrosis or apoptosis was significantly suppressed by glycyrrhizinic acid. The importance of the action of amino acids, glycine and cysteine contained in SNMC has not been completely explained, but they have been demonstrated to suppress increased aldosterone levels that are induced by glycyrrhizinic acid. Tarao *et al.* (24) reported that high aminotransferase level resulted in an increase of an HCC recurrence rate in patients with HCC. From the viewpoint of these anti-inflammatory activities, SNMC may be considered to only postpone the time of HCC appearance in the clinical course of cirrhosis. Because the entire process of hepatocellular carcinogenesis from the initial transformation of a hepatocyte to a detectable growth of cancer is considered to take at least several years, the influence of glycyrrhizin on the carcinogenesis rate will not be evaluated in a short period. Although several reports suggested a relationship of anti-hepatitis B core antibody or hepatitis B surface antibody with carcinogenesis (25–27), we could not show the association because of insufficient available data.

Because current data were obtained from a retrospective cohort analysis, dose of glycyrrhizin per time, times of injection per week, and duration of therapy varied in each patient in the treated group. To elucidate the cancer preventive effect of glycyrrhizin therapy in active HCV-related liver disease, we should further stratify the treated patients or perform much more detailed statistical procedures. Future studies should, therefore, aim at defining the basic oncogenic mechanisms and roles of long-term administration of glycyrrhizin in carcinogenesis in patients with cirrhosis caused by HCV.

In conclusion, a long-term intermittent glycyrrhizin therapy for a few years or more successfully reduced hepatocellular carcinogenesis in patients with HCV-related chronic liver disease. A randomized control study with a larger number of cases, with or without glycyrrhizin therapy, is expected to confirm the effectiveness of this therapy.

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Glycyrrhizin injection therapy prevents hepatocellular carcinogenesis in patients with interferon-resistant active chronic hepatitis C

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Aim: There is no useful and effective treatment for patients with non-sustained response to interferon, from the viewpoint of cancer prevention. Our aim was to elucidate the influence of a glycyrrhizin therapy on hepatocarcinogenesis rate in interferon-resistant hepatitis C

Methods: We retrospectively analyzed 1249 patients with chronic hepatitis with or without cirrhosis. Among 346 patients with high alanine transaminase values of twice or more of the upper limit of normal, 244 patients received i.v. glycyrrhizin injection and 102 patients did not, after judgment of interferon resistance.

Results: Crude carcinogenesis rates in the treated and untreated group were 13.3%, 26.0% at the fifth year, and 21.5% and 35.5% at the 10th year, respectively ($P = 0.021$). Proportional hazard analysis using time-dependent covariates disclosed that fibrotic stage, gender and glycyrrhizin treatment were significantly associated with future carcinogenesis. A long-term glycyrrhizin injection therapy decreased the hepatocarcinogenesis rate (hazard ratio, 0.49; 95% confidence interval, 0.27–0.86, $P = 0.014$) after adjusting the background features with significant covariates. Cancer preventive activity was also found in a subgroup of older patients of 60 years or more.

Conclusions: Glycyrrhizin injection therapy significantly decreased the incidence of hepatocellular carcinoma in patients with interferon-resistant active chronic hepatitis C, whose average aminotransferase value was twice or more of the upper limit of normal after interferon.

Key words: cancer prevention, chronic hepatitis, glycyrrhizin, hepatitis C virus, hepatocellular carcinogenesis

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most common cancers in the world. Until recently, hepatitis C virus (HCV) has been reported to be a causative agent of HCC aside from hepatitis B virus (HBV).[1–5] The annual incidence of HCC in patients with HCV RNA-positive cirrhosis ranges 5–7%.[5–7] The carcinogenesis rate was higher in those patients with cirrhosis caused by HCV than in those with HBV-related cirrhosis.[5]

Interferon (IFN) is effective in reducing HCC rate through suppression of necroinflammatory process serum alanine aminotransferase (ALT) and in eliminating HCV in some patients with chronic HCV and cirrhosis. Although IFN proves to be valuable in suppression of the risk of carcinogenesis, it is not effective in every patient with HCV-related disease. Oka *et al.*[8] reported in a randomized controlled trial that a kind of medicinal herb, "Sho-saiko-to", could significantly decrease hepatic carcinogenesis rate in patients without hepatitis B surface antigen (HBsAg)-negative cirrhosis. Tarao *et al.*[9] showed that the HCC appearance rate was significantly higher in HCV-related cirrhotics with a high ALT value of 80 IU or more than that of those with lower ALT value, and also suggested that treatment of cirrhosis and prevention of HCC should be directed to suppress the necroinflammation of HCV-related hepatitis.

In Japan, a glycyrrhizin-containing herbal medicine, Stronger Neo-Minophagen C (SNMC), is widely used in Japan for the treatment of chronic hepatitis. It is used in the form of an i.v. solution, comprised of 0.2% glycyrrhizin, 0.1% cysteine and 0.2% glycine in physiological solution. It is made by dissolving glycyrrhizin (200 mg),

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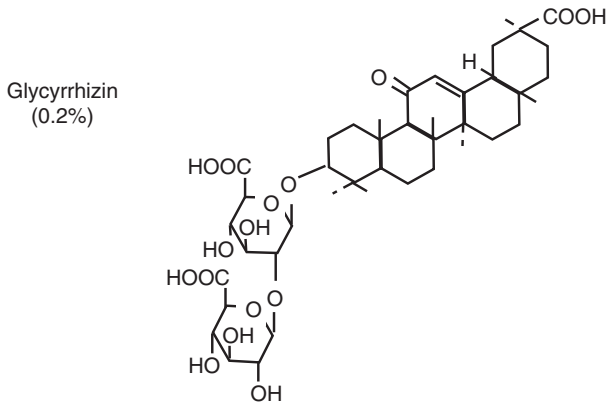

Figure 1 Chemical structure of glycyrrhizin and Stronger Neo-Minophagen C in physiological saline solution.

cysteine (100 mg), glycine (2 g) in 100 mL of physiological saline (Fig. 1). Glycyrrhizin is an aqueous extract of licorice root (*Glycyrrhizae radix*), which is anti-allergic and has detoxicating effects. As has been reported, the anti-inflammatory mechanism of glycyrrhizin is believed to be due to its protective effect on the hepatic cellular membrane, which may explain its ability to lower the serum transaminase level in patients with chronic hepatitis. Because glycyrrhizin has an anti-inflammatory action and favorable effect on ALT and histology in patients with chronic viral hepatitis,[10–15] we analyzed its effect on HCC in those patients with chronic HCV.[16]

In order to elucidate whether glycyrrhizin suppress the carcinogenesis rate in patients with IFN-resistant chronic hepatitis C, we retrospectively assessed a cohort of 1249 patients without sustained virological response.[17]

METHODS

Patients

A TOTAL OF 1249 consecutive Japanese patients with chronic hepatitis or cirrhosis type C were examined, who could not eradicate HCV RNA with previous IFN therapy. There were 778 men and 471 women aged 18–81 (median age, 53 years) in the study. They were diagnosed as having liver cirrhosis by peritoneoscopy, liver biopsy or both between 1987 and 2002 at Toranomon Hospital, Tokyo, Japan. All the patients had a history of receiving IFN therapy once or more. A total of 347 patients showed a normal ALT for at least 6 months after cessation of IFN (biochemical responders), and the other 902 patients showed abnormal ALT at 6 months after the end of IFN therapy.

Glycyrrhizin therapy

Of 1249 patients with IFN-resistant chronic liver disease, 453 patients underwent glycyrrhizin injection therapy (SNMC) and the remaining 796 patients did not receive glycyrrhizin therapy until the end of observation. The purpose for the introduction of glycyrrhizin therapy was to suppress high ALT levels and to prevent disease progression in all the patients. F1 stage hepatitis was significantly more often found in the untreated group than in the glycyrrhizin group ($P < 0.001$, χ^2 test). Both AST and ALT medians were significantly higher in the glycyrrhizin group than in the untreated group ($P < 0.001$).

When glycyrrhizin was regarded as effective from an aminotransferase viewpoint, treatment was usually continued for as long a period as possible. As a result, a median daily dose of 100 mL of glycyrrhizin was administered thrice weekly during a median period of 4.3 years (range, 0.1–14.5 years) in the treated group. Two (0.44%) of the 453 treated patients withdrew from glycyrrhizin injection therapy because of side-effects: one from hypertension and one from skin rash without itching.

Background and laboratory data of patients with and without therapy

Table 1 summarizes the profiles and data of the patients at the time of diagnosis of chronic hepatitis, with or without cirrhosis.

The male : female ratio was not different between the two groups. Median age was older by 2 years in the treated group than in the untreated group ($P < 0.001$). F1-stage hepatitis was found significantly more often in the untreated group than in the glycyrrhizin group ($P < 0.001$, χ^2 test). Median levels of both AST and ALT were significantly higher in the treated group than in the untreated group ($P < 0.001$). The rate of HCV serological group 1 was significantly higher in the glycyrrhizin group than in the untreated group ($P = 0.032$).

Follow up of the patients

Follow up of the patients was made on a monthly basis after the judgment of IFN resistance by monitoring hematological, biochemical, and virological data.

Table 1 Patients profiles and laboratory data at the time of judgment of interferon-resistance

	Glycyrrhizin group ($n = 453$)	Untreated group ($n = 796$)	*P*-value
Demography			
Sex (M/F)	283/170	495/301	0.92‡
Age (year)†	54 (25–81)	52 (18–77)	<0.001
Liver histology			
F1/F2/F3/F4	146/193/38/69	502/192/52/38	<0.001‡
Laboratory data†			
Aspartic transaminase (IU/L)†	81 (19–446)	54 (11–355)	<0.001
Alanine transaminase (IU/L)†	122 (12–630)	83 (10–822)	<0.001
HCV serological group 1/2	360/73	582/165	0.032‡

†Expressed by median (range). ‡χ^2 test or Mann–Whitney *U*-test.

Imaging diagnosis with ultrasonography (US) and/or computerized tomography (CT) was made three or more times per year in a majority of patients with cirrhosis, and once a year in patients without cirrhosis.

The numbers of cases lost to follow up were 121 (9.7%): 28 patients (6.2%) in the glycyrrhizin group and 93 (11.7%) in the untreated group. Because the eventual outcomes regarding appearance of HCC were not identified in these patients, they were dealt as censored data in the following statistics. Death unrelated to HCC was also classified as withdrawal and regarded as a censored case. The median observation period of the total number of patients was 5.7 years with a range of 0.1–16.1 years.

Statistical analysis

Non-parametric procedures were employed for the analysis of background characteristics of the patients, including Mann–Whitney *U*-test and χ^2 method. HCC appearance rates were calculated from a period between the judgment of IFN ineffectiveness and the appearance of HCC in each group, using the Kaplan–Meier technique.[17] The differences in carcinogenesis curves were tested using the log-rank test. Independent factors associated with the appearance rate of HCC were studied using time-dependent Cox regression analysis.[18] An interaction term of IFN treatment and "waiting time" to the therapy was introduced in the analysis as a time-dependent covariate. The independence of treatment factor from "waiting time" was also confirmed by a log-minus-log plot of a proportional hazard model.

All data analysis was performed using the computer program SPSS version 11 (SPSS, Chicago, IL, USA).

RESULTS

Initial aminotransferase and carcinogenesis rates

BECAUSE AMINOTRANSFERASE LEVEL is likely to affect future disease progression, entire patients of the cohort were classified into six categories according to average ALT value during the first year after cessation of IFN therapy: (i) normal ALT; (ii) less than 1.5 times of upper limit of normal (ULN); (ii) 1.5–2 times of ULN; (iv) 2–3 times of ULN; (v) 3–4 times of ULN; and (vi) more than 4 times of ULN. Hepatocellular carcinogenesis rates were 2.5%, 5.0%, 8.1%, 11.8%, 12.0% and 12.7% at the end of the fifth year, and 6.6%, 7.2%, 19.6%, 15.1%, 21.0% and 39.3% at the 10th year, respectively. There was a significant statistical difference among the six subgroups (log-rank test, $P < 0.0001$). The higher the average ALT, the higher the carcinogenesis rate was.

Glycyrrhizin therapy was usually performed in patients with a high ALT value and high hepatitis activity. In this retrospective study, average ALT values were significantly different between the treated and the untreated groups: (i) normal average ALT was found in 38 among patients with glycyrrhizin therapy and in 188 among patients without therapy; (ii) ALT of less than 1.5 times of ULN was found 42 and 331; (iii) 1.5 times to 2 times of ULN 84 and 138; (iv) 2–3 times of ULN in 143 and 92; (v) 3–4 times in 53 and 29; and (vi) ALT of more than 4 times of ULN in 93 of the glycyrrhizin group and 18 of the untreated group, respectively. The rate of a high ALT value of twice or more of ULN in the glycyrrhizin treated group (64.2%, 289/453) was significantly higher than that of the untreated group (16.2%, 129/796).

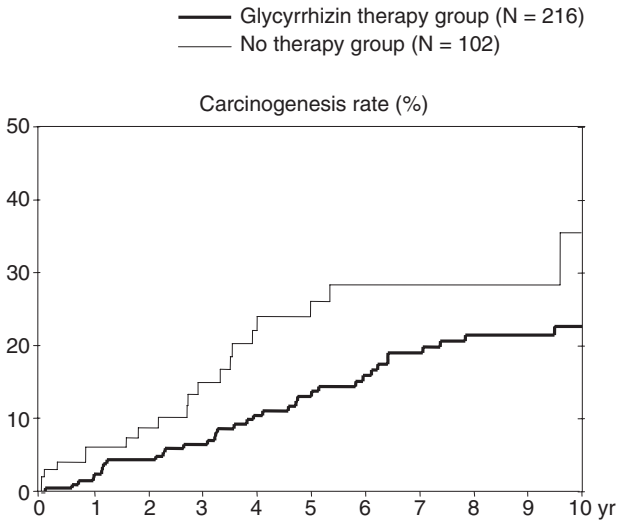

Figure 2 Carcinogenesis rates in patients with active chronic hepatitis showing high average alanine transferase (ALT) values of twice or more of upper limit of normal. Carcinogenesis rate in patients with a sufficient period of glycyrrhizin treatment was significantly lower than that of the untreated patients (log-rank test, $P = 0.021$).

Carcinogenesis in patients with high aminotransferase

Of the 418 patients with a high average ALT in both groups, 68 patients showed a normal ALT value for at least 6 months just after IFN therapy (biochemical response). because biochemical response with normal ALT for a certain period after IFN was likely to affect carcinogenesis rates in those patients, biochemical responders were excluded in the following analyses on the influence of glycyrrhizin on carcinogenesis: after all, 244 patients with glycyrrhizin therapy and the 102 patients without therapy were assessed.

Cumulative hepatocellular carcinogenesis rates were calculated in these 346 patients with high average ALT values, excluding biochemical responders from both groups. Carcinogenesis rates in the glycyrrhizin group and the untreated group were 6.5% and 13.3% at the end of the third year, 13.3% and 26.0% at the end of the fifth year, 17.7% and 28.3% at the end of the seventh year, and 21.5% and 35.5% at the 10th year, respectively (Fig. 2). In the stratified and selected patient group, the carcinogenesis rate of the glycyrrhizin-treated group was significantly lower than that of the untreated group (log-rank test, $P = 0.021$).

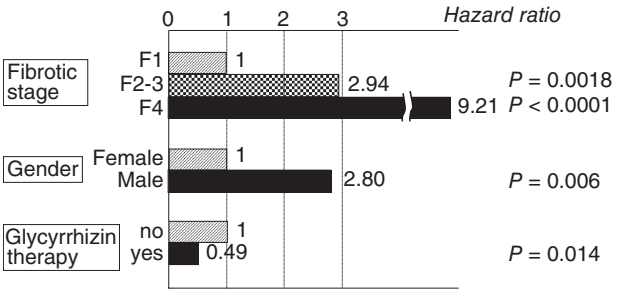

Figure 3 Independent risk factors affecting hepatocellular carcinogenesis (time-dependent Cox proportional hazard analysis).

Impact of glycyrrhizin therapy on carcinogenesis

In the selected patients with active hepatitis with an average ALT value of twice ULN or higher, multivariate analysis was performed to explore associating factors with carcinogenesis, using a time-dependent Cox proportional hazard model. Time between the judgment of IFN ineffectiveness and initiation of glycyrrhizin therapy was set as a time-dependent variable, in order to clarify the significance of glycyrrhizin therapy in the clinical course of HCV-related chronic liver diseases. Patients with biochemical response with a normal ALT value sustained for at least 6 months after IFN therapy were also excluded in the analysis.

In multivariate analysis, the following three factors influenced the carcinogenesis: (i) fibrotic staging; (ii) sex ($P = 0.006$); and (iii) glycyrrhizin therapy ($P = 0.014$) (Fig. 3). When a hazard of F1-stage fibrosis for carcinogenesis was set as 1 in the model, the hazard ratio of F2 to F3 stage fibrosis was calculated as 2.94 ($P = 0.018$), and that of F4 (cirrhosis) was estimated as 9.21 ($P < 0.001$). Similarly, the hazard ratio for carcinogenesis of male gender was 2.80, compared to female. Use of glycyrrhizin independently decreased the carcinogenesis rate with a hazard ratio of 0.49, in patients with active chronic hepatitis after IFN therapy. The following factors did not affect the HCC appearance rate significantly: age, association of diabetes mellitus, serological grouping of HCV, HCV RNA concentration, AST, ALT at the time before IFN therapy, and bilirubin.

Carcinogenesis in elderly patients

Cumulative carcinogenesis rates were compared between patients with and without glycyrrhizin therapy, in a subgroup of older patients of 60 years old or more. Carcinogenesis rates in the treated ($n = 58$) and

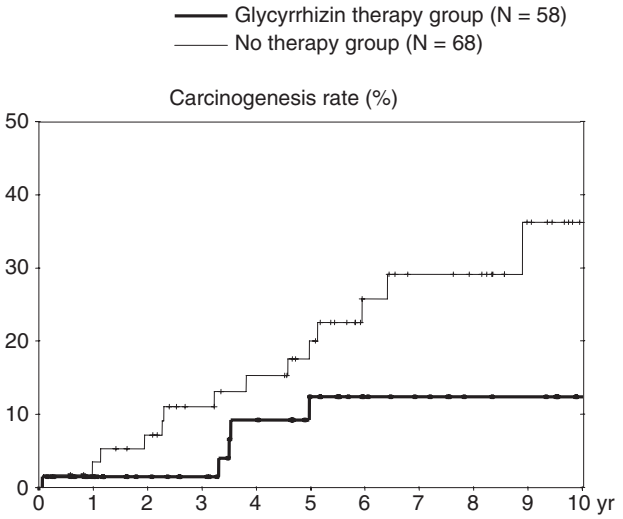

Figure 4 Carcinogenesis rates in elderly patients of 60 years or more.

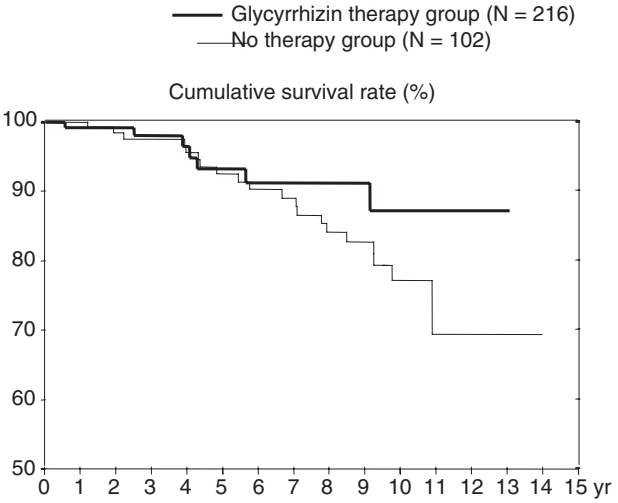

Figure 5 Crude survival rates in patients with and without glycyrrhizin treatment.

untreated groups ($n = 68$) were 12.4% and 20.0% at the end of the fifth year, and 12.4% and 36.2% at the 10th year, respectively (Fig. 4). The carcinogenesis rate in the glycyrrhizin injection group apparently decreased, but marginal statistical difference was observed (log-rank test, $P = 0.052$).

Survival rate

Cumulative survival rates after cessation of IFN therapy were calculated in the treated and untreated groups. Five-year survival rates in patients with and without glycyrrhizin injection therapy were 93.3% and 92.5%, and 10-year rates were 87.2% and 77.1%, respectively (Fig. 5). Although statistical significance was not obtained in the survival rates between the two groups, it showed higher rates in the treated group than in the untreated group.

DISCUSSION

YAMAMOTO *ET AL.*[10] first treated patients with chronic hepatitis with glycyrrhizin (SNMC) and found a distinct improvement in their ALT levels. Suzuki *et al.*[12] confirmed its ability to suppress serum aminotransferase in patients with chronic hepatitis in a randomized controlled trial. Hino *et al.*[13] and Yasuda *et al.*[14] also proved glycyrrhizin to be useful in the improvement of transaminase and liver histology. We once reported that glycyrrhizin was beneficial in carcinogenesis rate in patients with chronic hepatitis type C when it was administered for 10 years or longer.[19] In this study,[20] we assessed the role of glycyrrhizin in the prevention of hepatocellular carcinogenesis in patients with INF-resistant chronic hepatitis C.

Because it requires at least 5 years to show a statistical difference in carcinogenesis rate from hepatitis or cirrhosis between glycyrrhizin-treated and "untreated" groups, a prospective randomized trial using untreated control patients is actually difficult from both ethical and medical viewpoints in Japan, where glycyrrhizin injection therapy is covered by standard medical insurance and it is already regarded as a usual salvaging procedure for IFN-ineffective patients. Therefore, we attempted to carry out a retrospective cohort study, with a statistical adjustment using possible covariates explored in multivariate analysis.

When crude carcinogenesis rates were compared between the treated and untreated patient group, the hepatocellular carcinogenesis rate in the glycyrrhizin therapy group was higher than that of the untreated group (data not shown). Because anti-inflammatory therapy using glycyrrhizin was usually performed for those patients with high ALT values and more active hepatitis, it seemed a quite convincing result that the carcinogenesis rate of the treated group was higher than that of the untreated group. Actually, the treated group consisted of significantly more numbers of patients with high ALT values of twice or more of ULN. When carcinogenesis rates were assessed only in patients with high ALT values of twice or more ULN, the rate of the treated group became slightly higher than that of the

untreated group. Of patients in the treated group, some of them received glycyrrhizin injection therapy several months or a few years after judgment of IFN ineffectiveness. In order to elucidate the cancer preventive activity of glycyrrhizin in active HCV-related liver disease, we further stratified the treated patients into two groups: (i) early treatment group of glycyrrhizin within 2 years after judgment of IFN ineffectiveness; and (ii) late treatment group after 2 years. Because the latter patients were observed without therapy for a considerable period inspite of the "treated group", they were regarded as partly and insufficiently treated with glycyrrhizin from a viewpoint of the entire observation period. We therefore compared the carcinogenesis rates between the treated and untreated patients, excluding those patients of a late treatment group.

The hepatocellular carcinogenesis rate of the patients with a sufficient period of glycyrrhizin injection was significantly lower than that of those without therapy ($P = 0.038$). In the treated group, median ALT values significantly decreased after initiation of the glycyrrhizin injection, suggesting that suppression of the necroinflammatory process was the principal mechanism of the anti-carcinogenic activity of the medicine. The current study dealing with a large cohort ($n = 1249$), showed that the carcinogenesis rate reduces when glycyrrhizin therapy is started at an early time after judgment of IFN ineffectiveness. Cancer preventive activity of glycyrrhizin was also found in a subgroup of elderly patients 60 years or older. Because glycyrrhizin therapy has few side-effects, it should be taken into account for the treatment of aged patients with chronic hepatitis C, from the viewpoint of cancer prevention. Survival rate is likely to increase in those patients undergoing long-term glycyrrhizin injection therapy through suppression of aggressive necroinflammatory process and suppression of liver-related morbidity and mortality.

CONCLUSIONS

AS CARCINOGENESIS IS not a single-step event, but a complex, multistep process, the exact mechanism of the glycyrrhizin activity in suppression of liver carcinogenesis still remains unknown. One of the principal roles of long-term administration of glycyrrhizin in decreasing the carcinogenesis rate seemed to be anti-inflammatory ones, which would retrieve an active carcinogenic process with ALT elevation and continuous hepatic necroinflammation. Glycyrrhizin may only postpone the time of HCC appearance in the clinical course of cirrhosis. Because the entire process of hepatocellular carcinogenesis from initial transformation of a hepatocyte to detectable growth is considered to take at least a few years, the influence of glycyrrhizin on the carcinogenesis rate will not be evaluated in a short period of a few years. Future studies should therefore be aimed at defining the basic oncogenic mechanisms and roles of long-term administration of glycyrrhizin in carcinogenesis in patients with cirrhosis caused by HCV.

In conclusion, a long-term intermittent glycyrrhizin therapy for a few years or more successfully reduced hepatocellular carcinogenesis in patients with HCV-related chronic liver disease. A randomized control study with a larger number of cases, with or without glycyrrhizin therapy, is expected to confirm the effectiveness of this therapy.

CONFLICT OF INTEREST

NO CONFLICT OF interest statement has been received from the author.

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Research Article

Inhibitory Effects of Glycyrrhetinic Acid on DNA Polymerase and Inflammatory Activities

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We investigated the inhibitory effect of three glycyrrhizin derivatives, such as Glycyrrhizin (compound **1**), dipotassium glycyrrhizate (compound **2**) and glycyrrhetinic acid (compound **3**), on the activity of mammalian pols. Among these derivatives, compound **3** was the strongest inhibitor of mammalian pols α, β, κ, and λ, which belong to the B, A, Y, and X families of pols, respectively, whereas compounds **1** and **2** showed moderate inhibition. Among the these derivatives tested, compound **3** displayed strongest suppression of the production of tumor necrosis factor-α (TNF-α) induced by lipopolysaccharide (LPS) in a cell-culture system using mouse macrophages RAW264.7 and peritoneal macrophages derived from mice. Moreover, compound **3** was found to inhibit the action of nuclear factor-κB (NF-κB) in engineered human embryonic kidney (HEK) 293 cells. In addition, compound **3** caused greater reduction of 12-*O*-tetradecanoylphorbol-13-acetate-(TPA-) induced acute inflammation in mouse ear than compounds **1** and **2**. In conclusion, this study has identified compound **3**, which is the aglycone of compounds **1** and **2**, as a promising anti-inflammatory candidate based on mammalian pol inhibition.

1. Introduction

The human genome encodes at least 15 DNA polymerases (pols) that conduct cellular DNA synthesis [1, 2]. Eukaryotic cells contain 3 replicative pols (α, δ, and ε), 1 mitochondrial pol (γ), and at least 11 nonreplicative pols [β, ζ, η, θ, ι, κ, λ, μ, ν, terminal deoxynucleotidyl transferase (TdT) and REV1] [3, 4]. Pols have a highly conserved structure, which means that their overall catalytic subunits show little variance among species. Enzymes with conserved structures usually perform important cellular functions, the maintenance of which provides evolutionary advantages. On the basis of sequence homology, eukaryotic pols can be divided into 4 main families, termed A, B, X, and Y [4]. Family A includes mitochondrial pol γ, as well as pols θ and ν. Family B includes 3 replicative pols (α, δ, and ε) and pol ζ. Family X comprises pols β, λ, and μ, as well as TdT, and lastly, family Y includes pols η, ι, and κ, in addition to REV1.

We have been screening for selective inhibitors of each pol derived from natural products, in particular from traditional medical plants, food materials and food additives, for more than 15 years [5, 6]. Licorice (fabaceae and glycyrrhiza), a well-known herb plant with biological properties, has been widely used in food additives (sweetener

and flavoring agent), nutraceuticals (liver protection), and the treatment of various inflammatory diseases since ancient times [7]. Licorice root has been used since ancient Egyptian, Greek, and Roman times in the West, and since the Former Han era (the 2nd-3rd century B.C.) in ancient China in the East. In traditional Chinese medicine, licorice is one of the most frequently used drugs. There are many reports about its pharmacological actions and physiological functions, such as detoxification, induction of decreased blood glucose, and antitumor, anti-inflammatory, hypocholesterolemic, antiestrogen, antihistamine, antiallergic hepatitis, antileukemia, anticancer, and antibiotic-like effects [8].

The seed stock of licorice (*Glycyrrhiza glabra* L.) has 6 varieties, and licorice production is widely distributed over the Eurasian continent, South Europe, Central Asia, China and Russia. In particular, *G. inflata*, *G. uralensis,* and *G. glabra* are utilized in the production of sweeteners, cosmetics, and medicines. The major constituents of licorice root are triterpenoid saponins, such as glycyrrhizin (3–8%). Glycyrrhizin (glycyrrhizic acid and glycyrrhizinic acid) is a sweet-tasting compound, and is 30–50 times as sweet as sucrose. Glycyrrhizin (compound **1**) consists of one molecule of glycyrrhetinic acid (compound **3**) and two molecules of glucuronic acid (Figure 1), and it is converted to these constituents by acidic hydrolysis [9].

In our studies of pol inhibitors, we have found that pol λ-selective inhibitors, such as curcumin derivatives [10–12], have anti-inflammatory activity against 12-*O*-tetradecanoylphorbol-13-acetate-(TPA-) induced inflammation [13–15]. Although tumor promoters, such as TPA, are classified as compounds that promote tumor formation [16], they also cause inflammation and are commonly used as artificial inducers of inflammation in order to screen for anti-inflammatory agents [17]. Tumor promoter-induced inflammation can be distinguished from acute inflammation, which is exudative and accompanied by fibroblast proliferation and granulation. The tumor promoter TPA is frequently used to search for new types of anti-inflammatory compound. TPA not only causes inflammation, but also influences mammalian cell growth [18], suggesting that the molecular basis of the inflammation stems from pol reactions related to cell proliferation. This relationship, however, needs to be investigated more closely.

In this study, we have investigated the inhibitory effects of glycyrrhizin (compound **1**) and its derivatives, including the potassium salt of compound **1** (dipotassium glycyrrhizate and compound **2**) and glycyrrhetinic acid (compound **3**) (Figure 1), on mammalian pol activity and inflammatory responses both *in vitro* and *in vivo*. In particular, we demonstrate that these compounds exert inhibitory effects against TNF-α production and NF-κB activation in cell culture models of inflammatory response. The relationship between the inhibition of pols and the anti-inflammatory action of the glycyrrhizin derivatives is discussed.

2. Materials and Methods

2.1. Materials. Glycyrrhizin (3-*O*-(2-*O*-β-D-glucopyranuronosyl-α-D-glucopyranuronosyl)-18β-glycyrrhetinic acid, compound **1**), dipotassium glycyrrhizate (compound **2**) and glycyrrhetinic acid (compound **3**) were obtained from Maruzen Pharmaceuticals Co., Ltd. (Onomichi, Hiroshima, Japan), and each compound was purified to special grade purity. These structures are shown in Figure 1. Chemically synthesized DNA templates, such as poly(dA), was purchased from GE Healthcare Bio-Sciences (Little Chalfont, UK). Radioisotope-labeled nucleotides, such as [^{3}H]-deoxythymidine 5′-triphosphate (dTTP) (43 Ci/mmol), was purchased from MP Biomedicals, LLC (Tokyo, Japan). The oligo(dT)$_{18}$ DNA primer was customized by Sigma-Aldrich Japan K.K. (Hokkaido, Japan). Lipopolysaccharide (LPS) and TPA were purchased from Sigma-Aldrich. All other reagents were of analytical grade and were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

2.2. Mammalian Pol Assays. Pol α was purified from calf thymus by immunoaffinity column chromatography as described by Tamai et al. [19]. The human pol γ catalytic gene was cloned into pFastBac (Invitrogen Japan K.K., Tokyo Japan). Histidine-tagged enzyme was expressed using the BAC-TO-BAC HT Baculovirus Expression System according to the supplier's manual (Life Technologies, MD) and purified using ProBound resin (Invitrogen Japan K.K.) [20]. A truncated form of pol κ (residues 1–560) with a 6xHis tag attached at the C-terminus was overproduced in *E. coli* and purified as described previously [21]. Recombinant human His-pol λ was overexpressed and purified according to a method described previously [22].

The reaction mixture for calf pol α was described previously [23, 24]. The reaction mixture for human pol γ was previously described by Umeda et al. [20]. The reaction mixtures for mammalian pols κ and λ were the same as that for calf pol α. The components of the pol assay were poly(dA)/oligo(dT)$_{18}$ (A/T = 2/1) and dTTP as the DNA template-primer and 2′-deoxynucleoside 5′-triphosphate (dNTP) substrate, respectively. The glycyrrhizin derivatives (i.e., compounds **1**–**3**) were dissolved in distilled dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 sec. The sonicated samples (4 μL) were mixed with 16 μL of each enzyme (final amount, 0.05 units) in 50 mM Tris-HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol, and 0.1 mM EDTA, and kept at 0°C for 10 min. These inhibitor enzyme mixtures (8 μL) were added to 16 μL of each of standard enzyme reaction mixture (50 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, 1 mM $MgCl_2$, 15% glycerol, 10 μM poly(dA)/oligo(dT)$_{18}$, and 10 μM [^{3}H]-dTTP), and incubation was carried out at 37°C for 60 min. Activity in the absence of inhibitor was considered to be 100%, and the activity remaining at each concentration of inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol dNTP (dTTP) into the synthetic DNA template-primer (poly(dA)/oligo(dT)$_{18}$, A/T = 2/1) in 60 min at 37°C under normal reaction conditions for each enzyme (scintillation counts: approximately 1 pmol of incorporated radioactive nucleotide = 100 cpm) [23, 24].

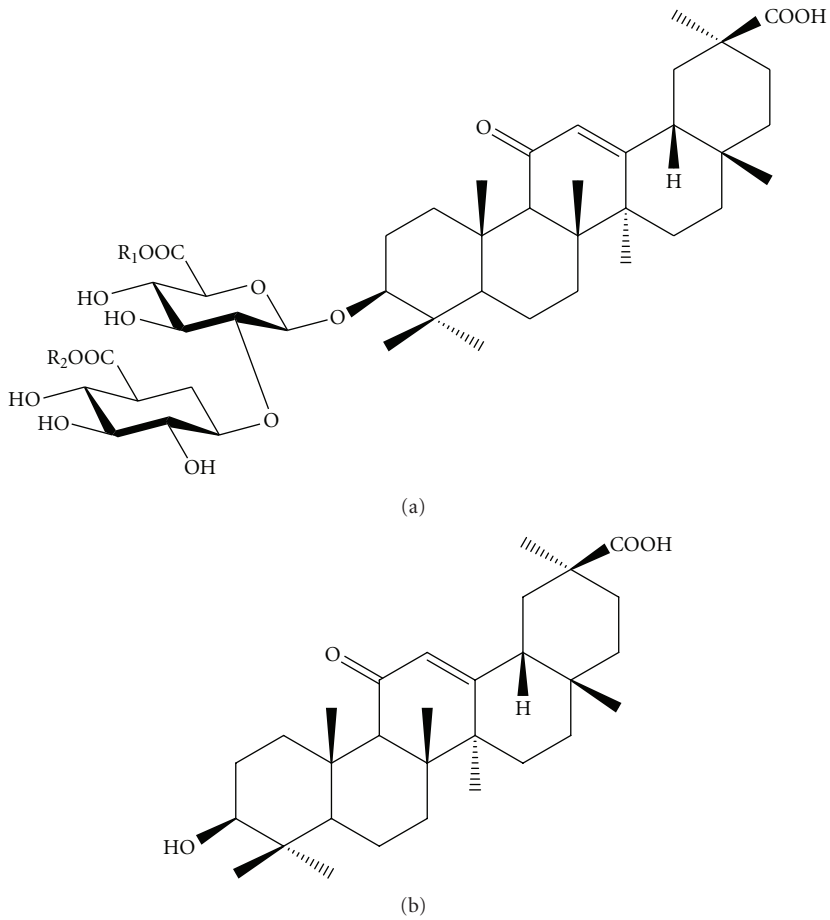

Figure 1: Structure of the glycyrrhizin derivatives. (a) Glycyrrhizin (3-*O*-(2-*O*-*β*-D-glucopyranuronosyl-*α*-D-glucopyranuronosyl)-18*β*-glycyrrhetinic acid; compound **1:** R_1 and R_2 = H) and dipotassium glycyrrhizate (compound **2**: R_1 and R_2 = K^+). (b) Glycyrrhetinic acid (compound **3**).

2.3. Animal Experiments. All animal studies were performed according to the guidelines outlined in the "Care and Use of Laboratory Animals" of Kobe University. The animals were anesthetized with pentobarbital before undergoing cervical dislocation. Female 8-week-old C57BL/6 mice that had been bred in-house with free access to food and water were used for all experiments. All of the mice were maintained under a 12-h light/dark cycle and housed at a room temperature of 25°C.

2.4. Cell Culture. A mouse macrophage cell line, RAW264.7, was obtained from American Type Culture Collection (ATCC) (Manassas, Va, USA). The cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 4.5 g of glucose per liter plus 10% fetal calf serum, 5 mM L-glutamine, 50 units/mL penicillin and 50 units/mL streptomycin. HEK-Blue hTLR4 cells were purchased from InvivoGen (San Diego, Calif, USA), and were maintained in complete Dulbecco minimal essential medium with selective antibiotics, in accordance with the manufacturer's instructions (InvivoGen). The cells were cultured at 37°C in standard medium in a humidified atmosphere of 5% CO_2–95% air.

2.5. Preparation of Peritoneal Macrophages. Female C57BL/6 mice were injected intraperitoneally with phosphate-buffered saline (PBS), and the peritoneal cavity of the mice was washed with PBS. The PBS was collected**,** and peritoneal macrophages were separated from the PBS by centrifugation at 1,500 ×g for 10 min.

2.6. Measurement of Cytotoxicity on a Cell-Culture Medium. Approximately 1×10^4 cells per well were inoculated into 96-well microplates, and then compounds **1–3** were diluted to various concentrations and applied to each well. After incubation for 24 h, the survival rate of RAW264.7 cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [25].

2.7. Measurement of TNF-α in a Cell-Culture Medium. RAW264.7 cells or peritoneal macrophages were placed in a 12-well plate at 5×10^4 cells/well and incubated for 24 h. The cells were pretreated with various concentrations of compounds **1–3** for 30 min before the addition of 100 ng/mL LPS. After stimulation with LPS for 24 h, the cell culture medium was collected to measure the amount of TNF-*α*

secreted. The concentration of TNF-α in the culture medium was quantified by using a commercially available enzyme-linked immunosorbent assay (ELISA) development system (Bay Bioscience Co., Ltd., Kobe, Japan) in accordance with the manufacturer's protocol.

2.8. Measurement of the Nuclear Translocation of NF-κB in HEK293 Cells. HEK-Blue hTLR4 cells are engineered human embryonic kidney (HEK) 293 cells that stably coexpress human Toll-like receptor 4 (TLR4) and an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. These cells were placed in a 96-well plate at 1×10^4 cells/well and incubated for 24 h. The cells were pre-incubated with various concentrations of compounds **1–3** for 30 min before the addition of 1 ng/mL LPS. After stimulation with LPS for 24 h, NF-κB-induced SEAP activity was assessed by using QUANTI-Blue (a medium used for the detection and quantification of SEAP; InvivoGen) and by reading the absorbance at 650 nm by means of an ELISA plate reader.

2.9. TPA-Induced Anti-Inflammatory Assay in Mouse. The mouse inflammatory test was performed according to Gschwendt's method [26]. In brief, an acetone solution of compounds **1–3** (250 or 500 μg in 20 μL) or 20 μL of acetone as a vehicle control was applied to the inner part of the mouse ear. Thirty minutes after the test compound was applied, a TPA solution (0.5 μg/20 μL of acetone) was applied to the same part of the ear. To the other ear of the same mouse, methanol, followed by TPA solution, was applied as a control. After 7 h, a disk (6 mm diameter) was obtained from each ear and weighed. The inhibitory effect (IE) is presented as a ratio of the increase in weight of the ear disks: IE: {[(TPA only) − (tested compound plus TPA)]/[(TPA only) − (vehicle)] × 100}.

3. Results

*3.1. Effect of Glycyrrhizin Derivatives (Compounds **1–3**) on Mammalian Pol Activity.* Initially, we investigated the *in vitro* biochemical action of glycyrrhizin (compound **1**) and its derivatives (compounds **2** and **3**). The inhibition of four mammalian pols, namely, calf pol α, human pol γ, human pol κ, and human pol λ, by each compound at 20 and 100 μM was investigated. Pols α, γ, κ, and λ were used as representatives of the B, A, Y, and X families of pols, respectively [1–3]. As shown in Figure 2, at 100 μM these compounds inhibited the activity of all mammalian pols tested, because the relative pol activity was less than 50% after incubation with these compounds. At 20 μM, compound **3** also inhibited the activity of these pols, whereas compounds **1** and **2** had no effect; therefore, the inhibitory effect of these compounds on mammalian pols was ranked as follows: compound **3** > compound **1** = compound **2**. Compound **3** showed almost the same strength of inhibition among the four mammalian pols tested; that is, the concentration of compound **3** giving 50% inhibition of pols α, γ, κ, and λ was 16.1, 19.3, 15.8, and 13.7 μM, respectively. When activated

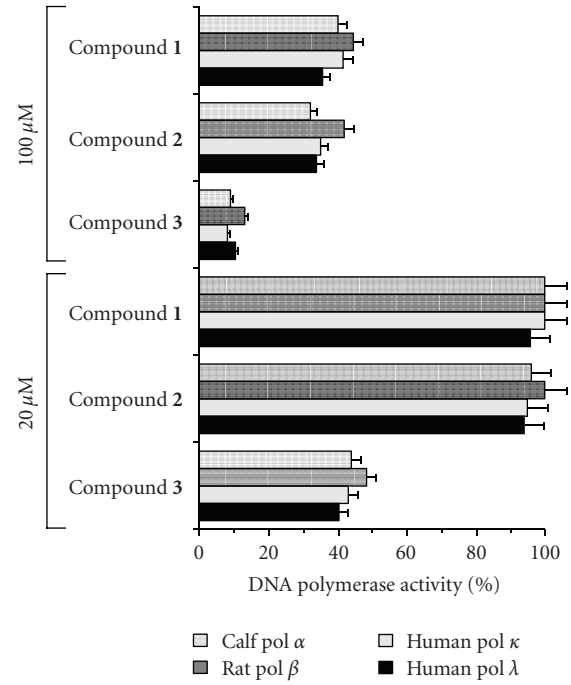

Figure 2: Inhibitory effects of glycyrrhizin derivatives (compounds **1–3**) on the activity of mammalian pols. Each compound (20 and 100 μM) was incubated with calf pol α (B-family pol), human pol γ (A-family pol), human pol κ (Y-family pol), and human pol λ (X-family pol) (0.05 units each). Pol activity in the absence of the compound was taken as 100%, and the relative activity is shown. Data are shown as the mean ± SE ($n = 4$).

DNA (i.e., bovine deoxyribonuclease I-treated DNA) and dNTP were used as the DNA template-primer and nucleotide substrate instead of synthesized DNA [poly(dA)/oligo$(dT)_{18}$ (A/T = 2/1)] and dTTP, respectively, the inhibitory effects of these compounds did not change (data not shown).

*3.2. Inhibitory Effect of Glycyrrhizin Derivatives (Compounds **1–3**) on LPS-Induced Inflammatory Responses in Cultured Cells.* Next, we investigated whether the three glycyrrhizin derivatives could inhibit both the reduction of TNF-α production and the nuclear translocation of NF-κB caused by LPS stimulation in cultured cells. The inflammatory cytokine TNF-α activates the NF-κB signaling pathway by binding to the TNF-α receptor (TNFR) and thereby initiates an inflammatory response, resulting in various inflammatory diseases [27]. In cultured macrophage RAW264.7 cells, no compound showed cytotoxicity at 25 to 250 μM (Figure 3); therefore, the LD_{50} values of compounds **1–3** were >250 μM. These compounds also had no effect on the cell proliferation of peritoneal macrophages (data not shown). As shown in Figure 4(a), RAW264.7 cells produced 693 pg/mL of TNF-α after LPS treatment. Compounds **1–3** slightly suppressed this LPS-stimulated production of TNF-α, showing dose-dependent inhibition at 100 and 200 μM. The suppression of TNF-α production by compound **3** was stronger than that by compounds **1** and **2**. Figure 4(b) shows the dose-dependent suppression of LPS-evoked TNF-α production in peritoneal

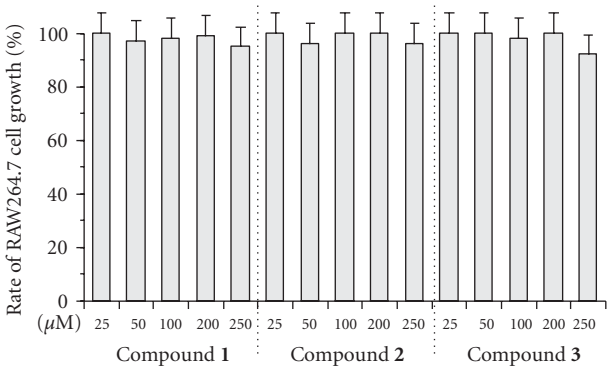

Figure 3: Effect of glycyrrhizin derivatives (compounds **1–3**) on the proliferation of the mouse macrophage RAW264.7 cell growth. The cells were added the indicated concentrations of each compound and incubated for 24 h, and the rate of cultured cell growth inhibition was determined by MTT assay [25]. Cell growth inhibition of the cells in the absence of the compound was taken as 100%. Data are shown as the mean ± SE ($n = 5$).

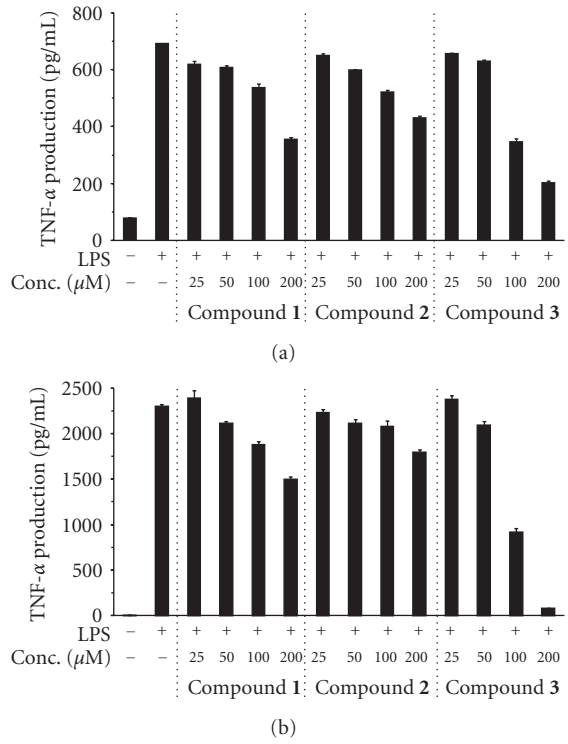

Figure 4: Inhibitory effects of glycyrrhizin derivatives (compounds **1–3**) on LPS-induced production of TNF-α in mouse macrophages. (a) The mouse macrophage cell line RAW264.7 was pretreated with the indicated concentrations of the glycyrrhizin derivatives for 30 min, and then treated with 100 ng/mL LPS for 24 h. (b) Peritoneal macrophages derived from mice were pretreated with the indicated concentrations of each compound for 30 min, and then with 100 ng/mL LPS for 24 h. The TNF-α concentration in the cell medium was measured by ELISA. Data are shown as the mean ± SE ($n = 5$).

macrophages derived from mice by the glycyrrhizin derivatives. The inhibitory effect of compounds **1–3** showed almost the same tendency in peritoneal macrophages as in the macrophage cell line RAW264.7 although compound **3** significantly suppressed the production of TNF-α in peritoneal macrophages. From these results, the strength of the inhibitory effect of these compounds can be ranked as follows: compound **3** > compound **1** = compound **2**.

NF-κB is known to be the rate-controlling factor in inflammatory responses. We, therefore, examined the inhibitory effect of compounds **1–3** on the LPS-induced nuclear translocation of NF-κB in RAW264.7 cells (Figure 5). In this experiment, we used HEK-Blue hTLR4 cells, which are HEK293 cells that have been engineered to report TLR4-linked NF-κB activation. In brief, this cell line is transfected with TLR4 and an NF-κB-inducible alkaline phosphatase reporter gene system. On interaction with the appropriate ligand, TLR4 transduces a signal that results in NF-κB activation. In this assay system, the amount of NF-κB undergoing nuclear translocation in the cells after LPS stimulation was significantly reduced by compound **3** at 100 and 200 μM. By contrast, compounds **1** and **2** had no effect on the LPS-stimulated nuclear translocation of NF-κB; thus, the ranking was compound **3** ≫ compound **1** = compound **2**. These results demonstrate that compound **3** can strongly suppress the nuclear translocation of NF-κB by inhibiting the production of TNF-α. The effects of glycyrrhizin derivatives on the molecular mechanism underlying inflammatory responses will be addressed in future studies.

3.3. Effect of Glycyrrhizin Derivatives (Compounds 1–3) on TPA-Induced Anti-Inflammatory Activity. In a previous study of pol inhibitors, we found that there is a relationship between pol λ inhibitors and TPA-induced acute anti-inflammatory activity [6, 13, 14]. Thus, using the mouse ear inflammatory test, we examined the anti-inflammatory activity of the glycyrrhizin derivatives. Application of TPA (0.5 μg) to the mouse ear induced edema, resulting in a 241% increase in the weight of the ear disk 7 h after application. As shown in Figure 6, pretreatment with compounds **1–3** dose-dependently suppressed inflammation, and the effect of these compounds was ranked as follows: compound **3** > compound **1** = compound **2**. Thus, these *in vivo* data from the mouse ear study showed almost the same trend as the LPS-induced inflammatory response data from cultured cells (Figure 5). Furthermore, the anti-inflammatory effect of these compounds showed the same tendency as their inhibitory effect on mammalian pols including pol λ, which was strongly inhibited by compound **3** (Figure 2). These results suggest that inhibition of pol λ inhibitory activity has a positive correlation with the anti-inflammatory activity observed.

4. Discussion

We have shown here that glycyrrhetinic acid (compound **3**) was the strongest inhibitor of mammalian pols α, γ, κ, and λ (Figure 2), and this compound prevented the inflammatory response among the three glycyrrhizin derivatives (compounds **1–3**) tested (Figures 4 to 6). Compound **3** is the agly-

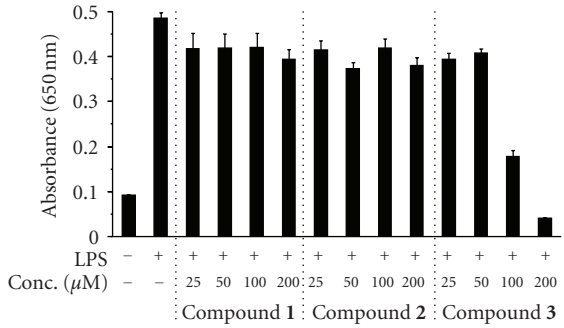

Figure 5: Inhibitory effects of glycyrrhizin derivatives (compounds **1**–**3**) on nuclear translocation of NF-κB in engineered HEK293 cells. HEK-Blue hTLR4 cells were pretreated for 30 min with the indicated concentrations of the glycyrrhizin derivatives and then treated with 1 ng/mL LPS for 24 h. NF-κB-induced SEAP activity was assessed by using QUANTI-Blue and by reading the absorbance at 650 nm via an ELISA plate reader. Data are shown as the mean ± SE ($n = 5$).

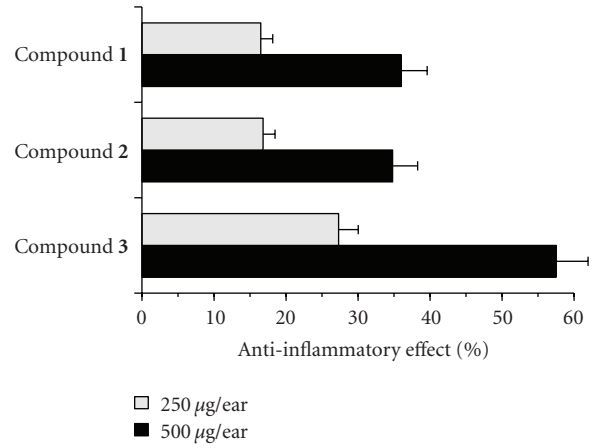

Figure 6: Anti-inflammatory activity of glycyrrhizin derivatives toward TPA-induced edema on mouse ear. Each compound (250 μg, gray bar; and 500 μg, black bar) was applied individually to one ear of a mouse, and after 30 min TPA (0.5 μg) was applied to both ears. Edema was evaluated after 7 h. The inhibitory effect is expressed as a percentage of edema. Data are shown as the means ± SE ($n = 6$).

cone of compound **1**, and the pentacyclic triterpenoid structure must be important for the inhibitory activity shown by these compounds. Glycyrrhizin (compounds **1**) and dipotassium glycyrrhizate (compound **2**), which is the dipotassium salt of compound **1**, are triterpenoid saponin glycosides, and they both have two molecules of glucuronic acid (Figure 1). Compound **2** is a major sweet-tasting food additive, and it is 5-fold sweeter than compound **1** [7]. The inhibitory effect of compound **1** on pol activity and inflammation showed the same tendency as that of compound **2**; therefore, the salt form had no effect on the inhibitory activities of glycyrrhizin.

Eukaryotic cells reportedly contain 15 pol species belonging to four families: namely, family A (pols γ, θ, and ν), family B (pols α, δ, ε, and ζ), family X (pols β, λ, and μ and TdT) and family Y (pols η, ι, and κ and REV1) [3, 4]. As reported previously, the phenolic compound curcumin, which is a known anti-inflammatory agent, is a pol λ-specific inhibitor [6, 13, 14]. Intriguingly, on the basis of compound **3**, the principle molecular target of the glycyrrhizin derivatives is also pol λ. Among the X family of pols, pol λ has an unclear biochemical function, although it seems to work in a similar way to pol β [28]. Pol β is involved in the short-patch base excision repair (BER) pathway [29–32], as well as playing an essential role in neural development [33]. Recently, pol λ was found to possess 5′-deoxyribose-5-phosphate (dRP) lyase activity, but no apurinic/apyrimidinic (AP) lyase activity [34]. Pol λ is able to substitute for pol β during *in vitro* BER, suggesting that pol λ also participates in BER. Northern blot analysis indicated that transcripts of pol β are abundantly expressed in the testis, thymus, and brain in rats [35], whereas pol λ is efficiently transcribed mostly in the testis [36]. Bertocci et al. reported that mice in which pol λ expression is knocked out are not only viable and fertile, but also display a normal hypermutation pattern [37].

As well as causing inflammation, TPA influences cell proliferation and has physiological effects on cells owing to its tumor-promoting activity [18]. Therefore, anti-inflammatory agents are expected to suppress DNA replication/repair/recombination in nuclei in relation to the action of TPA. Because pol λ is a repair/recombination-related pol [28], our finding—that pol λ is the molecular target of glycyrrhizin derivatives—is in good agreement with this expected mechanism of anti-inflammatory agents. As a result, any pol λ inhibitor might also be an inhibitor of inflammation.

Compound **1** has been reported to possess various pharmacological properties such as anti-inflammatory activity [38], inhibition of prostaglandin E2 production in rat macrophages [39], antiallergic activity [40], antiviral activity [41, 42], and induction of interferon-γ [43]. In Japan, a preparation of compound **1**, Stronger-Neo Minophagen C, has been used extensively to treat chronic hepatitis for more than 30 years. Compound **3** is also known to have wide pharmacological effects such as anti-inflammatory [44, 45], antitumor [46], and antihepatotoxic [47] activities, and inhibition of the growth of mouse melanoma [48]. In 1989, it was reported that compound **3** strongly inhibits renal 11β-hydroxysteroid dehydrogenase in rat [49]; this inhibition has been regarded as a cause of the pseudoaldosteronism that is occasionally induced by the administration of a compound **3** preparation or Carbenoxolone. However, the mechanisms underlying the therapeutic effects of the glycyrrhizin derivatives remain unknown.

In this study, therefore, we investigated the inhibitory effect of the glycyrrhizin derivatives on mammalian pols, which are responsible for DNA replication leading to cell proliferation and DNA repair/recombination, as well as the relationship between the degree of the suppression of LPS-evoked TNF-α production and anti-inflammatory activity. The molecular mechanism that links the LPS-induced in flammatory response and anti-inflammatory activity in the model of TPA-induced ear edema is unknown. Because activated NF-κB has been observed in a model of TPA-induced ear edema [50], the anti-inflammatory effects of compound **3** may be, at least in part, dependent on

the inhibition of NF-κB activation. Our study indicates that compound **3** is useful as an NF-κB inhibitor and may be a potent chemopreventive agent against inflammation. As a result, we found a positive correlation between the pol inhibitory activity and anti-inflammatory activity of compound **3**. The relationship between these activities, namely, pol λ inhibition and anti-inflammatory action, may be useful as a new and convenient *in vitro* assay to screen for novel anti-inflammatory compounds.

5. Conclusions

Our study is the first to demonstrate that glycyrrhetinic acid (compound **3**), which is the aglycone of glycyrrhizin (compounds **1**), potently inhibited the activity of mammalian pols including pol λ. Compound **3** also reduced TNF-α production and NF-κB activation and suppressed mouse ear inflammation stimulated by TPA. Thus, compound **3** could be an anti-inflammatory agent based on pol λ inhibition.

Abbreviations

pol:	DNA polymerase (E.C. 2.7.7.7)
TPA:	12-*O*-tetradecanoylphorbol-13-acetate
TNF:	Tumor necrosis factor
NF:	Nuclear factor
LPS:	Lipopolysaccharide
TdT:	Terminal deoxynucleotidyl transferase
dTTP:	2′-deoxythymidine 5′-triphosphate
DMSO:	Dimethylsulfoxide
PBS:	Phosphate buffered saline
ELISA:	Enzyme-linked immunosorbent assay
HEK:	Human embryonic kidney
TRL:	Toll-like receptor
SEAP:	Secreted embryonic alkaline phosphatase
IE:	Inhibitory effect
BER:	Base excision repair
dRP:	5′-deoxyribose-5-phosphate
AP:	Apurinic/apyrimidinic.

Conflict of Interests

There is no conflict of interests related to this work.

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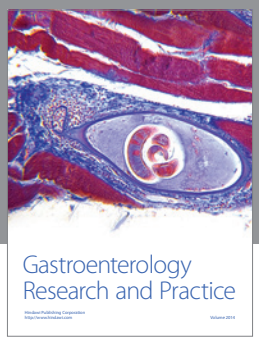

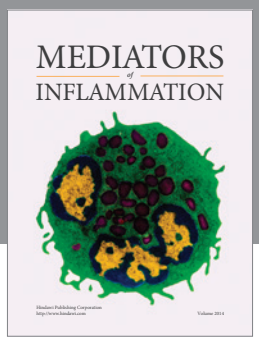

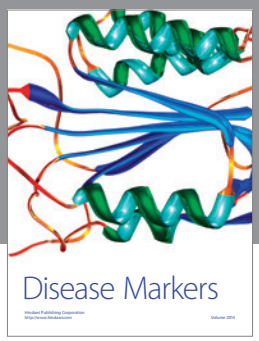

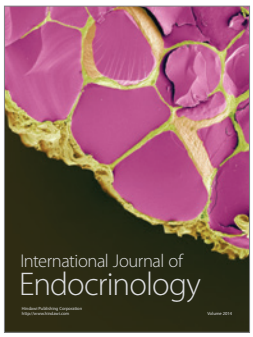

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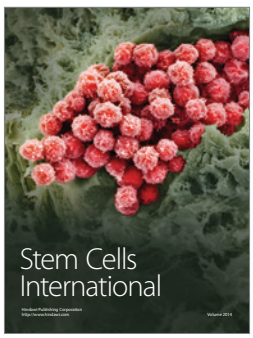

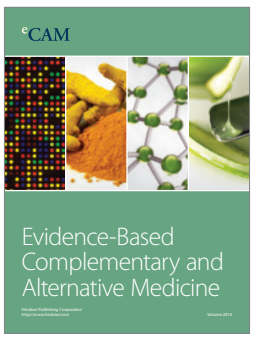

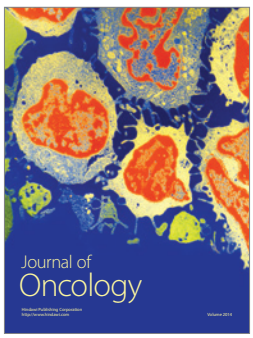

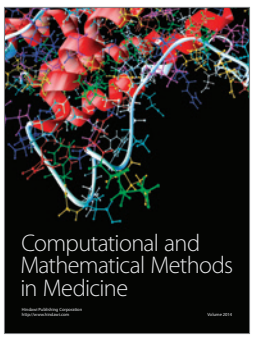

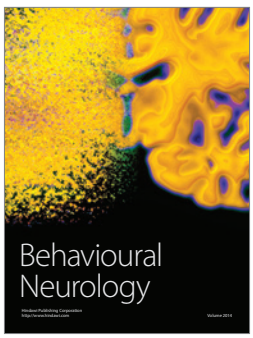

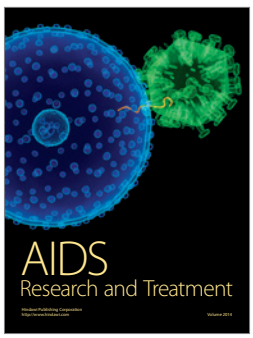

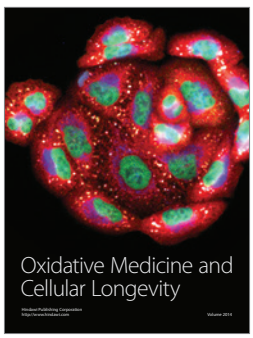

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Induction of inducible nitric oxide synthase expression by 18β-glycyrrhetinic acid in macrophages

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Abstract Glycyrrhizin (GL), a triterpenoid saponin fraction of licorice, is reported to have anti-viral and anti-tumor activities and is metabolized to 18β-glycyrrhetinic acid (GA) in the intestine by intestinal bacteria. However, the mechanism underlying its effects is poorly understood. To further elucidate the mechanism of GA, the aglycone of GL, we investigated the effects of GA on the release of nitric oxide (NO) and at the level of inducible NO synthase (iNOS) gene expression in mouse macrophages. We found that GA elicited a dose-dependent increase in NO production and in the level of iNOS mRNA. Since iNOS transcription has been shown to be under the control of the transcription factor nuclear factor κB (NF-κB), the effects of GA on NF-κB activation were examined. Transient expression assays with NF-κB binding sites linked to the luciferase gene revealed that the increased level of iNOS mRNA, induced by GA, was mediated by the NF-κB transcription factor complex. By using DNA fragments containing the NF-κB binding sequence, GA was shown to activate the protein/DNA binding of NF-κB to its cognate site, as measured by electrophoretic mobility shift assay. These results demonstrate that GA stimulates NO production and is able to up-regulate iNOS expression through NF-κB transactivation in macrophages.

Key words: 18β-Glycyrrhetinic acid; Macrophage; Inducible nitric oxide synthase; Nuclear factor κB

1. Introduction

In immunocompetent hosts, the innate and adaptive arms of the immune system are relatively efficient at containing and killing microbial pathogens. Nitric oxide (NO) is a radical messenger molecule produced by the enzyme NO synthase (NOS) [1]. Three NOS isoforms have been characterized: the constitutively expressed neuronal NOS, endothelial NOS, and the inducible isoform of NOS (iNOS). iNOS expression is significantly induced by lipopolysaccharide (LPS) or cytokines in a variety of immune cells, including macrophages [1]. Moreover, NO has been identified as the major effector molecule involved in the destruction of microorganisms and tumor cells by activated macrophages during the non-specific host defense of the immune system [2–5]. In contrast, with these host protective actions, NO has also been implicated as a mediator of tissue injury. As a host defense molecule, NO also inhibits the proliferation of viruses, such as ectromelia virus, coxsackie virus B3, cytomegaloviruses, and hepatitis B virus [5–10]. In macrophages, nuclear factor κB (NF-κB) in cooperation with other transcription factors has been found to coordinate the expression of genes encoding iNOS. Moreover, NF-κB plays a critical role in the activation of immune cells by up-regulating the expression of many cytokines essential for the immune response [11].

Licorice (*Glycyrrhizza glabra* L.) and its main water-soluble constituent glycyrrhizin (GL), a pentacyclic triterpene derivative of the β-amyrin type (oleanane), have been widely used as an antidote, demulcent and as a folk medicine for generations in Asia and Europe, and it is currently used as a flavoring and sweetening agent in food products. After oral administration or intravenous injection, GL has been shown to be hydrolyzed by the glucuronidase in intestinal bacteria to its active principle aglycone, 18β-glycyrrhetinic acid (GA), which is then absorbed into the blood [12]. GL and GA have been shown to possess several beneficial pharmacological activities, which include an anti-ulcerative effect, anti-inflammatory activity, interferon (IFN)-γ induction, and anti-hepatotoxicity effect [13–15]. Moreover, GL has also been described as an anti-viral agent [16–18], and to have anti-tumor activity [19,20]. GL is extensively used in Japan and is being examined in Europe in patients with active and chronic hepatitis [21,22]. However, the details of its mechanism remain unclear.

It is well known that the inducible production of NO by macrophages inhibits the growth of many pathogens, including bacteria, fungi, viruses, and parasites. Thus, it is possible that GA-derived NO production may mediate the anti-viral and anti-tumor activities of GA. To test this hypothesis, we investigated the effects of GA on NO production and the molecular mechanisms underlying this effect.

2. Materials and methods

2.1. Chemicals

Chemicals and cell culture materials were obtained from the following sources: GA, *Escherichia coli* 0111:B4 LPS and polymyxin B sulfate from Sigma Co.; BAY 11-7082 from Biomol; MTT-based colorimetric assay kit from Roche Co.; LipofectAMINE Plus, RPMI 1640, fetal bovine serum, and penicillin–streptomycin solution from Life Technologies, Inc.; pGL3-4κB-Luc and the luciferase assay system from Promega; pCMV-β-gal from Clontech; and AmpliTaq® DNA polymerase from Perkin Elmer. Other chemicals were of the highest commercial grade available.

2.2. Animals

Specific pathogen-free BALB/C mice (female, 5–7 weeks old) were

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obtained from KRIBB (South Korea). Animals were housed under normal laboratory conditions, i.e. at 21–24°C and 40–60% relative humidity under a 12 h light/dark cycle with free access to standard rodent food and water.

2.3. Preparation of peritoneal macrophages and cell cultures

Peritoneal macrophages were isolated from mice and cultured as described previously [23]. RAW 264.7 cells, a mouse macrophage cell line, were obtained from the American Type Culture Collection (Bethesda, MD, USA), and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator. GA was dissolved in dimethylsulfoxide and added directly to the culture media. Control cells were treated only with solvents, the final concentration of which never exceeded 0.1%, and this concentration did not show any effect on the assay systems.

2.4. Cell viability

Cell viability was assessed using a MTT-based colorimetric assay kit (Roche Co.), according to the manufacturer's instructions.

2.5. Nitrite assay

Peritoneal macrophages (2×10^5 cells/ml) or RAW 264.7 cells (5×10^5 cells/ml) were cultured in 48-well plates. After incubating for 24 h, NO synthesis was determined by assaying the culture supernatants for nitrite, the stable reaction product of NO with molecular oxygen, using Griess reagent as described previously [23].

2.6. Endotoxin assay

An E-Toxate test (*Limulus* amebocyte lysate; Sigma Chemical Co.) was used to assay GA for the presence of Gram-negative bacterial endotoxin (LPS), according to the manufacturer's instructions.

2.7. RNA preparation and iNOS mRNA analysis by reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 cells were cultured with GA at a density of 1×10^6 cells/ml for 6 h. Total cellular RNA was isolated by the acidic phenol extraction procedure of Chomczynski and Sacchi [24]. cDNA synthesis, semiquantitative RT-PCR for iNOS and β-actin mRNA, and the analysis of results were performed as described previously [23]. cDNA was synthesized from 2 μg of total RNA using an Omniscript RT-PCR kit as instructed. A cycle number was used that fell within the exponential range of response for iNOS (754 bp, 35 cycles) and β-actin (153 bp, 17 cycles). PCR reactions were electrophoresed through a 2.5% agarose gel and visualized by ethidium bromide staining and UV irradiation. Gel images were captured on a Gel Doc Image Analysis System (Kodak) and the yield of PCR products was normalized to β-actin after quantitative estimation using NIH Image software (Bethesda, MD, USA). The relative expression levels were arbitrarily set at 1.0 in the control group.

2.8. Transfection and luciferase and β-galactosidase assays

RAW 264.7 cells (5×10^5 cells/ml) were plated in each well of a 12-well plate, and 12 h later transiently co-transfected with the plasmids pGL3-4κB-Luc and pCMV-β-gal, using LipofectAMINE Plus according to the manufacturer's protocol. Briefly, the transfection mixture containing 0.5 μg of pGL3-4κB-Luc and 0.2 μg of pCMV-β-gal was mixed with the LipofectAMINE Plus reagent and added to cells. After 18 h, the cells were treated with GA or LPS for 12 h, and then lysed. Luciferase and β-galactosidase activities were determined as described previously [23]. Luciferase activity was normalized using β-galactosidase activity and was expressed relative to the activity of the control.

2.9. Electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described [25]. Two double-stranded deoxyoligonucleotides containing the NF-κB binding site (5′-GGGGACTTTCC-3′) [11] were end-labeled with [γ-^{32}P]dATP. Nuclear extracts (5 μg) were incubated with 2 μg of poly(dI-dC) and the ^{32}P-labeled DNA probe in binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin and leupeptin) for 10 min on ice. DNA was separated from the free probe using a 4.8% polyacrylamide gel in 0.5×TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA). Following electrophoresis, the gel was dried and subjected to autoradiography.

2.10. Statistical analysis

All experiments were repeated at least three times. Student's *t*-test was used to assess the statistical significance of differences. A confidence level of <0.01 was considered significant.

3. Results and discussion

As a host defense molecule, the inducible production of NO by macrophages appears to be important in the elimination of viruses and tumors [2–10]. Moreover, since GL is known to have anti-viral and anti-tumor activity [16–20], we investigated the effects of GA on the NO production and its effects on the level of iNOS gene expression in mouse macrophages. Our results demonstrate that GA stimulates NO production and is able to up-regulate iNOS expression through NF-κB transactivation. GA-induced NO production was assessed after incubating for 24 h using the Griess reaction. The basal level of NO in medium from untreated peritoneal macrophages was found to be less than 2 μM (Fig. 1). However, upon GA stimulation, NO release by peritoneal macrophages increased in a dose-dependent manner in the range 1–20 μM, and showed a cytotoxic action upon macrophages at concentrations exceeding 30 μM (Fig. 1). The potent macrophage activator LPS (0.5 μg/ml), when used as immunostimulator, increased NO production compared to the control. Consistent

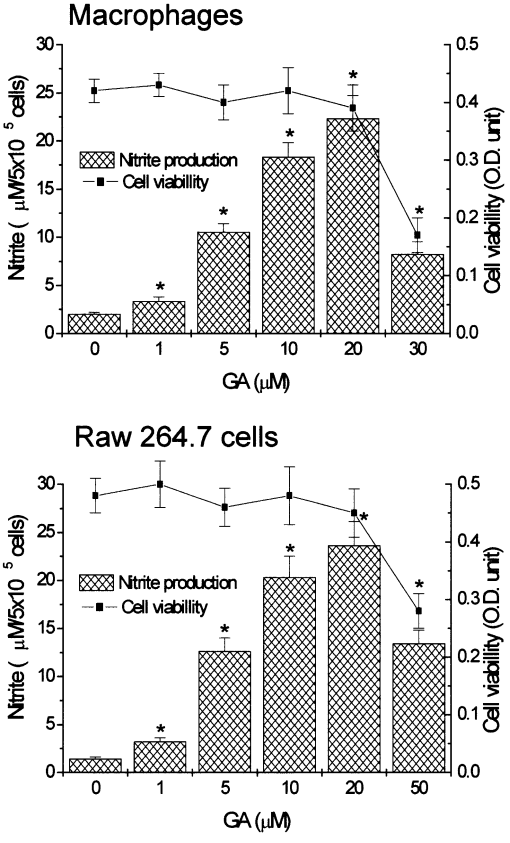

Fig. 1. Effects of GA on NO production. Murine peritoneal macrophages (2×10^5 cells/ml) or RAW 264.7 cells (5×10^5 cells/ml) were cultured for 24 h in the presence of media alone, with the indicated concentrations of GA. NO production was determined by measuring the accumulation of nitrite in the culture medium. Cell viability was assessed by MTT assay. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. $^*P<0.01$, significantly different from the control.

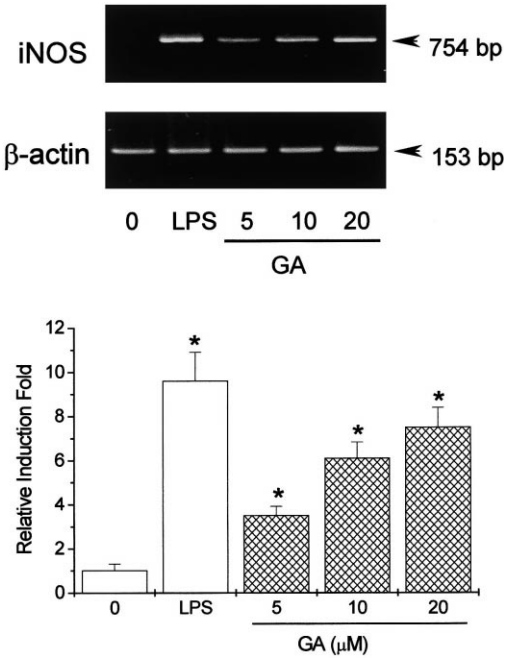

Fig. 2. Effects of GA on iNOS mRNA expression. RAW 264.7 cells (1×10^6 cells/ml) were cultured for 6 h in the presence of media alone, with the indicated concentrations of GA, or with LPS (0.5 μg/ml). Cells were lysed and total RNA was prepared for the RT-PCR analysis of gene expression. PCR amplification of the housekeeping gene, β-actin, was performed for each sample. The PCR amplification products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide. One of three representative experiments is shown. The ratio of the RT-PCR products of iNOS to β-actin was calculated. Induction-fold represents the mean ± S.D. of three separate experiments. $^*P<0.01$, significantly different from the control.

with these findings, GA also induced NO generation in a dose-dependent manner in RAW 264.7 cells (Fig. 1). Based on these results, and the relationship between NO and the anti-viral and anti-tumor functions of macrophages, we suggest that these effects of GA might be mediated in part through the activation of NO production.

Previous studies have shown that although GL alone did not induce NO from resting or unstimulated macrophages, NO production was enhanced in IFN-γ- or LPS-activated macrophages isolated from GL-treated mice [20,26], which suggested that GL has difficulty inducing NO production in the absence of some other stimulation. This may be because

Table 1
Effects of polymyxin B on NO secretion by GA and LPS

Treatment[a]	Nitrite (μM)[b]
Control	1.98 ± 0.23*,**
GA	19.62 ± 2.23*
GA+polymyxin B	20.24 ± 2.27*
LPS	42.38 ± 6.31**
LPS+polymyxin B	8.43 ± 0.93*,**

[a]RAW 264.7 cells (5×10^5 cells/ml) cultured with GA (10 μM) or LPS (0.5 μg/ml), in the presence or absence of polymyxin B (10 μg/ml).
[b]Supernatants were harvested after being cultured for 24 h and assayed for NO. Values are the means ± S.D. of three individual experiments, performed in triplicate.
$^*P<0.01$, significantly different from the LPS. $^{**}P<0.01$, significantly different from the GA.

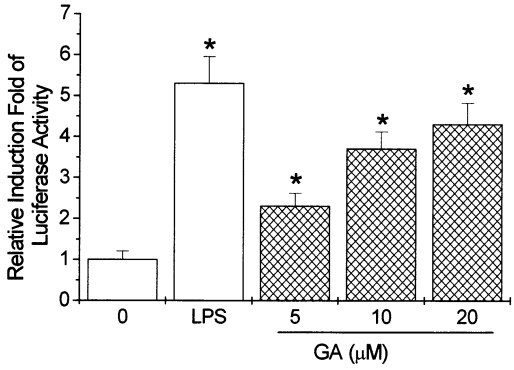

Fig. 3. Effects of GA on NF-κB-dependent luciferase gene expression. RAW 264.7 cells (5×10^5 cells/ml) were transiently co-transfected with pGL3-4κB-Luc and pCMV-β-gal. After 18 h, cells were treated with the indicated concentrations of GA or LPS (0.5 μg/ml) for 12 h. Cells were then harvested, and their luciferase and β-galactosidase activities determined. Luciferase activities are expressed relative to the control. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. $^*P<0.01$, significantly different from the control.

GL works synergistically with IFN-γ to induce NO production of macrophages or because of the low sensitivity of the assay. Moreover, it has been reported that GL enhances IFN-γ production in mice [14]. We also observed that GL slightly increased NO production in resting macrophages (data not shown). In the present study, however, GA, the aglycone of GL, significantly elicited a dose-dependent increase in NO production in the absence of any stimulator. Unlike as in previous studies [20,26], we did not need to co-stimulate with IFN-γ or LPS to generate NO with GA. Moreover, this GA-induced NO production was reversed when cells were treated with both GA and with *N*-nitro-L-arginine methyl ester, a competitive inhibitor of NOS (data not shown). Therefore, GA, unlike GL, has the ability to increase NO production alone in resting macrophages. The biological significance of the different effects of GA and GL on NO production in the resting and stimulated state needs to be determined.

Macrophages can be induced to produce NO by LPS or cytokines [11]. To confirm that the observed ability of GA to induce NO was not due to LPS contamination, the GA was tested for the presence of contaminating LPS by using the *Limulus* amebocyte lysate test. The level of LPS in GA was found to be below the detection limit, which is typically below 12.5 pg/ml (data not shown). Polymyxin B sulfate has been used previously as an LPS inhibitor in macrophage cultures [27], and although GA contained no detectable activity in the *Limulus* amebocyte lysate assay, we rechecked for possible LPS contamination in GA by adding polymyxin B (10 μg/ml) to cell cultures treated with GA (10 μM). As shown in Table 1, polymyxin B effectively inhibited the NO production induced by LPS, but had no effect on the induction by GA, which demonstrated that the production of NO by GA was unlikely to have resulted from LPS contaminating the GA.

As stated above, GA induced macrophage secretion of NO. In order to determine whether GA regulates NO production at the mRNA level, an RT-PCR assay was conducted. LPS was used as a positive control. Consistent with the results obtained from the NO assay, iNOS mRNA levels were markedly increased by GA treatment (Fig. 2). This result indicates

that GA up-regulates NO accumulation in macrophages in a dose-dependent manner. Therefore, we believe that increased NO production by GA is regulated through transcriptional activation.

Activated macrophages have the capacity to produce relatively large quantities of NO and NO-derived species, such as $NO_2^{\bullet}$, NO_2^-, N_2O_3, N_2O_4, *S*-nitrosothiols, and peroxynitrite ($ONOO^-$). Moreover, DNA and proteins are targets of reactive nitrogen intermediates. In addition, nitrogen intermediates and reactive oxygen intermediates can synergistically interact through the formation of peroxynitrites [28]. The reactive nitrogen intermediates formed by NO play a significant role in tumoricidal and microbiocidal activities [3]. Cysteine proteases are critical for virulence or replication of many viruses, bacteria, and parasites, and *S*-nitrosylation of pathogen cysteine proteases may be a general mechanism of the antimicrobial host defenses [29]. In addition, NO has been reported to interfere with specific stages in the life cycles of viruses. For example, NO inhibits DNA synthesis of the vaccinia virus and herpes simplex virus type 1, late protein translation, and virion assembly [30,31]. One specific viral target of NO has been identified; NO can inhibit the function and expression of the Epstein–Barr virus immediate early transactivator Zta [32]. Since NO can inhibit a variety of viruses, it is possible that NO also inhibits the cellular processes necessary for viral replication.

NF-κB is a member of the Rel family, and is a common regulatory element in the promoter region of many cytokines. In activated macrophages, NF-κB, in synergy with other transcriptional activators, plays a central role in coordinating the expression of genes encoding iNOS, tumor necrosis factor-α, and interleukin (IL)-1 [11]. To further investigate the role of GA on iNOS gene expression, we assessed the effect of GA on NF-κB-dependent gene expression by using the luciferase reporter gene assay. RAW 264.7 cells were transiently transfected with a plasmid containing four copies of the NF-κB binding sites, and the luciferase activities were measured. LPS, an immunostimulatory agent, was used as a positive control. When cells were stimulated with LPS a near five-fold increase in luciferase activity was observed versus the unstimulated control cells. Consistent with NO production and iNOS mRNA expression, GA also significantly increased NF-κB-dependent luciferase activities in a dose-dependent manner (Fig. 3). To further investigate the putative mechanism by which GA activates iNOS, the effect of GA on the activation of a family of transcription factors was monitored by electrophoretic mobility gel shift assay. NF-κB binding activity was examined in the light of its critical role in the regulation of iNOS. The results demonstrated that GA induced a marked increase in NF-κB binding at its conserved site, which was visualized as a distinct band (Fig. 4). Recently, it has been reported that GL treatment augmented IL-12 p40 mRNA expression in mice and that this effect may be associated with NF-κB activation [33]. To further confirm the role of NF-κB in iNOS expression by GA, we used BAY 11-7082, an inhibitor of IκBα kinase, which specifically inhibits NF-κB activation by inhibiting the phosphorylation and the subsequent degradation of IκBα, the endogenous inhibitor of NF-κB [34]. As shown in Fig. 5, pretreated RAW 264.7 cells with BAY 11-7082 effectively inhibited the NO production induced by GA. Although we demonstrated the up-regulatory effect of GA on iNOS gene expression through NF-κB transactivation in macrophages, the mechanism by which GA activates NF-κB is unknown, such as the activation of Raf-1 and mitogen-activated protein kinases [35]. Additional studies are needed to answer these questions and further elucidate the mechanisms involved.

GL has been widely and effectively prescribed as a therapy for chronic hepatitis [21,22]. Recently, it was reported that the long-term treatment with GL for chronic hepatitis C effectively inhibited liver carcinogenesis [36]. Cellular immune response has recently been shown to play an important role in patients who have recovered from hepatitis C virus infection

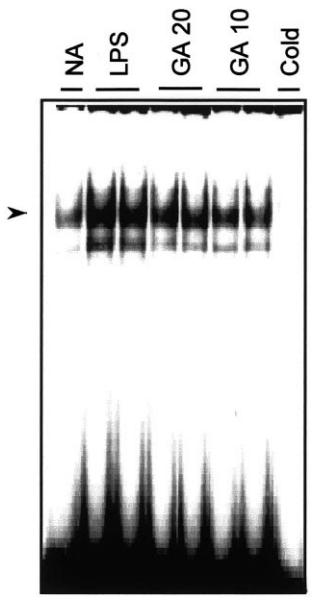

Fig. 4. Effects of GA on NF-κB binding. RAW 264.7 cells were treated with LPS (0.5 μg/ml) or GA (10, 20 μM) for 1 h. Nuclear extracts were isolated and used in an electrophoretic mobility shift assay with ^{32}P-labeled NF-κB oligonucleotide as a probe, as described in Section 2. The arrow indicates the NF-κB binding complex. Cold: 200-fold molar excess of non-labeled NF-κB probe. One of three representative experiments is shown.

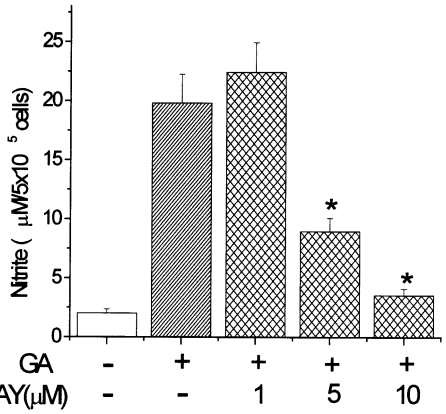

Fig. 5. Effects of NF-κB inhibition on NO production. RAW 264.7 cells (5×10^5 cells/ml) were pretreated with BAY 11-7082 for 1 h and then cultured for 24 h in the presence of media alone, with the indicated concentrations of GA. NO production was determined by measuring the accumulation of nitrite in the incubation medium. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. $^*P<0.01$, significantly different from the control.

[37]. Therefore, GL may activate certain immune functions, and for this reason, it is important to elucidate the mechanisms associated with the immunomodulatory activities of GL. NO was investigated in the current study to confirm the possibility that GA might be an immunostimulator, and as a result, GA was found to elicit NO production. This result supports the notion that NO induction by GA may contribute in vivo to the immunomodulatory, anti-viral and anti-tumor activity of GA. Biological response modifiers are widely used in immunochemotherapy to potentiate therapeutic efficacy or to alleviate the toxicity of cytotoxic anti-cancer agents. Further studies on GA are needed to prove its immunochemotherapeutic usefulness and its exact mechanism.

In summary, our results show for the first time that GA stimulates macrophage-derived NO production, and is able to up-regulate iNOS expression through NF-κB transactivation in murine macrophages. These actions may provide a mechanistic basis for the anti-viral and anti-tumor properties of GA.

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RESEARCH ARTICLE Open Access

Hepatoprotective effect of licorice, the root of *Glycyrrhiza uralensis* Fischer, in alcohol-induced fatty liver disease

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Abstract

Background: Our previous study suggested that licorice has anti-inflammatory activity in lipopolysaccharide-stimulated microglial cells and anti-oxidative activity in *tert*-butyl hydroperoxide–induced oxidative liver damage. In this study, we evaluated the effect of licorice on chronic alcohol-induced fatty liver injury mediated by inflammation and oxidative stress.

Methods: Raw licorice was extracted, and quantitative and qualitative analysis of its components was performed by using LC–MS/MS. Mice were fed a liquid alcohol diet with or without licorice for 4 weeks.

Results: We have standardized 70 % fermented ethanol extracted licorice and confirmed by LC-MS/MS as glycyrrhizic acid (GA), 15.77 ± 0.34 μg/mg; liquiritin (LQ), 14.55 ± 0.42 μg/mg; and liquiritigenin (LG), 1.34 ± 0.02 μg/mg, respectively. Alcohol consumption increased serum alanine aminotransferase and aspartate aminotransferase activities and the levels of triglycerides and tumor necrosis factor (TNF)-α. Lipid accumulation in the liver was also markedly induced, whereas the glutathione level was reduced. All these alcohol-induced changes were effectively inhibited by licorice treatment. In particular, the hepatic glutathione level was restored and alcohol-induced TNF-α production was significantly inhibited by licorice.

Conclusion: Taken together, our data suggests that protective effect of licorice against alcohol-induced liver injury may be attributed to its anti-inflammatory activity and enhancement of antioxidant defense.

Keywords: Licorice, Alcohol-induced liver injury, Glutathione, TNF-α

Background

Licorice is the root of *Glycyrriza uralensis* Fischer, *Glycyrrhiza glabra* Linné or *Glycyrrhiza inflata* Batalin (Fabaceae), which has been used as traditional medicine since ancient times. In particular, licorice was used as a medical raw material for multiple purposes such as antidote, antitussive expectorant, relaxant, to relieve pain that occurs because of a sudden nervous breakdown of muscle or tissue, to reduce weight gain, to increase white blood cell count, and also because of its diuretic and anti-inflammatory effects [1]. Although licorice has been used in both Eastern and Western medicine to treat a wide variety of diseases from common cold to liver disease, more scientific evidence is needed to prove its potential preventive and therapeutic benefits. The biologically active components of licorice are liquiritins (LQ), liquiritigenin (LG), glycyrrhizic acids (GAs), and flavones (Fig. 1). Various biological effects of these compounds and pharmacokinetics of glycyrrhizic acid have been reported [2–4]. In addition, studies have been performed to analyze and characterize primary and secondary metabolites of licorice [5, 6].

Accumulating lines of evidence show that licorice has anti-inflammatory, anticancer, antioxidant, and antimicrobial effects [1, 4, 7–9]. In particular, recent studies on hepatoprotective effects of licorice suggest that it can reduce liver injury by enhancing antioxidant and anti-

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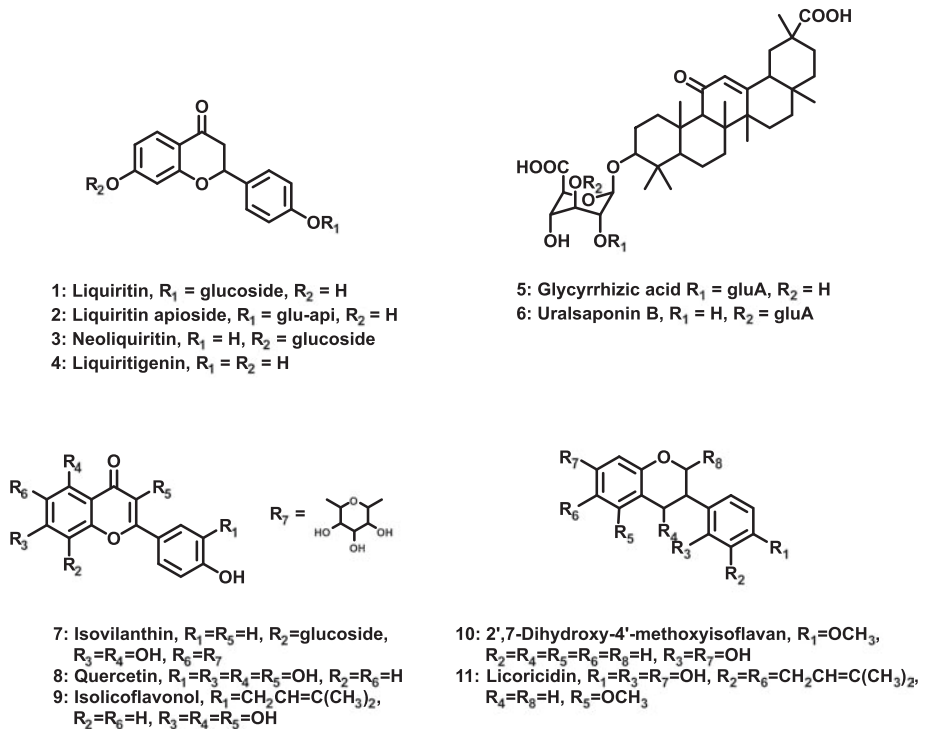

Fig. 1 Structures of Liquiritins (1 ~ 4), Glycyrrhizic acids (5 ~ 6), and Flavones (7 ~ 11) in licorice

inflammatory capacity [7, 10]. Administration of licorice extract prevented CCl_4-induced hepatotoxicity by increasing antioxidant enzyme activity and decreasing TNF-α production [11]. Jung et al. [12] investigated the hepatoprotective effects of 18β-glycyrrhetinic acid, one of the active compounds in licorice, in a CCl_4-induced liver injury model. Treatment with 18β-glycyrrhetinic acid inhibited the increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and hepatic lipid peroxidation in a dose-dependent manner. In addition, 18β-glycyrrhetinic acid significantly protected against glutathione (GSH) depletion. Although these studies show a promising effect of licorice in preventing liver injury, their limitation was that the chemically induced acute hepatotoxicity model used was not very relevant to clinical situations.

Alcohol abuse causes a range of acute and chronic health problems worldwide, which lead to morbidity and mortality. Depending on overall alcohol consumption and drinking patterns, chronic exposure to alcohol is harmful to the central nervous system and many organs, including the liver. Among alcohol-induced liver diseases, fatty liver is the most common histopathologic condition in drinkers. Although alcohol-induced fatty liver is widely considered to be benign and to have a very low risk of progression, clinical studies have provided evidence that it is an important pathogenic factor in the development of liver disease [13–15]. Specifically, the authors suggested that both oxidative stress and inflammation as second hits are critical factors in the pathological progression from simple fat accumulation to liver disease. Recently, we reported that licorice extract had an anti-inflammatory effect in lipopolysaccharide-stimulated microglial cells and acted as an antioxidant in a *tert*-butyl hydroperoxide–induced oxidative liver injury model [16]. Therefore, it was of interest to examine the effects of licorice on chronic alcohol-induced fatty liver, which is more relevant to clinical situations. In this study, we examined the preventive effect of licorice in alcoholic fatty liver by administering its extract to mice exposed to alcohol for 4 weeks.

Methods

Extraction

Glycyrrhiza uralensis Fisher (Fabaceae) was cultivated in Jecheon, Chungbuk Province, Korea. The raw material has been provided by the Korea Licorice Farming Association in 2013 and its extraction was produced by Tecos Co., Ltd (Chuncheon, Korea). Prof. Min Hye Yang of the Pusan National University identified plant material and a voucher specimen (PNU-0020) has been deposited in the Medicinal Herb Garden, Pusan National University (Busan, Korea). The analysis of biological component and microbiological test were confirmed by Novarex Co., Ltd (Ochang, Korea). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO,

USA) and Wako Pure Chemical Industries (Osaka, Japan). The raw material of licorice (the root of *Glycyrrhiza uralensis* Fisher, 400 kg) was extracted for 3 h using a reflux circulation of 70 % aqueous ethanol (2800 L). The extracts was cooled at 30 ~ 35 °C and filtered using 75 μm cartridge and then the residue of raw materials was removed through subject of a centrifuge. The residue was concentrated *in vacuo* under reduced pressure (10 atm, 55 ~ 58 °C) to reach 10 ~ 20 brix materials (52 ~ 64 kg). The residue was blended with dextrin and sterilized at 95 °C for 30 min and then it was spray-dried (liquid temperature: 75 ~ 80 °C, the blowing temperature of 180 °C, atomizer 18,000 rpm) to provide a licorice extract powder (90 kg, 11.3 %). To establish bulk scale production of licorice extracts, we confirmed manufacturing process based on experimental pilot condition using Jecheon domestic licorice in Korea (Fig. 2).

Analysis of licorice extract

To confirm two index components such as triterpenoid saponin series GA and flavonoids LQ, we performed quantitative and qualitative analysis through HPLC and HPLC-MS/MS based on United States Pharmacopoeia and Korean Pharmacopeia as standard analytical methods (Fig. 3).

Analytical condition of LC-MS/MS

We used digoxin as internal standard in order to quantitative analysis of major components GA, LQ, and LG of licorice extract. In addition, we performed material separation for each component of the material using LUNA C_{18} column (2.0 × 150 mm, 5 μm). Solvent A was water with 1.0 % acetic acid and solvent B was acetonitrile with 1.0 % acetic acid. The gradients of solvents were as followings: 0 min, 10 % B; 1 min, 10 % B; 6.5 min, 90 % B; 8 min, 90 % B; 8.5 min, 10 % B; 15 min, 10 % B. Samples were dissolved in 50 % acetonitrile and the injection volume of each sample was 5 μl. Detailed condition for LC-MS/MS analysis is in the Table 1.

Animals and treatments

Male C57BL/6 mice were purchased from Orient Bio (Sungnam, Korea). The use of animals was in compliance with the guidelines established and approved by the Institutional Animal Care and Use Committee in Pusan National University (PNU-2014-0568). Animals were acclimated to temperature (22 ± 2 °C) and humidity (55 ± 5 %) controlled rooms with a 12-h light/dark cycle for 1 week prior to use. The diets were purchased from Dyets Inc. (Bethlehem, PA, USA). Mice were fed a Lieber–DeCarli liquid diet with or without ethanol for 4 week. For the control liquid diet, 35 % of energy was derived from fat, 18 % from protein, and 47 % from carbohydrates; the liquid ethanol diet contained 35 % of energy from fat, 18 % from protein, 11 % from carbohydrates, and 36 % from ethanol.

Serum biochemistry and histopathologic evaluation

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total serum triglyceride (TG) were measured using Automated Chemistry Analyzer (Prestige 24I; Tokyo Boeki Medical System, Tokyo, Japan). For histopathologic evaluation, a cross section of the left lateral lobe of the liver was sliced at 10 μm, immersed in propylene glycol for 5 min, and

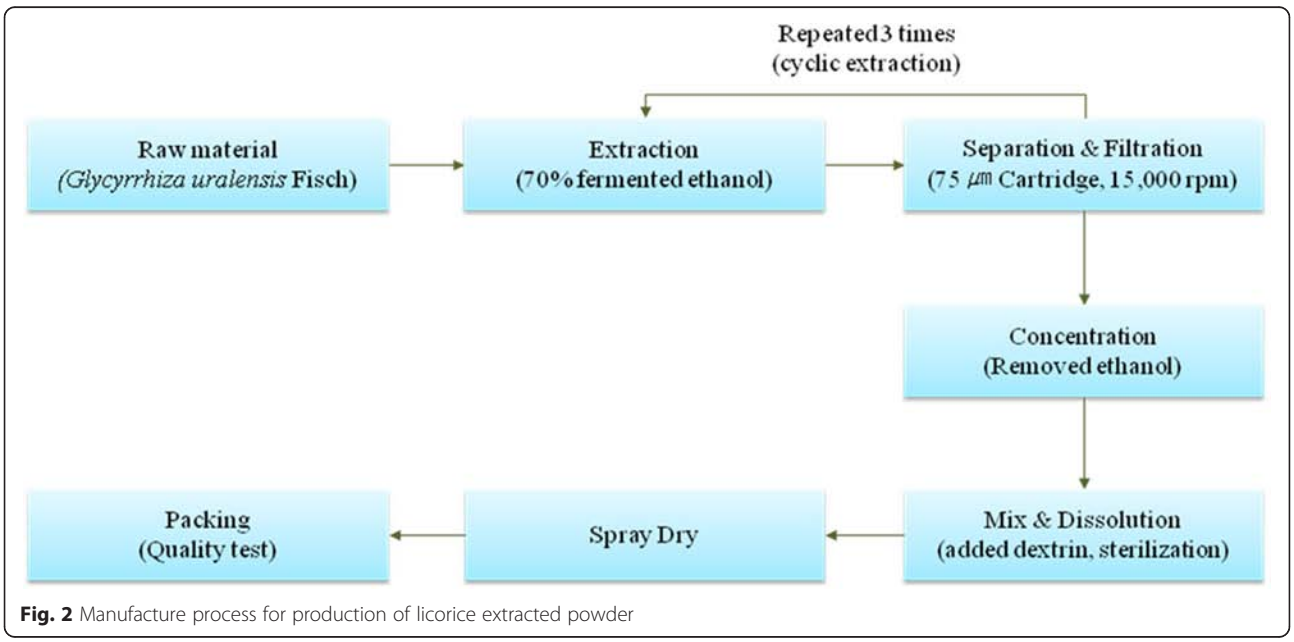

Fig. 2 Manufacture process for production of licorice extracted powder

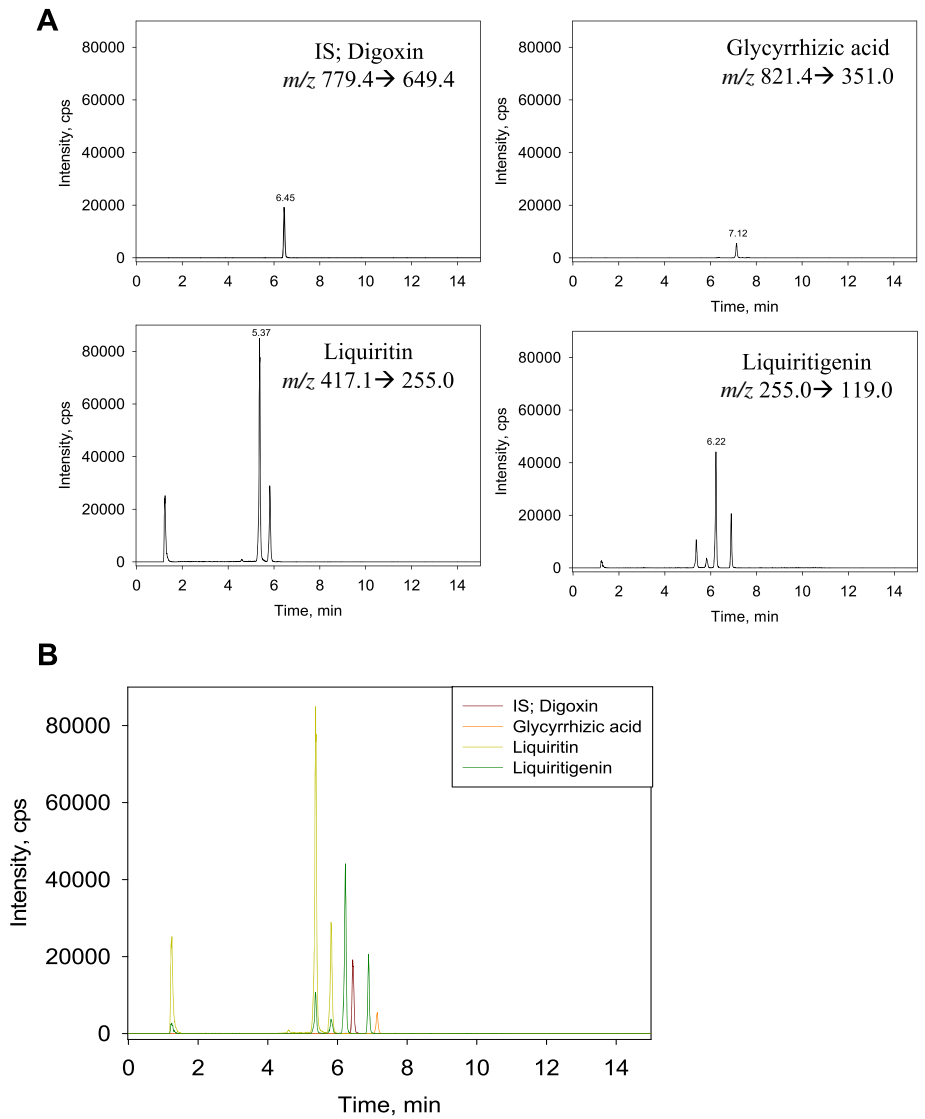

Fig. 3 LC-MS/MS spectrum of standard (**a**) and licorice extract (**b**)

stained with Oil red O for 7 min. After rinsing with 85 % propylene glycol and distilled water, the sections were counterstained with hematoxylin for 2 min before microscopic examination.

Measurement of serum TNF-α

The levels of serum TNF-α were determined by enzyme-linked immunosorbent assay using a commercially available kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction.

Determination of hepatic triglyceride contents

Total lipids of the liver were extracted from homogenate prepared from 100 mg of mouse liver using the mixture of chloroform/methanol (2:1, v/v). Triglycerides in total lipid were determined enzymatically using a commercially available enzymatic kit (Sigma Chemical Co.) according to the manufacturer's protocol.

Measurement of hepatic glutathione (GSH)

Liver was homogenized in a four-fold volume of ice-cold 1 M perchloric acid. After the denatured protein was removed by centrifugation at 10,000 g for 10 min, the supernatant was assayed for the total GSH concentration using a HPLC separation/fluorometric detection method [17].

Real time RT-PCR

Total RNA was purified from liver tissue using the RNeasy kit (Qiagen, Valencia, CA, USA). cDNA synthesis was accomplished with iScript™ cDNA Synthesis system (Bio-Rad, Hercules, CA, USA). Real time RT-PCR was performed using Thunderbird SYBR qPCR mix

Table 1 Condition for LC-MS/MS analysis of Licorice extracts

A.	
HPLC Condition	
Column	Luna C_{18} RP column (2.0 × 150 mm, 5 μm)
Flow rate	0.3 mL/min
Injection volume	5 μL
Column temperature	40 °C
Autosampler temperature	4 °C
B.	
Mass condition	
Ion source	Turbo spray (Negative)
Curtain Gas	30 psi
Collision Gas	N_2 (Medium)
Ionspray Voltage	- 4.0 kV
Source temperature	400 °C
Gas 1	40 psi
Gas 2	50 psi

(Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol. Relative values of gene expression were normalized to 18S ribosomal RNA. Primer sequences and full name of the genes are provided in Additional file 1: Table S1.

Statistical analysis

All results expressed as mean ± s.d. were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple range test (parametric). The acceptable level of significance was established at $P < 0.05$.

Results

We analyzed licorice extract composition based on testing condition using a Shiseido HPLC system coupled to an AB Sciex electrospray-ionization (ESI) mass-spectrometer (Table 2). For quantitative LC-MS/MS analysis of each component, we established optimal conditions for the precursor ion and product ion by adjusting collision energy, cone voltage, and cone temperature of the ion source (Table 1). The instrument was operated in the multiple-reaction-monitoring (MRM) mode.

We prepared the calibration curves for GA, LQ, and LG according to the concentration-dependent ESI method in LC-MS/MS analysis. We found that the correlation coefficient (r^2) values were 0.9999, 0.9997, and 0.9998, respectively, which showed good linearity of the calibration curves (Table 2). The limit of detection (LOD) was 4.29 ng/mL, 1.27 ng/mL, and 0.54 ng/mL, whereas the limit of quantitation (LOQ) was 13.99 ng/mL, 3.86 ng/mL, and 1.64 ng/mL, respectively.

We performed quantitative analysis of GA, LQ, and LG. The MRM conditions were m/z 821.4 (precursor ion) → 351.0 (product ion) for GA, m/z 417.1 (precursor ion) → 255.0 (product ion) for LQ, and m/z 255.0 (precursor ion) → 119.0 (product ion) for LG. We set up the amount of digoxin as internal standard at m/z 779.4 (precursor ion) → 649.4 (product ion). For quantitative analysis, we used the calibration curves to calculate the ratios of compounds in the analyzed material to respective standards. We diluted licorice extract 1/20 to ensure that the concentrations of its components are within the quantitative ranges of the calibration curves, and then multiplied the obtained concentrations by 20 (the dilution factor). The results of quantitation of the licorice extract components were as follows: GA, 15.77 ± 0.34 μg/mg; LQ, 14.55 ± 0.42 μg/mg; and LG, 1.34 ± 0.02 μg/mg (Table 3).

To test the effect of licorice on alcohol-induced fatty liver, a dose-dependence study was performed in mice fed a standard Lieber–DeCarli liquid diet supplemented with ethanol for 4 weeks (Fig. 4). Different dosage of licorice ranging from 25 mg/kg body weight to 200 mg/kg body weight was orally administered every day from the beginning of the liquid diet. At the end of the treatment period, lipid accumulation in the liver was evaluated by Oil Red-O staining (Fig. 4a). Serum ALT and AST activities were also determined (Fig. 4b and c). The dose of 25 to 50 mg/kg body weight was not effective, but treatment with doses exceeding 100 mg/kg significantly reversed hepatic lipid accumulation and serum ALT, AST activities (Fig. 4).

We compared the effect of licorice (100 mg/kg body weight) on alcoholic fatty liver with that of silymarin (100 mg/kg body weight; Sigma Chemical Co., Cat S0292), which is a well-known compound that alleviates alcohol-induced liver injury. Using staining with Oil Red-O and evaluation of hepatic triglyceride, we found

Table 2 Linearities, regression equation, correlation coefficients, limit of detection (LOD), and limit of quantitation (LOQ) for glycyrrhizic acid (GA), liquiritin (LQ) and liquiritigenin (LG)

Compounds	Linear range (ng/ml)	Regression equation[a]	Correlation coefficient	LOD[b] (ng/ml)	LOQ[c] (ng/ml)
Glycyrrhizic acid (GA)	12.5–5000	Y = 0.0004x − 0.0055	0.9999	4.62	13.99
Liquiritin (LQ)	12.5–1000	Y = 0.0059x + 0.0451	0.9997	1.27	3.86
Liquiritigenin (LG)	12.5–500	Y = 0.0289x + 0.1574	0.9998	0.54	1.64

[a]y: Analyte area / IS area; x: concentration (ng/mL) of compounds
[b]LOD = 3.3 x δ /S (δ; standard deviation, S; slope of the calibration curve)
[c]LOQ = 10 x δ /S (δ; standard deviation, S; slope of the calibration curve)

Table 3 Analytical LC-MS/MS data of licorice extracts

Compounds		Peak area	IS area	Analytes/IS ratio	Calculation	μg/mg	Ave.	S.D.
Glycyrrhizic Acid (GA)	1/20 dil.1	23,132	74,555	0.31	789.42	15.79	15.77	0.34
	1/20 dil.2	23,226	73,402	0.32	804.80	16.10		
	1/20 dil.3	23,016	75,956	0.30	771.29	15.43		
Liquiritin (LQ)	1/20 dil.1	316,205	74,555	4.24	711.21	14.22	14.55	0.42
	1/20 dil.2	328,810	73,402	4.48	751.61	15.03		
	1/20 dil.3	326,100	75,956	4.29	720.03	14.40		
Liquiritigenin (LG)	1/20 dil.1	153,592	74,555	2.06	65.84	1.32	1.34	0.02
	1/20 dil.2	154,374	73,402	2.10	67.33	1.35		
	1/20 dil.3	159,391	75,956	2.10	67.16	1.34		

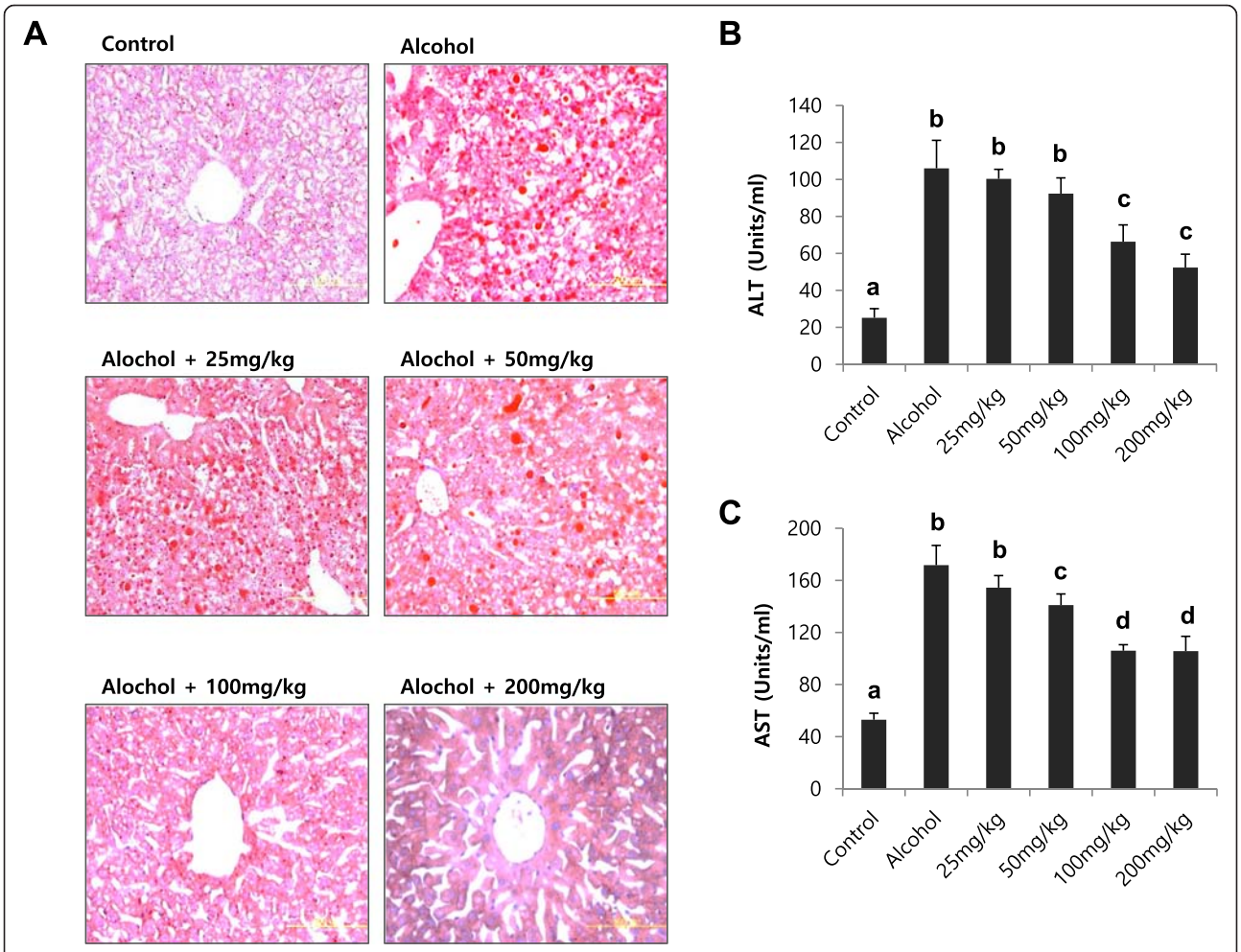

Fig. 4 Dose-dependence study of licorice in alcohol-induced fatty liver. Hepatic lipid accumulation (**a**), serum ALT (**b**) and AST (**c**) activities in mice fed different diet with or without licorice. The livers were stained with Oil red O for histopathological examination. Each value represents the mean ± SD for 6 mice. Values with different letters are significantly different from one another (ANOVA followed by Newman-Keuls multiple range test $P < 0.05$)

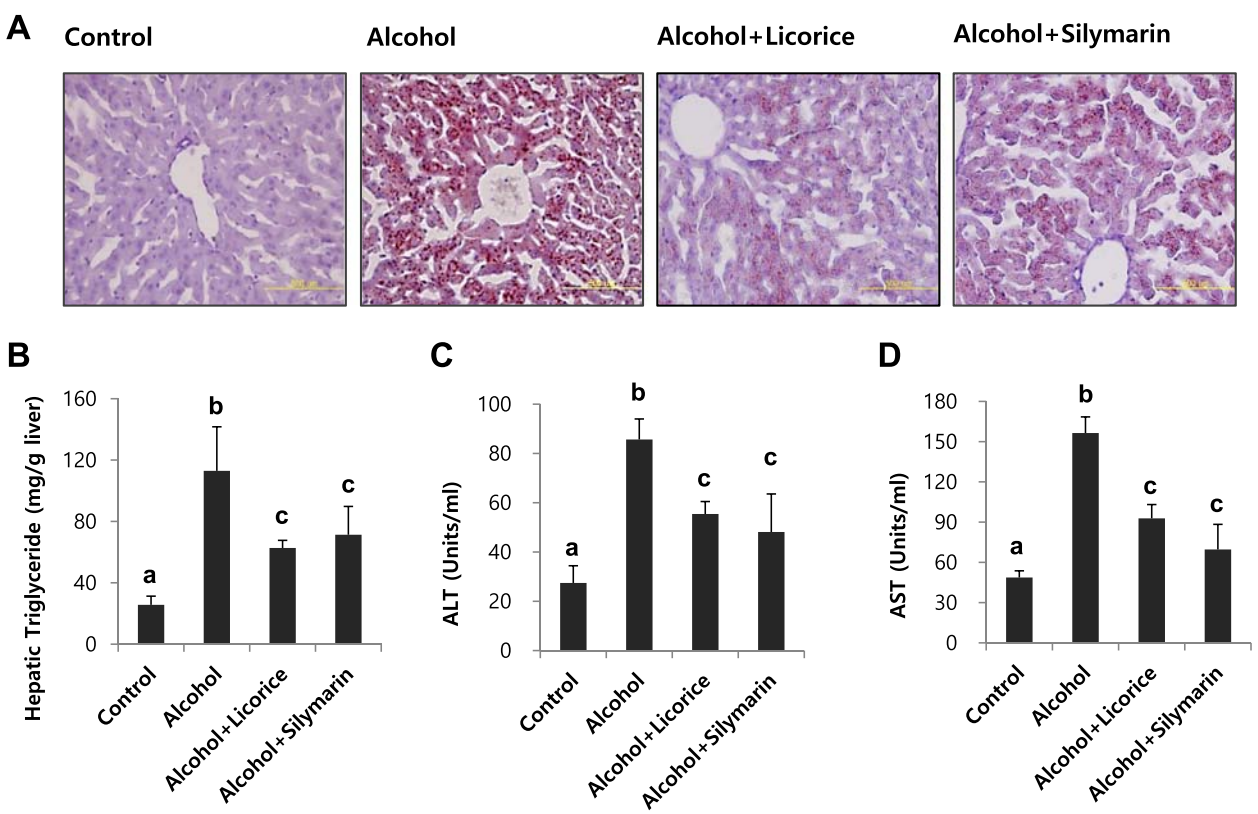

Fig. 5 Effect of licorice on hepatic lipid accumulation and serum ALT, AST compared with silymarin as a positive control. Hepatic lipid accumulation (**a**) and triglyceride level (**b**), serum ALT (**c**) and AST (**d**) activities in mice fed different diet with or without licorice. The livers were stained with Oil red O for histopathological examination. Each value represents the mean ± SD for 6 mice. Values with different letters are significantly different from one another (ANOVA followed by Newman-Keuls multiple range test $P < 0.05$)

that licorice was a promising candidate to alleviate alcoholic fatty liver in comparison with the inhibitory effect of silymarin on fat accumulation induced by chronic alcohol ingestion (Fig. 5a and b).

Biochemical analyses of serum ALT, AST activities, and liver triglyceride levels corresponded to the histopathologic findings: licorice administration significantly attenuated the effects of ethanol on triglyceride accumulation in the liver and ALT and AST activities in serum (Fig. 5c and d). The hepatic GSH content in ethanol-treated mice was significantly lower than that in the control mice. Licorice treatment restored GSH to its original level (Fig. 6a). Licorice also significantly decreased the level of serum TNF-α (Fig. 6b).

To find detailed mechanism for the hepatic lipid lowering effect of licorice, we analyzed the hepatic expression levels of the lipogenic genes such as *Srebf1* and *Fasn*, and the genes involved in lipid transportation such as *Mttp, Apob, Cd36, Lpl, Ldlr, Fatp1, Fatp2, Fatp3, Fatp4, and Fatp5* (Fig. 7). Chronic alcohol drinking significantly enhanced *Srebf1* expression and the mRNA level of *Cd36* and *Lpl, Fatp4* related to lipid uptake. However, licorice supplement significantly prevented the induction of these genes expression.

Discussion

Although the mechanisms of the induction of fatty liver by alcohol appear to be complicated, accumulating lines of evidence suggest contribution of both oxidative stress and inflammation. On the basis of our recent findings that licorice protects cells against inflammation and oxidative stress [16], we hypothesized that licorice would alleviate alcohol-induced fatty liver injury.

In mice fed a standard Lieber–DeCarli alcohol diet for 4 weeks, hepatic triglyceride levels increased and GSH content decreased with concomitant increases in serum ALT and AST activities, triglycerides, and TNF-α. Supplementation of the alcohol diet with licorice for the same time period significantly reversed the changes in liver injury markers and effectively abrogated fat accumulation. Thus, we suggest that the hepatoprotective

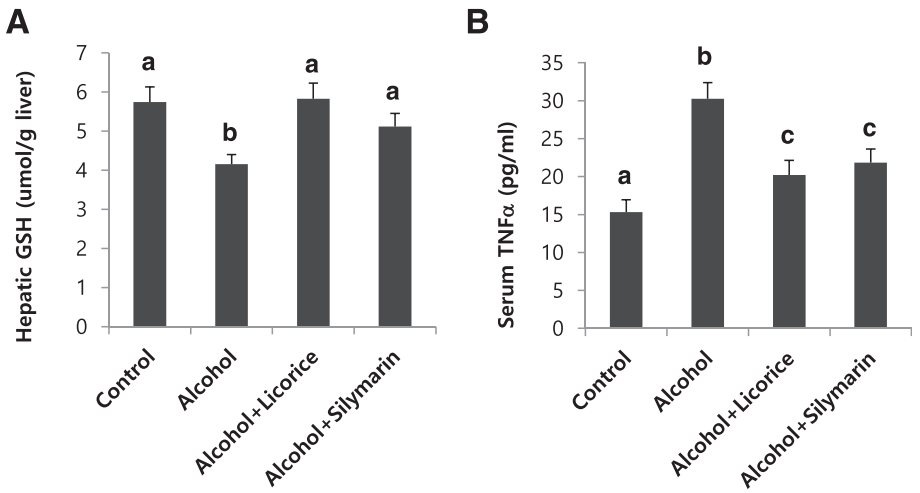

Fig. 6 Effect of licorice on the level of hepatic GSH (**a**) and serum TNFα (**b**) in alcohol-induced fatty liver. Each value represents the mean ±SD for 6 mice. Values with different letters are significantly different from one another (ANOVA followed by Newman-Keuls multiple range test $P < 0.05$)

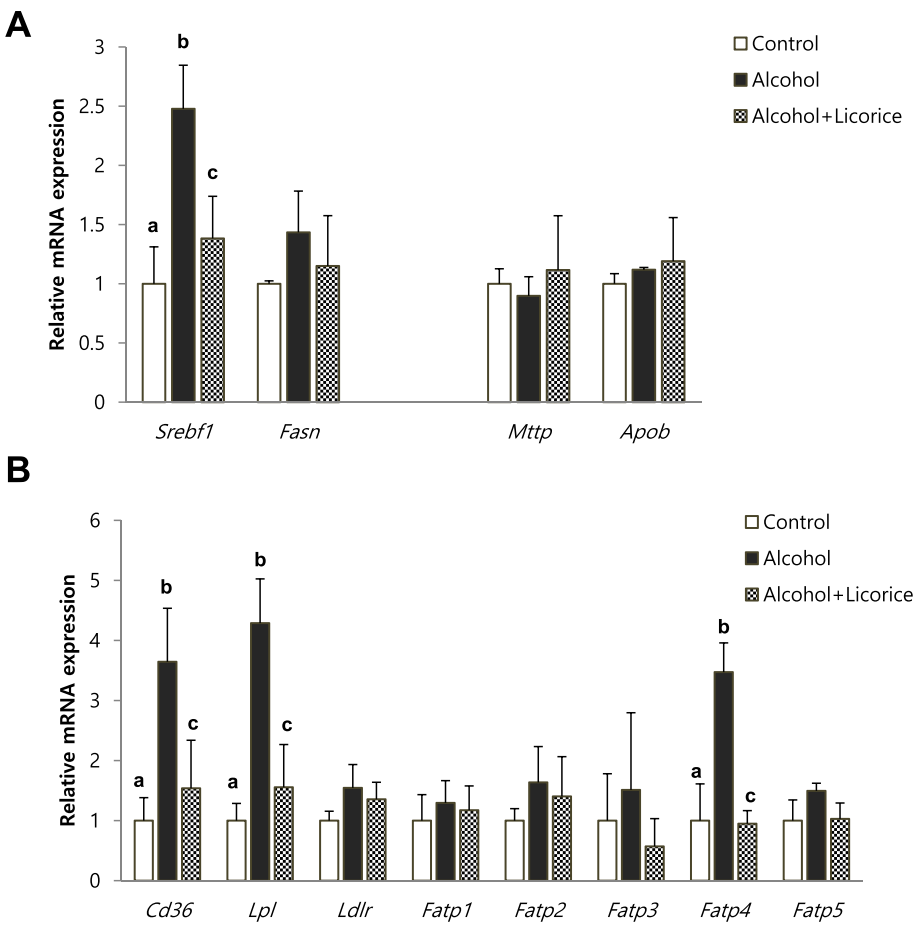

Fig. 7 Effect of licorice on the level of hepatic mRNA expression in alcohol-induced fatty liver. mRNA expression of *Srebf1* and *Fasn* for lipogenesis, and *Mttp* and *Apob* for lipid export in the liver (**a**). Expression of mRNA related to lipid uptake in the liver (**b**). Each value represents the mean ± SD for 6 mice. Values with different letters are significantly different from one another (ANOVA followed by Newman-Keuls multiple range test $P < 0.05$)

effect of licorice is associated with an augmentation of antioxidant defense and anti-inflammatory response.

GSH, a thiol-containing tripeptide, plays a major antioxidant and detoxification role in the liver. Alcohol increases the levels of intracellular reactive oxygen species and depletes mitochondrial GSH, and therefore induces oxidative stress [18]. Although the contribution of oxidative injury to the development of alcoholic fatty liver remains to be elucidated, enhancement of antioxidant capacity using some compounds ameliorates alcoholic fatty liver [19–22]. In line with these results, overexpression of superoxide dismutase prevents the accumulation of lipid droplets in hepatocytes, whereas double knockout of glutathione peroxidase-1 and catalase aggravates alcohol-induced liver injury [23–25]. Our results thus indicate that an improvement in the antioxidant capacity in alcohol-fed mice via recovery of the hepatic GSH pool could make licorice valuable in the treatment of alcoholic liver disease.

Direct inflammatory and cytotoxic effects of TNF-α in alcoholic liver disease are well characterized. Chronic drinking of alcohol increases the level of bacterial endotoxin, which stimulates resident liver macrophages to produce free radicals and cytokines [26]. NADPH oxidase plays critical roles in the generation of oxidants in resident liver macrophages after alcohol intake. Activation of NF-kB by oxidant generation leads to an increase in the TNF-α level, which causes tissue injury [27]. Moreover, TNF-α is suggested to induce lipolysis in adipose tissue followed eventually by fatty liver. Earlier studies showed that TNF-α causes free fatty acid release from adipocytes, stimulates lipogenesis in the liver, and inhibits β-oxidation of free fatty acids [28–30]. Moreover, in a more recent report, TNF-α was suggested to increase intrahepatic fat deposition by affecting hepatic lipogenic metabolism that involves SREBP-1c [31]. Indeed, TNFR1 knockout almost completely blocks the development of alcohol-induced fatty liver [32] . In agreement with these reports, the present study demonstrated that licorice significantly inhibited up-regulation of *Srebf1* by chronic alcohol drinking. Importantly, increase of gene expression involved in lipid uptake such as *Cd36, Lpl,* and *Fatp4* is also effectively reduced by licorice treatment. Considering the importance of TNF-α in the development of alcoholic fatty liver, suppression of TNF-α secretion by licorice may contribute to its overall preventive effect in alcoholic liver injury.

Conclusion

We found that licorice is effective in preventing alcoholic fatty liver in mice. An important issue in the management of alcoholic liver disease is the progression of simple fat accumulation to alcoholic hepatitis. Licorice treatment restored hepatic GSH content and inhibited TNF-α secretion, and also inhibited lipid accumulation in the liver of chronic alcohol-fed mice. Therefore, licorice is a promising candidate to prevent the progression of alcoholic liver injury, which probably acts by enhancing anti-oxidative and anti-inflammatory capacity.

Additional file

Additional file 1: Table S1. List of murine primers used for real time RT-PCR. (DOCX 17 kb)

Competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' contributions

JCJ, YHL, and YSJ designed the study and prepared the manuscript. JCJ, YHL, SHK, KJK, KMK, and SO carried out experiment. All authors have read and approved the final version of this manuscript.

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Modulation of genotoxicity of oxidative mutagens by glycyrrhizic acid from *Glycyrrhiza glabra* L.

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Background: The chemopreventive effects of certain phytoconstituents can be exploited for their use as functional foods, dietary supplements and even as drugs. The natural compounds, acting as anti-genotoxic and free radical scavenging compounds, may serve as potent chemopreventive agents. These can inhibit DNA modulatory activities of mutagens and help preventing pathological processes. **Objectives:** Present study on *Glycyrrhiza glabra* L., a promising medicinal plant, widely used in traditional medicine, focused on the bioassay-guided fractionation of its extracts for the isolation of certain phytochemicals with anti-genotoxic potential against oxidative mutagens. **Materials and Methods:** The methanol extract of *Glycyrrhiza glabra* rhizomes was subjected to column chromatography, and isolated fraction was evaluated for its anti-genotoxic and antioxidant potential using SOS chromotest, Comet assay, and DPPH radical scavenging assay. **Results:** GLG fraction, which was characterized as Glycyrrhizic acid, inhibited the genotoxicity of oxidative mutagens *viz.*, H_2O_2 and 4NQOquite efficiently. In SOS chromotest, using *E.coli* PQ37 tester strain, it inhibited induction factor induced by H_2O_2 and 4NQO by 75.54% and 71.69% at the concentration of 121.46 μM,respectively. In Comet assay, it reduced the tail moment induced by H_2O_2 and 4NQO by 70.21% and 69.04%, respectively, at the same concentration in human blood lymphocytes. The isolated fraction also exhibited DPPH free radical scavenging activity and was able to scavenge 85.95% radicals at a concentration of 120 μM. **Conclusion:** Glycyrrhizic acid is a potential modulator of genotoxins as well as efficient scavenger of free radicals.

Key words: Chemoprevention, *Glycyrrhiza glabra* L., glycyrrhizic acid, H_2O_2, oxidative mutagens, 4NQO

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INTRODUCTION

Environmental pollution has introduced certain xenobiotic compounds, which act as mutagens and carcinogens as well as sources of reactive oxygen species production in various life forms. Research in the field of search for natural anti-mutagenic/antioxidant compounds has gained pace in the last few decades as the synthetic compounds often come with certain undesirable side-effects. Plant secondary metabolites are known to possess a wide range of pharmacological properties like acetylcholinesterase inhibitory effects, anti-microbial, anti-inflammatory, and anti-diabetic activities.[1,2] Similarly, several natural compounds exhibit anti-carcinogenic or anti-mutagenic activity against environmental carcinogens and mutagens.[3] The agents, which are able to interfere with mutation, have the potential to interfere with early stages of cancer. Various natural compounds, evaluated for chemopreventive potential, have been found to regulate cellular signaling of proliferation and death, and thus conferring a preventive benefit to the host.[4]

Glycyrrhiza glabra L. (Fabaceae), commonly known as 'Licorice,'is considered as the oldest and most widely used herbal drugs around the world. Traditionally, it is used for its anti-viral effects as well as for respiratory, gastrointestinal, cardiovascular, genitourinary, eye and skin disorders.[5] The antioxidant, anti-genotoxic and anti-inflammatory activities of *G. glabra* leaves have been documented.[6] The anti-ulcerogenic action[7] as well as the anti-ulcer[8] of standardized extract of *G. glabra* has also been reported. The major bioactive constituents of *G. glabra* consists of flavonoids and pentacyclic triterpene saponins, which include isoliquiritigenin, glycyrrhizin, and glabridin.[9] The

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present study aimed to isolate potent anti-genotoxic/antioxidant phytoconstituents from *G. glabra* through bioactivity-guided fractionation and to evaluate for their protective effect against DNA damage induced by oxidative mutagens *viz.*, hydrogen peroxide (H_2O_2) and 4-Nitroquinoline 1-oxide (4NQO) by employing SOS chromotest and Comet assay and evaluating their free radical scavenging activity.

MATERIALS AND METHODS

Plant material

The rhizomes of *G. glabra* were purchased from a local market at Amritsar, India. Voucher specimen No. 0342-A-03/2006 (*G. glabra*) has been deposited in the herbarium of the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India.

Chemicals

Ethidium bromide, ortho-nitrophenyl-β-D-galactopyranoside (ONPG), nicotinamide adenine dinucleotide phosphate, (NADP), glucose-6-phosphate (G6P), normal melting point agarose (NMPA), and low melting point agarose (LMPA) were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Histopaque 1077 from Sigma Chemicals (St. Louis, MO, USA); *para*-nitrophenyl phosphate (PNPP) from Sisco Research Laboratories Pvt. Ltd.; polyethyleneglycol-4-tetraoctylphenolether (Triton X-100), hydrogen peroxide, dimethyl sulphoxide were procured from Qualigens Fine Chemicals, Mumbai, India. *Escherichia coli* PQ37 strain was purchased from Institute Pasteur, France. All the chemicals used were of analytical grade.

Extraction/fractionation of rhizomes of *Glycyrrhiza glabra* L.

The rhizomes of *G. glabra* (1 kg) were washed and dried in oven at 40°C. The dried material was powdered and extracted 5 times with 80% methanol each time (3 liter ×5). The extract was concentrated using rotary evaporator (Buchi) and was further lyophilized on a lyophilizer to get the methanol extract (MeOH-GG), which was subjected to dry column chromatography. Dry column chromatography of MeOH-GG yielded fractions recovered from chloroform: Methanol (75/25 fractions) and (0/100 fractions), which were pooled, dried, and after making slurry, were subjected to reverse phase column chromatography by using silica gel RP-18, (running solvent mixture of chloroform: EtOAc: Methanol: 40:40:20). Five fractions were collected (each of 100 ml). The fourth fraction yielded a crystalline compound named as 'GLG' after placing the liquid fraction at 4°C in refrigerator [Figure 1].

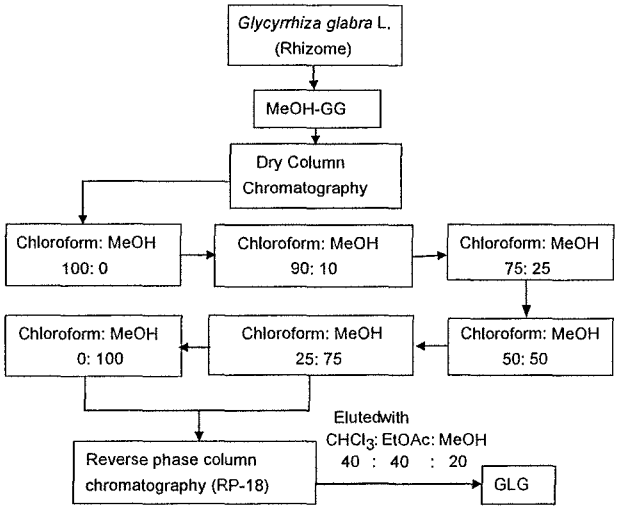

Figure 1: Schematic representation of isolation of GLG fraction from methanol extract of *G. glabra* L

SOS Chromotest

The ability to inhibit bacterial genotoxicity was tested in *Escherichia coli* PQ37 using SOS chromotest.[10] Hydrogen peroxide and 4NQO were used as mutagens to induce SOS inducing potency (SOSIP). Culture of *E. coli* PQ37 was grown at 37°C in Luria broth medium (1% bactotryptone, 0.5% yeast extract, 1% NaCl, and 20 μg/ml ampicillin) and diluted 1:9 into fresh medium subsequently. Aliquots of 100 μl were distributed into test tubes containing different concentrations of GLG fraction, making final volume to 0.6 ml. A positive control was prepared by exposure of the bacteria to either H_2O_2 or 4NQO. After 2 h of incubation at 37°C, with shaking, 300 μl samples were used for assay of β-galactosidase (β-gal) and alkaline phosphatase (Ap) activities. The different concentrations of GLG fraction samples were also tested in the absence of mutagens for their genotoxic effect. The induction factor (IF) was calculated as the ratio of Rc/Ro where Rc is equal to β-galactosidase activity/alkaline phosphatase activity determined for the test compound at concentration c, and Ro is equal to β-galactosidase activity/alkaline phosphatase activity in the absence of test compound. In the SOS chromotest assay, the compounds are classified as non-genotoxic, if the induction factor (IF) remains <1.5 and genotoxic, if IF exceeds 2.0. Anti-genotoxicity was expressed as percentage inhibition of genotoxicity according to the formula:

$$\text{Inhibition } (\%) = 100 - (IF_1 - IF_0 / IF_2 - IF_0) \times 100$$

Where,

IF_1 is the induction factor in the presence of the test compound and mutagen (H_2O_2/4NQO)

IF_2 the induction factor in the absence of the test compound (only mutagen) and

IF_0 is the induction factor of the blank.
Data was collected (mean±standard error) of triplicate experiments.

Comet assay

The alkaline comet assay was performed using human blood lymphocytes.[11] Heparinized blood samples were obtained by venipuncture from non-smoking, healthy male donor aged 25-40 years. Lymphocytes were isolated by the method of Boyum.[12] The blood was mixed with equal volume of phosphate buffer saline (PBS; pH 7.2). This mixture was then overlaid to double volume of histopaque 1077 and centrifuged at 1500 rpm for 20 min. White ring of lymphocytes, formed at the interface of plasma and histopaque, was aspirated very carefully with the help of pasteur pipette. The lymphocytes were then diluted in PBS and centrifuged at 2000 rpm for 15 min. The supernatant was discarded, and pellet was again suspended in PBS and centrifuged at 2000 rpm for 15 minutes. This step was repeated twice to remove the impurities from lymphocytes. Isolated lymphocytes were stored in heparinized eppendorf tubes at 4°C and their viability was determined by trypan blue dye exclusion analysis.[13]

Comet assay was performed with lymphocytes suspended in 1 ml PBS and incubated for 30 min at 37°C with 20 µl of mutagen (H_2O_2/4NQO) in the presence 20 µl of different concentrations of GLG fraction. Images of 100 randomly selected cells from each sample were obtained using an Epifluorescent Nikon microscope connected with a digital camera. Extent of DNA damage was evaluated in terms of Tail moment (an index of DNA damage, which considers both tail length and the fraction of DNA in comet tail) by analyzing images on a computerized image analysis system (Lucia Comet Assay Software 4.8 of Laboratory Imaging Ltd.). To check for toxicity or an effect on DNA, GLG fraction was tested alone (without mutagen) also. The percentage inhibition was calculated by the formula:

Inhibition (%)=$(T_1 - T_C)/(T_1 - T_0) \times 100$
Where,
T_1=Tail moment induced by H_2O_2/4NQO
T_C=Tail moment of different concentrations of GLG fraction in presence of H_2O_2/4NQO
T_0=Tail moment of negative control

The whole experiment was repeated thrice to attain significant data.

DPPH radical scavenging assay

The effect of GLG fraction on DPPH radical was estimated according to the method given by Blois.[14] 300 µl of different concentrations of GLG fraction were added in 2 ml of DPPH (0.1 mM methanolic solution). The mixture was shaken well, placed at room temperature for 15 min., and absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The percent DPPH decolorization of the sample was calculated by the equation:

$$\text{Radical scavenging activity} = \frac{A_0 - A_1}{A_0} \times 100$$

Where,
A_0 is the absorbance of DPPH solution.
A_1 is the absorbance of reaction mixture (containing test compound and DPPH solution)

Statistical analysis

The results are presented as the average and standard error of experiments performed in triplicates. The data were analyzed for statistical significance using analysis of variance (one-way ANOVA), and difference among average values was compared by high-range statistical domain (HSD) using Tukey's Test.[15] The statistical significance was checked at $P<0.05$. IC_{50} was calculated from the regression line.

RESULTS

Characterization of GLG fraction

The fraction 'GLG' isolated from MeOH-GG through reverse phase column chromatography was characterized using ^{1}H NMR, ^{13}C NMR, Distortionless Enhancement by Polarization Transfer (DEPT). TLC of this fraction gave one spot at Rf 0.28 comparable to that of standard glycyrrhizic acid in the solvent system *n*-butanol: Distilled water: Acetic acid:: 7: 2:1. GLG fraction was further analyzed by electrospray ionization tandem mass spectrometry (ESI-QTOF-MS/MS) in positive ion mode. ESI-MS of GLG fraction generated two major molecular ion peaks at m/z 823 $[M+H]^+$. Further mass fragmentation of m/z 823 generated the two major fragments at m/z 647 and 471 $[aglycone+H]^+$ due to the sequential glycosidic cleavage of two glucouronic units (176 U). The fragment at m/z 453 $[aglycone+H-H_2O]^+$ was observed due to the loss of one H_2O unit from the aglycone part. Therefore, on the basis of mass spectral data and comparison with standard glycyrrhizic acid, the GLG fraction was identified as 'Glycyrrhizic acid' (GA) [Figure 2].

Anti-genotoxic potential

In the SOS chromotest assay, induction factor of different concentrations of GA remained below 1.5, revealing that it was not genotoxic to the indicator bacteria. The doses of 1.0 mM of H_2O_2 and 20 µg/ml of 4NQO

induced a significant SOS response without affecting the alkaline phosphatase activity. GA inhibited IF caused by H_2O_2 and 4NQO by 75.54% and 71.69%, respectively, at a concentration of 121.46 μM. The fraction showed moderate reduction in induction factor, even at lower test doses [Table 1].

In Comet assay, GA dose dependently inhibited the induced DNA damage in human blood lymphocytes. Among the viability test, the lymphocytes showed a viability ranging from 93% to 97%. The GA, at a concentration of 3.78 μM, inhibited the H_2O_2-induced DNA damage by reducing the tail moment by 11.04%, which dose-dependently increased up to 70.21% at concentration 121.46 μM [Figure 3]. A dose-response relationship showed significance at $P \leq 0.05$. The Tail moment induced by 4NQO was reduced by 69.04% at maximum concentration tested [Figure 4].

Antioxidant activity

The ability of compounds to donate hydrogen ion to stabilize the DPPH radical is a measure of their antioxidant activity. GA showed a very strong activity by effectively scavenging the free radicals by 85.96% at 120 μM concentration. The dose-response relationship was observed, and a logarithmic behavior was followed by the concentrations used for the present study at $P \leq 0.05$ [Figure 5].

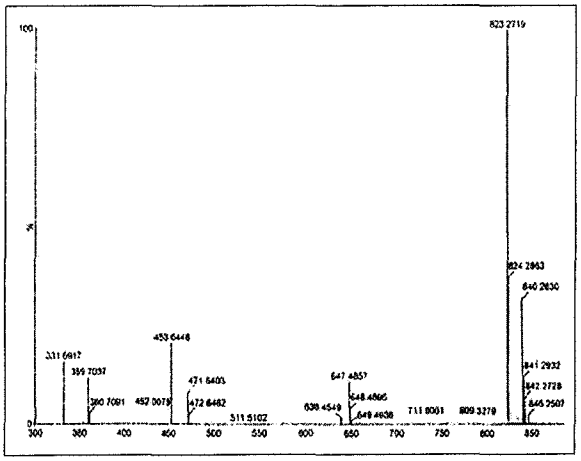

Figure 2: ESI-QTOF-MS/MS of GLG fraction isolated from *G. glabra* L

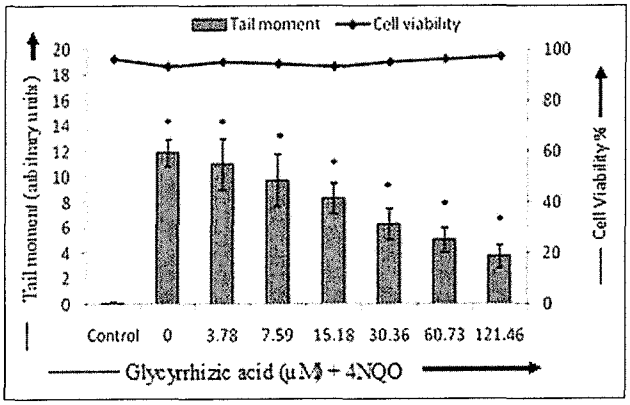

Figure 4: Decrease in tail moment by Glycyrrhizic acid from *G. glabra*. in human blood lymphocytes in Comet assay. Level of statistical significance; $P \leq 0.05$ compared to positive control (4NQO)

DISCUSSION

Dietary compounds, including vitamins, minerals, carotenoids, and a large class of phytochemicals, have been acknowledged as potential chemopreventive agents. These prevent carcinogens to hit their cellular targets by various mechanisms as scavenging reactive oxygen species and enhancing DNA repair. Triterpenoids, a large and diverse group of naturally-occurring compounds, possess important pharmacological properties. These have been reported to exhibit anti-cancer[16] and anti-viral[17] and anti-HIV[18] activities. Triterpenoids isolated from methanolic extract of *Eucalyptus camaldulensis* Dehnh. fruits have been found to inhibit proliferation of the A2780 human ovarian cancer cell line.[19]Triterpenoids from the roots of *Astilbe myriantha* Diels have found to be anti-fungal agents.[20]

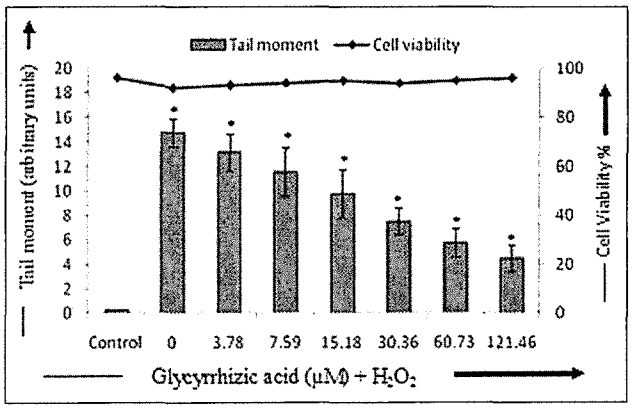

Figure 3: Decrease in tail moment by Glycyrrhizic acid from *G. glabra*. in human blood lymphocytes in Comet assay. Level of statistical significance; $P \leq 0.05$ compared to positive control (H_2O_2)

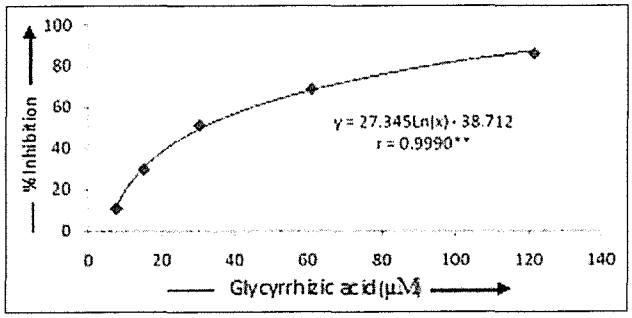

Figure 5: Effect of Glycyrrhizic acid isolated from *G. glabra*in DPPH assay. $^{**}P \leq 0.05$

Table 1: Effect of Glycyrrhizic acid from *G. glabra* L. on genotoxicity of H_2O_2 and 4NQO in SOS chromotest using *E. coli* PQ37 tester strain

Treatment	Dose (μM)	β-Galactosidase units	Alkaline phosphatase units	Induction factor (IF)	Percent inhibition
		Mean±SD	Mean±SD		
Positive Controls					
H_2O_2	1 mM	4.48±0.02	12.98±0.11	7.50	–
4NQO	20 μg/ml	4.26±0.01	13.11±0.21	7.04	–
Negative Control	0.00	0.60±0.03	13.00±0.20	1.00	–
	3.78	0.69±0.02	13.00±0.39	1.15	–
	7.59	0.67±0.03	12.99±0.09	1.12	–
	15.18	0.66±0.06	12.98±0.22	1.10	–
	30.36	0.67±0.04	13.01±0.22	1.12	–
	60.73	0.68±0.06	13.05±0.21	1.13	–
	121.46	0.67±0.04	13.05±0.23	1.12	–
H_2O_2+GA	3.78	4.12±0.05	13.02±0.13	6.86*	09.85
	7.59	3.88±0.10	13.02±0.15	6.41*	16.77
	15.18	3.30±0.08	13.09±0.11	5.48*	31.08
	30.36	2.59±0.05	13.03±0.09	4.30*	49.24
	60.73	2.12±0.10	13.09±0.05	3.50*	61.54
	121.46	1.55±0.08	13.00±0.07	2.59*	75.54
4NQO+GA	3.78	4.02±0.11	13.10±0.20	6.65*	06.06
	7.59	3.84±0.05	13.09±0.25	6.36*	11.26
	15.18	3.30±0.11	13.12±0.13	5.45*	26.33
	30.36	2.92±0.08	13.13±0.22	4.82*	36.76
	60.73	1.98±0.12	13.07±0.28	3.28*	62.26
	121.46	1.65±0.13	13.10±0.18	2.71*	71.69

SD = Standard deviation, Data shown are mean ± SD of three independent experiments, Level of statistical significance, *P<0.05 with respect to positive control

4NQO, a quinoline derivative, is reported to be tumorigenic to lung, esophagus, stomach, skin and other organs and also as a co-carcinogen in the liver.[21] 4-HAQO, the 4-electron reduction product of 4-NQO metabolism, is alleged to be a contiguous carcinogen as it reacts spontaneously with DNA in the presence of oxygen and can get metabolized to DNA-reactive esters.[22] H_2O_2 causes extensive oxidative damage as it interacts with DNA through highly reactive oxygen and radical species.[23]

In the present study, the glycyrrhizic acid (GA), a triterpenoid glycoside, isolated from *G. glabra*, very efficiently suppressed the 4NQO and H_2O_2 induced genotoxicity in both SOS and Comet assay, respectively. It accounts for the sweet taste of licorice root. Among the natural saponins, GA is a molecule composed of a hydrophilic part, two molecules of glucuronic acid, and a hydrophobic fragment, glycyrrhetic acid.[24]

In SOS chromotest, GA reduced the induction factor of H_2O_2 with IC_{50} 34.61 μM. Earlier, there are reports revealing that GA protected human hepatoma cell line against aflatoxin-induced oxidative stress[25] and the chemopreventive activity of GA on 12-O-tetradecanoyl phorbol-13-acetate-induced cutaneous oxidative stress and tumor promotion in Swiss albino mice.[26] In Comet assay, it reduced the tail moment induced by H_2O_2 with IC_{50} 34.95 μM. In another study, comet assay was utilized to evaluate DNA protection capability of GA and revealed that intraperitoneal administration of GA to mice protected cellular DNA from gamma-radiation-induced damage.[27] The ability of silver nanoparticles-glycyrrhizic acid complex (SN-GLY) to protect against ionizing radiation using Swiss albino mice was assessed by employing the comet assay. It was found that immediately after 4 Gy gamma radiation exposures, the treatment of mouse blood leucocytes with SN-GLY *ex vivo* enhanced the rate of repair of cellular DNA damages.[28] GA has also been reported to prevent the lung injuries induced by short term exposures of benzo(a)pyrene,[29] protecting SKH-1 hairless mice against UVB radiation-induced skin tumor formation[30] and acting as anti-hyperglycemic and anti-dyslipidemic compound.[31]

GA exhibited IC_{50} of 34.41 μM in scavenging DPPH free radicals, which is in agreement with the reports revealing GA to be potent scavenger of reactive oxygen species and with anti-inflammatory activity.[32,33] Further, there are studies indicating GA and 18 β-glycyrrhetinic acid to possess anti-inflammatory activity. Both suppressed the expression of pro-inflammatory genes via inhibition of NF-kB and PI3K activity and thus decreased the excessive generation of NO, PGE2, and ROS.[34] Administration of *G. glabra* polysaccharides to mice, which were fed high-fat diet, significantly enhanced immune and antioxidant enzyme activities as compared to the control mice.[35] Similarly, the protective effects of GA on oxidative injury induced by tert-butyl hydroperoxide (t-BHP) leading

to apoptosis in cultured primary rat hepatocytes have also been demonstrated. GA modulated the critical end points of apoptosis induced by oxidative stress and thus GA could be beneficial against liver diseases.[36] In our earlier studies, we have reported GA to be a significant inhibitor of COX-2. It showed 72.60% and 95.08% COX-2 inhibition at concentrations 1 μM and 10 μM, respectively.[37]

CONCLUSION

The present study provides an insight into the role of glycyrrhizic acid as a strong modulator of genotoxic oxidative mutagens and a potent scavenger of free radicals.

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REVIEW ARTICLE

Oxidative stress—implications, source and its prevention

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Abstract Oxidative stress has been a major predicament of present day living. It has been the product of imbalance between the processes involved in free radical generation and their neutralization by enzymatic and non-enzymatic defence mechanisms. The oxidative stress has been contributed by numerous factors including heavy metals, organic compound-rich industrial effluents, air pollutants and changing lifestyle pattern focussing mainly on alcohol consumption, dietary habits, sun exposure, nuclear emissions, etc. The most common outcome of oxidative stress is the increased damage of lipid, DNA and proteins that resulted in the development of different pathologies. Among these pathologies, cancer is the most devastating and linked to multiple mutations arising due to oxidative DNA and protein damage that ultimately affect the integrity of the genome. The chemopreventive agents particularly nutraceuticals are found to be effective in reducing cancer incidences as these components have immense antioxidative, antimutagenic and antiproliferative potentials and are an important part of our dietary components. These secondary metabolites, due to their unique chemical structure, facilitate cell-to-cell communication, repair DNA damage by the downregulation of transcription factors and inhibit the activity of protein kinases and cytochrome P450-dependent mixed function oxidases. These phytochemicals, therefore, are most appropriate in combating oxidative stress-related disorders due to their tendency to exert better protective effect without having any distinct side effect.

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Introduction

In recent years, considerable attention has been focussed on the exploration of phytotherapeutic agents for the treatment of oxidative stress and mutation-related disorders (Matés et al. 2009). Oxidative stress has been imposed due to the imbalance of biochemical processes that involves the generation of reactive oxygen and nitrogen species (ROS and RNS) and their neutralization by the inherent antioxidant (enzymatic or non-enzymatic) defence system of the cells (Matés et al. 2012a). It represents a disturbance in the equilibrium status of prooxidant and antioxidant reactions in living organisms and is the result of those metabolic reactions that utilizes oxygen (Valko et al. 2006). Biochemically, oxidative stress is the result of successive stimulation of mitochondrial electron transport chain, NAD(P)H by cytokines and xanthine oxidase.

The reactive species are the products of normal cellular metabolism, i.e. mitochondrial electron transport chain (ETC.), and are generated by tightly regulated enzymes such as NO synthase (NOS) and NAD(P)H oxidase isoforms, xanthine oxidase and xanthine dehydrogenase, respectively (Valko et al. 2007). ROS including superoxide anion radicals ($O_2{\cdot}^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO·) are generated by intracellular reduction of molecular oxygen. $O_2{\cdot}^-$ is the primary free radical and is produced in the mitochondria during energy transduction reactions in which there is a leakage of a small number of electrons from the electron transport chain particularly from the Fe–S cluster of complex I to molecular oxygen (Miller et al. 1990; Valko et al. 2004; Kovacic et al. 2005). The other sources of $O_2{\cdot}-$ are NAD(P)H, oxidases of phagocytes, 5-lipoxygenase and

xanthine oxidase. O_2·– is a short-lived radical and is transformed to H_2O_2 and molecular oxygen by superoxide dismutase. HO· is the neutral form of hydroxide ion and is a high-reactive radical with a half life of 1 ns. It is produced by the Haber–Weiss reaction and in the Fenton's reaction in the presence of iron, copper, cobalt and chromium (Henkler et al. 2010). Another reactive oxygen species include ROO· or HOO· and are the protonated form of O_2·– and derived from hydroperoxyl radicals (Ghafourifar and Cadenas 2005; Pastor et al. 2000; Valko et al. 2007). RNS include nitric oxide radicals (NO·), $ONOO^-$ and nitrogen dioxide radicals (NO_2·). NO· is produced in biological tissues by specific nitric oxide synthase (NOSs). It has a half life of a few seconds in an aqueous environment and leads to nitrosylation reactions that alter the structure of proteins and inhibit their normal functions. NO· on reaction with O_2·– results in the production of peroxynitrite anion ($ONOO^-$) that is a potent oxidising agent causing DNA fragmentation and lipid oxidation (Carr et al. 2000).

ROS and RNS maintain the redox state of the cell and are within the strict physiological limits under normal conditions due to the functioning of enzymatic and non-enzymatic defence systems. The various enzymes including glutathione reductase, glutathione peroxidase, catalase, superoxide dismutase, etc. and non-enzymatic components including glutathione, α-tocopherol (vitamin E), ascorbic acid (vitamin E), carotenoids, flavonoids and nonprotein thiol are important constituents of the antioxidant defence system prevailing in living organisms (Matés et al. 2012a). The most important endogenous antioxidant of cells is thiol containing glutathione and is abundant in cytosol, mitochondria and nucleus. It is synthesized by the sequential action of glutamate-cysteine ligase and glutathione synthetase. In the nucleus, GSH maintains the redox state of sulfydryl groups of proteins necessary for the repair and expression of DNA. It is also a co-factor for several detoxifying enzymes such as glutathione peroxidase and glutathione transferase, participates in amino acid transport through the plasma membrane, act as a scavenger of HO· and singlet oxygen, detoxifies H_2O_2 and lipid peroxides and helps in the regeneration of vitamins C and E (Masella et al. 2005). Due to these actions, enzymatic and non-enzymatic components regulate the equilibrium status of prooxidant/antioxidant reactions and thus maintain the delicate balance between beneficial and harmful effects of free radicals due to redox regulation (Droge 2002; Halliwell and Gutteridge 2007).

Reactive species are involved in a number of cellular signalling pathways, provide protection against infectious agents and induce mitogenic response at low concentration (Droge 2002; Valko et al. 2006). The signalling pathways that respond to the imbalance of redox state of the cell include the transcription factors such as activator protein 1 (AP-1), nuclear factor kappa B (NK-κB), protein tyrosine phosphatase, mitogen-activating protein kinases (MAPKs), insulin receptor kinases, cytokines, interleukins, tumor necrosis factor-α, angiotensin II, platelet-derived growth factor (PDGFs), nerve growth factor (NGF), transforming growth factor-β1 (TGF-β1), granulocyte macrophage colony-stimulating factor (GM-CSF) and fibroblast growth factor (FGF-2). The endogenous level of ROS regulates the functioning of these signalling pathways by acting on different levels in signal transduction cascades and, thus, checks the proliferation, differentiation and division of the cell (Valko et al. 2007). However, disruption of equilibrium balance imparts oxidative stress, and when present beyond the permissible limit, these reactive species interfere with the physiological functions of cells either directly by damaging biomolecules including DNA, lipids, proteins and carbohydrates or indirectly by inducing mutations as a result of base modifications in DNA or crosslinking it with other biomolecules. These species also alter the expression of genes activated by redox mechanisms and regulating proliferation, apoptosis and cell differentiation (Kitchin and Ahmad 2003).

A number of exogenous agents including chemical carcinogens, UV and ionizing radiations and bacterial or viral infections are known to intensify the production of ROS and RNS. A recent investigation explored the protective properties of plants and related them to their chemical composition (Matés et al. 2012a). The phytochemicals, present in plants, not only enable the plant to withstand harsh environmental conditions but also are helpful in life expectancy improvement strategies (Nobili et al. 2009). The remedies, based on the use of herbs and their bioactive principles, are currently being developed as part of a protective mechanism to render the organism more resistant to mutagens and oxidative stress that is being induced by rapid advancement in industrialisation. Moreover, they are pharmacologically safe as well as easily metabolized by the body, without exerting harmful effects, which are additional advantages linked to the consumption of these phytochemical-based remedies (Sangwan et al. 1998; Ma and Kineer 2002; De Flora and Ferguson 2005; Anetor et al. 2008). Keeping this in view, an attempt has been made in the following sections to review the implications of oxidative stress on various biomolecules, different oxidative stress-inducing agents along with the possible role of secondary metabolites to counteract the effect of oxidative stress.

Implications of oxidative stress and its source

Implications of oxidative stress

The oxidative stress imposed by the overproduction of ROS and RNS ultimately influences living organisms as these reactive species interact with their macromolecules (DNA, proteins and lipids) and might result in mutations due to the

negative gene–environment interactions (Mucci et al. 2001). Rheumatoid arthritis, cardiovascular diseases, neurodegenerative diseases and diabetes are the most common outcomes of increased oxidative DNA, protein and lipid damage, but the pattern of increased cancer risk seems unusual (Halliwell 2002). Recent statistics has also laid emphasis on the vulnerability of cancer as it is the second most common cause of death after heart disease in the world and accounts for nearly 23 % of the total deaths even in a developed country like the USA (Jemal et al. 2007). Besides genetic factors, dietary habits also influenced the incidences of cancer to a great extent, and it was estimated that approximately 30–35 % of cancer deaths were linked to diet (Doll and Peto 1981). The excessive use of pesticides in agriculture is one of the factors that make our diet unsafe for healthy living. The nitrates, nitrosamines and dioxins in food or food additives and the production of heterocyclic amines (HCAs) during cooking of beef, pork, fowl and fish are also sources of carcinogens (Felton and Knize 1991; deMeester and Gerber 1995; Williams et al. 1999; Steck et al. 2007; Shishu and Kaur 2009). In 2000, it was reported by Lichtenstein and colleagues that about 60 % of cancers are attributable to the risk factors present in the environment (Lichtenstein et al. 2000). Anand et al. (2008) shared a vision that only 5–10 % of cancer cases could be attributed to genetic defects, while the remaining cases (90–95 %) were induced and proliferated by environment and lifestyle patterns. The potential linkage between the environmental carcinogens and different forms of cancers is illustrated in Fig. 1, and furthermore, the effect of reactive species on macromolecules is discussed in the following sections.

Oxidative DNA damage

DNA damage mediated by ROS and RNS involves structural alterations due to single- or double-stranded DNA breaks; modification of purine, pyrimidine or deoxyribose; and formation of DNA crosslinks through oxidation, methylation, depurination and deamination reactions. The presence of ROS leads to DNA scission preferentially in the internucleosomal linker region and, thus, resulted in the production of ladders similar to apoptosis. ROS generated during ETC. in the

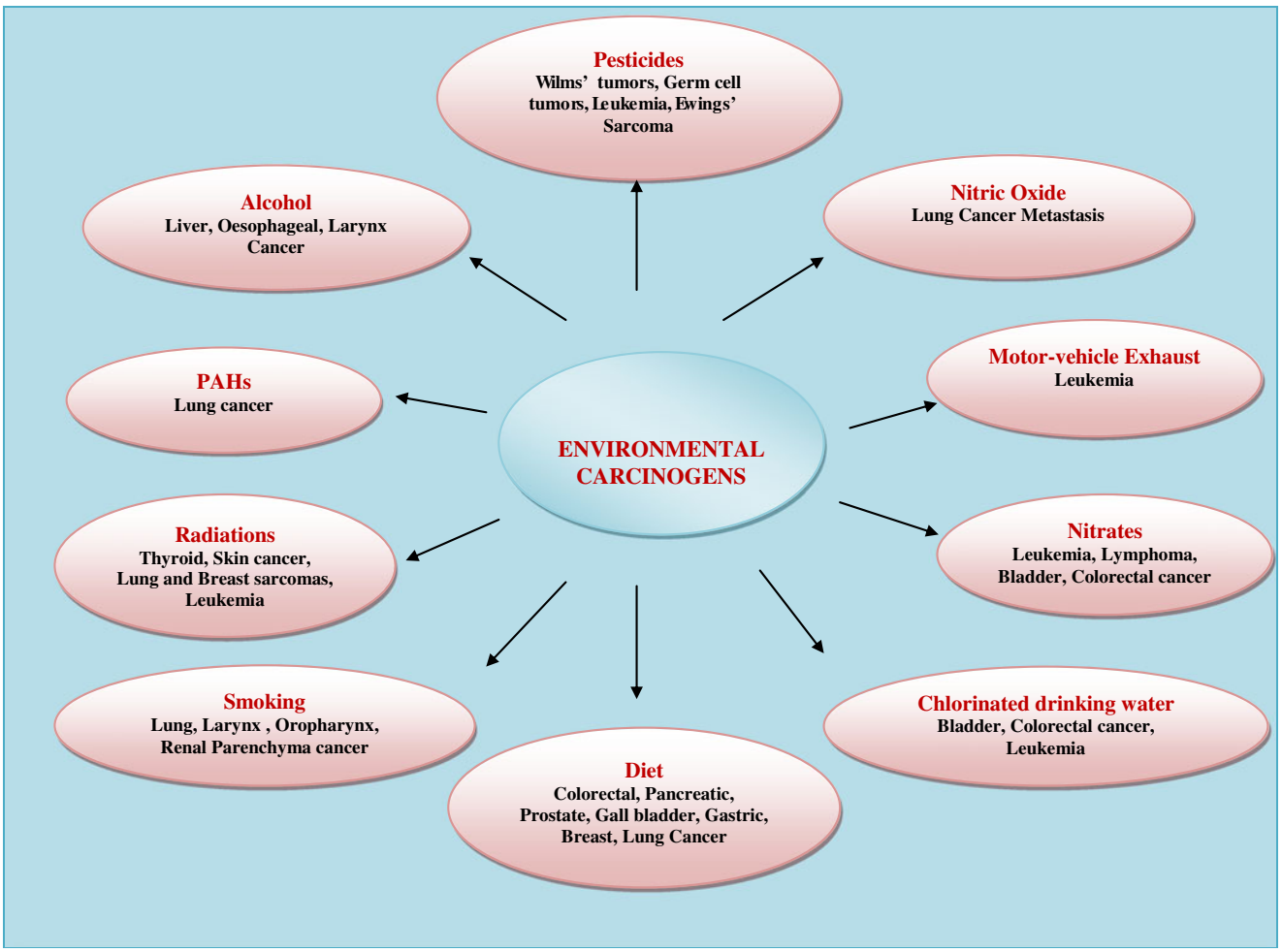

Fig. 1 Potential linkage of different environmental carcinogens to various forms of cancers (Anand et al. 2008)

mitochondria resulted in damage to mitochondrial DNA along with nuclear DNA and thus contributed to neurodegenerative diseases and artheriosclerosis (Wisemen and Halliwell 1996). HO· are known to induce DNA point mutation in GC base pairs and N-terminal deletions in genes leading to the activation of certain oncogenes K-ras and C-Raf-1 (Jackson 1994). HO· are also resulted in the generation of thymine radicals and sugar radicals as a result of hydrogen abstraction from the methyl group of thymine and C-atoms of deoxyribose, respectively. The reactions of base and sugar radicals generate a variety of modified bases and sugars, base free sites, strand breaks and DNA−protein crosslinks (Dizdaroglu et al. 2002; von Sonntag 1987; Valko et al. 2004).

DNA damage is known to play an important role in mutagenesis, ageing and carcinogenesis as oxidative DNA alters the sequence of various nucleotides along with modification of bases (Cadet et al. 2002; Olinski et al. 2002; Bjelland and Seeberg 2003; Barja 2004). DNA mutation is a critical step in carcinogenesis as it results in either arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis (Marnett 2000; Wang and Shi 2001; Valko et al. 2006). The level of DNA lesions is found to enhance in different tumors. 8-Hydroxy-2-deoxyguanosine (8-OHdG) is the most important DNA lesion that is formed due to the attack of ROS at C-8 position of 2-deoxyguanosine and is also considered as the product of promutagenic base modification. 8-OHdG altered the enzyme-catalyzed methylation of cytosines that are important for the regulation of gene expression (Thompson 2004; Valko et al. 2007). The frequency of such modifications is high in oxidative stress and is beyond their repair by base excision repair mechanisms. The replication of DNA prior to the repair of this modified base (8-OHdG) results in GC to TA transversion mutations that are also reported in the genes whose dysfunction is involved in the genesis of cancer (Grollman and Moriya 1993; Henderson et al. 2002).

The p53 tumor suppressor gene and the ras family of proto-oncogenes are known to be important cancer-related genes. The GC to TA transversion mutations in these genes diminish their tumor-suppressing activity and the fact is justified due to its occurrence in more than 50 % of human cancers (Halliwell 2000). ROS are also involved in the modification of other bases, and the resultant bases such as 2-hydroxyadenine (2-OH-Ade), 8-hydroxyadenine (8-OH-Ade), 5-hydroxycytosine (5-OH-Cyt) and 5-hydroxyuracil (5-OH-Ura) are also found to be promutagenic due to miscoding potential (Olinski et al. 2002). In view of the above-mentioned facts and the induction of somatic mutations as a result of DNA adduct formation, oxygen free radicals might be considered as an important class of carcinogens.

Oxidative protein damage

The excessive production of ROS and RNS in living organisms not only damages DNA but also makes proteins susceptible to oxidative insults. It has been reported by Stadtman (2004) that side chain of amino acids, basic elements of proteins, is more prone to oxidation. The amino acids including cysteine and methionine are more susceptible to oxidation, whereas the frequency of oxidative damage is less in intact proteins as compared to misfolded ones (Valko et al. 2007). Oxidation of cysteine residue might lead to reversible formation of mixed disulfides between protein thiol group (−SH) and low molecular weight thiol (GSH). Oxidative attack to proteins resulted in oxidative scission, loss of histidine residues, bityrosine crosslinks and formation of protein centered alkyl R·, alkoxyl RO· and alkyl peroxyl ROO· radicals. The formation of these oxidised side chains of amino acid resulted in the loss of catalytic activity of proteins and their increased susceptibility to proteolytic degradation (Eaton and Qian 2002; Stadtman 1990). The activities of DNA repair enzymes and fidelity of DNA polymerase are altered as a result of oxidative damage-mediated structural modifications of the proteins. The frequency of close proximity double mutations in different genetic stress is related to the altered conformation of DNA polymerase, involved in replication and repair processes (Feig et al. 1994; Madzak and Sarasin 1991). The efficacy of different proteins and aromatic amino acid residues to participate in the signal transduction mechanisms is altered by several RNS ($ONOO^-$ and NO_2·) (Beckman et al. 1994; Yermilov et al. 1995; Eiserich et al. 1994). Protein tyrosine phosphatases (PTPs) and protein kinases C (PKCs), member of serine/threonine kinases, are probably the best characterised direct targets of ROS, and these are the important components of redox control and cell signalling pathways that mediate important cellular functions such as proliferation and programmed cell death (Valko et al. 2007).

Oxidative lipid damage

The oxidative stress exerted by metal-induced ROS also has deleterious effects on polyunsaturated fatty acid residues of phospholipids (Siems et al. 1995). Lipid peroxidation (LPO) is a common cellular process, important in arteriosclerosis and inflammation, and becomes significant when cells are under oxidative stress. It consists of three stages, i.e. initiation, propagation and termination. In the initiation stage, ROS abstracts H from the methylene group in the lipid and results in the formation of fatty acid with unpaired electron. In the aerobic condition, fatty acid radical reacts with lipid to form lipo-peroxyl radicals (ROO·) that abstract H atom from neighbouring fatty acid molecule and thus propagate the reaction. The peroxyl radicals so formed can be rearranged

via cyclisation reaction to endoperoxides (Mao et al. 1999; Marnett 2000; Valko et al. 2007). These endoperoxides can decompose to form a range of secondary products like acrolein, crotonaldehyde, malondialdehyde (MDA) and trans-4-hydroxy-2-noenal (4-HNE) that are highly reactive and react with biological substrates including proteins, amines and DNA. Acrolein and crotanaldehyde are reported to be mutagenic in bacterial and mammalian cells (Cheesemen and Slater 1993; Kawanishi et al. 1998; Squadrito and Preyor 1998). HNE has an effect on signal transduction pathways that affect the phenotypic characteristics of the cell. 4-HNE is reported to be highly genotoxic to lymphocytes and hepatocytes and is found to disrupt the gap junction communications in cultured endothelial cells (Esterbauer 1993). The probable reason for the mutagenic effect of 4-HNE lies in the fact that it forms 4-HNE-dG adduct on interaction with DNA. The adduct, so formed, induces G:C to T:A mutations in human cells, and also, it is preferentially formed at codon 249 of the p53, a mutational hot spot in human cancers (Feng et al. 2004). MDA is reported to be mutagenic in mammalian cells and carcinogenic in rats as on interaction with DNA bases dG, dA and dC, it forms electrophilic adducts M_1G, M_1A and M_1C (Yau 1979; Basu and Marnett 1983; Mao et al. 1999; Valko et al. 2004).

Lipid peroxidation also results in the formation of exocyclic DNA adducts such as ethenoadducts (etheno-dA, etheno-dC and etheno-DG) and propaneadducts. Etheno-dA induces transition to G and etheno-dC induces transversion to A and transition to T. Propanoadducts are formed due to the reaction of DNA with acrolein and crotonaldehyde generated by lipid peroxidation and induce base pair substitution mutations (Valko et al. 2005). These adducts reveal various reactive groups of DNA that could participate in the formation of DNA−DNA interstrand crosslinks and DNA−protein crosslinks and thus have a potential role in human carcinogenesis particularly in hepatocellular carcinoma and cigarette smoke-induced lung cancer (Marnett 2000; Knight et al. 2003; Valko et al. 2004).

Mechanism of cancer development and progression

The mechanistic study of different forms of cancers revealed that cancer development and progression is linked to multiple mutations related to oxidative DNA damage that affect the integrity of genome and thus leading to malformations. The oxidative DNA damage involves a complex series of cellular and molecular changes mediated by a variety of endogenous and exogenous stimuli (Loeb and Loeb 2000; Hahn and Weinberg 2002; Fortini et al. 2003; Powell et al. 2005). The most important stimuli for oxidative DNA damage is the oxidative stress that is imposed by different environmental pollutants including metals and lifestyle factors (Ercal et al. 2001; Hirano et al. 2003; Shi et al. 2004; Valko et al. 2005; Anand et al. 2008). The oxidative DNA damage due to oxidative stress affects the signal transduction pathways by stimulating protein kinases and poly(ADP ribosylation) pathways and thus modulating the expression of genes and redox-sensitive transcription factors essential for cell proliferation and tumor promotion (Cerutti and Trump 1991). ROS and metal ions primarily interact with sulfydryl groups of cysteine residues which are oxidised for either intermolecular or intermolecular disulfide bonds and thus inhibit the activity of phosphoserine/threonine, phosphotyrosine and phospholipid phosphatases. These structural changes as a result of altered protein conformation lead to the upregulation of several signalling cascades, most importantly growth factor kinase-, src/Abl kinase-, MAPK- and PI3-kinase-dependent signalling pathways, and thus result in the activation of several redox-regulated transcription factors (AP-1, NF-B, p53, HIF-1, NFAT) (Valko et al. 2006). Out of these transcription factors, NF-κB, AP-1 and p53 are important factors related to cell growth, cell cycle regulation, DNA repair and differentiation. Out of these transcription factors, NF-κB is involved in inflammatory responses, AP-1 in cell growth and disruption and p53 protein guards cell cycle checkpoint and its inactivation leads to uncontrolled cell division. Nuclear factor of activated T cells (NFAT) regulates muscle growth, cytokine formation, differentiation, angiogenesis and adipogenesis and interacts with NF-κB and AP-1. HIF-1 is a heterodimer and is induced by the expression of oncogenes such as Src and Ras. It regulates the expression of many cancer-related genes including vascular endothelial growth factor (VEGF), aldolase, enolase, heme oxygenase 1 and lactate dehydrogenase A. The presence of H_2O_2 and metals induces the expression of HIF-1 and VEGF and thus stimulates tumor progression (Valko et al. 2006).

Activator protein (AP-1) comprises of several dimeric basic region-leucine zipper proteins (bZIP) such as Jun (c-Jun, Jun B, Jun D), Fos (Fos B, Fra-1, Fra-2), Maf and ATF subfamilies. These proteins have the ability to bind to tumor-promoting agents and cAMP response elements. The oxidative stress-inducing agents such as metals, xenobiotics and other lifestyle risk factors are involved in the activation of AP-1 and thus lead to proliferation, differentiation and apoptosis that furthermore have important roles in carcinogenesis (Evans et al. 2000; Ordway et al. 2003; Varfolomeev and Ashkenazi 2004). c-Fos and c-Jun are positive regulators of cell proliferation, and free radicals would result in the induced expression of these transcription factors and thus lead to increased cell division (Rusovici and Lavoie 2003). NF-κB is a DNA-binding protein and is an inducible and ubiquitously expressed transcription factor. It regulates several genes that are involved in cell transformation, proliferation and angiogenesis. Metal-mediated oxidative stress and ROS resulted in the activation

of NF-κB via tumor necrosis factor (TNF) and interleukin-1 and thus resulted in cell proliferation leading to cancer (Shakoory et al. 2004). Besides cancer, the overproduction of ROS and RNS has been implicated in various pathological conditions involving cardiovascular disease, hypertension; neurological disorders, diabetes, ischemia/reperfusion, rheumatoid arthritis, AIDS and ageing (Walzem et al. 1995; Kaneto et al. 1999; Dhalla et al. 2000; Sayre et al. 2001; Jenner 2003; Cooke et al. 2003; Mahajan and Tandon 2004; Crane et al. 2005; Dalle-Donne et al. 2006; Jung et al. 2006; Valko et al. 2007; Paravicini and Touyz 2008).

Sources of oxidative stress

Environmental pollutants including radiations, pesticides and xenobiotics serve as key factors in intensifying oxidative stress-mediated damage by disrupting the integrity and structures of cellular molecules such as DNA, proteins and lipid membranes (Kovacic and Jacintho 2001; Valko et al. 2001, 2006; Klatt and Lamas 2000; Ridnour et al. 2004). The exogenous agents along with their probable role in different pathologies are discussed in the succeeding sections.

Industrialisation—a prime source of carcinogens/oxidative stress

The industrialisation and synthesis of chemicals for technological advances that have been pacing up without the restraining laws and regulations are considered to be the prime source of carcinogens and oxidative stress-inducing agents (Montesano and Tomatis 1977; Gurjar et al. 1996; Infante and Pohl 2005). The overall impact of such a situation resulted in the contamination of basic elements of life, i.e. air, water and soil (Gurjar et al. 1996; Vidyasagar et al. 2004; Morra et al. 2006). The contamination of air, water and soil on such a large scale disrupts the ecological balance of our ecosystem due to the accumulation of contaminants and probability of their entrance in the food chain. Environmental pollution leads to the extinction of important flora and fauna of the earth's surface and also plays a key role in the etiology of several degenerative diseases (Zingde and Sabnis 1994; Govindan and Desai 1980; Cantillo et al. 1997; Quig 1998).

Oxidative stress induced by polluted water The pollution of water bodies is of serious concern as it has now reached a critical point with industrial effluents and sewage as its major contributing factors (Warhate et al. 2006; Zaman et al. 2008). Industrial effluents have a high proportion of heavy metals like chromium (Cr), nickel (Ni), arsenic (As), vanadium (V), cadmium (Cd), mercury (Hg), lead (Pb) and cobalt (Co) and transition metals like iron (Fe), zinc (Zn) and copper (Cu) (Singh and Chandel 2006; Warhate et al. 2006). These metals have been reported to induce tissue damage and carcinogenesis by invigorating DNA single-strand breaks, forming DNA −protein crosslinks and altering the function of enzymes involved in metabolism, repair and detoxification (Sunderman 1978; Tkeshelashvili et al. 1993; Wang and Shi 2001).

Transition metals are categorized into redox-active metals and redox-inactive metals. Fe, Cu, Co, Cr and V are redox-active metals that lead to the production of reactive species such as HO·, O_2·− and H_2O_2 in Fenton's reaction in mitochondria, microsomes and peroxisomes. The deleterious effects of these reactive species are discussed in the previous sections. The redox-inactive metals such as Cd, Hg, Pb, As and Ni impart toxicity as a result of their covalent binding with sulfydryl groups of proteins due to their electron-sharing affinities. These metals also interact with glutathione and resulted in its depletion within the cells. This, furthermore, leads to the inactivation of several glutathione-associated enzymes such as glutathione peroxidase and glutathione reductase. Certain enzymes have sulfydryl groups at their active sites, and in these enzymes, heavy metal exposure leads to the inactivation of these antioxidative enzymes and thus resulted in the disruption of cellular homeostasis (Valko et al. 2005, 2006). Among different redox inactive metals, Pb, As, Cd and Hg are the most toxic metals. Pb is a toxic metal that disrupts the functioning of important antioxidant enzymes. It interferes with the activity of glutathione reductase involved in the reduction of glutathione disulfide (GSSG) to GSH by interfering with disulfide bonds at its active site. Glutathione peroxidase requires selenium for its activity. Pb forms a complex with selenium and thus inhibits its activity. It also inhibits the synthesis of heme and thus results in the decreased activity of heme containing catalase enzyme. It also declines the activity of SOD by replacing Cu and Zn that are required for its activity (Ercal et al. 2001; Valko et al. 2005). Cadmium exerts its deleterious effects by depleting the GSH levels and enhancing lipid peroxidation. Arsenic in the form of dimethylarsine reacts with molecular oxygen and resulted in the generation of dimethylarsenic radicals and superoxide anion. The addition of another molecule of molecular oxygen results in the production of dimethylarsenic peroxyl radical and HO· due to its reactivity with cellular iron and other transition metals. Mercury is found in water in the form of elemental Hg and methylmercury (MeHg). It causes oxidative damage due to its covalent binding with sulfydryl groups of proteins. It also increases the production of O_2·− and H_2O_2 by impairing the efficiency of oxidative phosphorylation and electron transport in mitochondria. It disrupts calcium homeostasis and thus resulted in the activation of hydrolytic enzymes such as protease, endonuclease and phospholipase. These enzymes ultimately elevate the level of ROS within the cell. Hg in the form of Hg^{2+} displaces Fe and Cu from the intracellular binding site and thus increases the formation of ROS in Fenton's mediated reaction (Valko et al. 2005, 2006).

Industrial discharges also contain a variety of hazardous chemicals that act as mutagens/carcinogens either directly or transformed from procarcinogen to carcinogen via metabolic activation mediated by cytochrome P450 (phase I) enzymes (Ames et al. 1973; Shimada et al. 1996; Mabic et al. 1999; Jarukamjorn 2008; Mehta et al. 2008). These carcinogens alter cell growth and differentiation by causing mutations in DNA via epigenetic mechanisms that ultimately lead to uncontrolled cell growth by disabling the regulatory signals instructing the cell cycle (Sandstead and Alcock 1997; Antero et al. 2008).

Air pollution—another source of mutagens It was observed that polycyclic aromatic hydrocarbons (PAHs), aldehydes and ROS-inducing metals like Cr, Ni, As, Pb, etc. are the main air pollutants, produced by industries and motor vehicular exhausts. These pollutants are capable of adhering to the fine carbon particles present in the air that further penetrate the body through breathing, and the long-term exposure to these PAHs and air pollutants increases the risk of lung cancer by altering the expression of p53 genes (Kriek et al. 1993; Perera et al. 1994; Smith et al. 2000; Pope et al. 2002; Alfaro-Moreno et al. 2007).

Lifestyle patterns as risk factor

Besides environmental pollution, changing lifestyle patterns are also known to contribute a lot in making human beings more prone to degenerative diseases especially cancer. The lifestyle factors that serve as risk factors for the initiation, promotion and proliferation of cancer include alcohol, cigarette smoke, sun exposure, inclusion of fried foods and red meat in the diet, motor vehicular exhausts and nuclear emissions. Fungal and bacterial infections, obesity, stress as well as physical inactivity are also contributing a lot to different pathological conditions (Anand et al. 2008). Cigarette smoke, due to the presence of carcinogenic constituents, has been implicated as a major risk factor for the development of pulmonary, cardio and cerebrovascular diseases and cancer (Rahman et al. 1996; Genbacev-Krtolica 2005; USDHHS 1989). The commonly used pesticides (DDT, DDE, dieldrin and organochlorine) are also reported to augment the risk of brain tumors, Wilm's tumors, Ewing's sarcoma and germ line tumors in children and adults (Bradlow et al. 1995; Stellman et al. 2000; Snedeker 2001; Alavanja et al. 2003; Flower et al. 2004).

Ionizing and non-ionizing radiations are known to induce carcinogenesis and it is estimated that up to 10 % of total cancer incidences might be induced by radiations (Belpomme et al. 2007). The common sources of these radiations are UV rays, pulse electromagnetic fields, mines, radon and radon decay products in homes as well as X-rays used for therapeutic purposes. The radiations serve as the risk factor for different forms of cancer incidences depending upon the exposure time, physiological state, age, etc. (Anand et al. 2008; Little 2009). Exposure to non-ionizing radiations (UV radiations) is known to increase the risk of various types of cancer including basal cell carcinoma, squamous cell carcinoma and melanoma (Tornaletti and Pfeifer 1996; Cadet et al. 2005). The X-rays having therapeutic importance has the potential to induce lung and breast carcinoma, whereas radioactive nuclei are known to develop gastric cancer in rats (Cohen 2002; Ron 2003; Berrington de Gonzalez and Darby 2004; Kleinerman 2007). Low-frequency electromagnetic field like high-voltage power lines, transformers, electric train engines, and more generally, all types of electrical equipment are known to induce the risk of childhood leukemia, brain tumors and breast cancer as a result of clastogenic DNA damage (Demers et al. 1991; Tynes et al. 1992; Brainard et al. 2007).

Defence systems against oxidative stress

The human body, under normal physiological conditions, has several defence mechanisms comprising of non-enzymatic and enzymatic components to counterbalance the production of ROS and RNS and thus minimizing the macromolecular damage exerted by these entities. The non-enzymatic components include radical scavenging antioxidants like glutathione (GSH), ascorbic acid, tocopherols, vitamin E, β-carotene, etc., whereas enzymatic components include enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), glutathione reductase (GSSR) (Halliwell and Gutteridge 1995). Superoxide dismutase, located in the cytosol and mitochondria, is involved in the reduction of superoxide anions to hydrogen peroxide and water, whereas catalase (located in peroxisome) are involved in the removal of hydrogen peroxide by forming water and oxygen. GSH-Px (located in the cytosol and mitochondria) helps to remove excess of hydrogen peroxide in the presence of glutathione, with the formation of water and reduced glutathione. The latter is converted to glutathione with the help of glutathione reductase. The other enzymes that form part of the antioxidative enzyme system and help to maintain redox balance include ascorbate peroxidase, glucose-6-phosphate dehydrogenase (G-6-PDH) and the detoxifying enzymes like γ-glutamyl transpeptidase (GGT) (Halliwell and Gutteridge 1989). However, in case of oxidative stress, the redox balance maintained by different enzymatic and non-enzymatic antioxidant components is shifted towards cellular oxidants and thus increases the amount of free radicals that could not be utilized and neutralized completely by the inherent defence mechanism of the body. These ROS act on different oxidants as well as in different cellular compartments and, due to their devastating nature, are considered as a driving force in the processes of mutagenesis and carcinogenesis (Lal et al. 1999). In such a situation, the urge for additional antioxidants arises, derived from synthetic and natural sources. These antioxidants

unfetter the spiteful effect of free radicals either by acting as antimutagenic or antioxidative agents.

The term antimutagen refers to those agents that result in the reduction of the number of spontaneous or induced mutations. As evident from Fig. 2, these antimutagens either decline the mutation frequency by acting on the repair and replication processes of mutagen-damaged DNA (bioantimutagens) or prevent a mutagen from interacting with DNA by chemical or enzymatic inactivation (inhibiting functioning of cytochrome P450 enzymes such as indole-3-carbinol or by acting as inducer of phase II enzymes), reduce the adverse effects of mutagens by modulating cellular changes induced after DNA damage and also act as a scavenger of free radicals or electrophilic form that serve as mutagen/carcinogens (desmutagens) (Namiki 1990; Bronzetti 1994). On the other hand, antioxidants are substances that delay or prevent the oxidation of cellular oxidisable substrates. They exert their effects by scavenging ROS, activating a battery of detoxifying proteins or preventing the generation of ROS by terminating the chain reactions involved in their production (Halliwell et al. 1992). Tea polyphenols, including (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)epicatechin (EC) and ascorbic acid, are such secondary metabolites that have been extensively studied for their antioxidant and antimutagenic effects. These compounds are reported to exert antimutagenic activity by inhibiting the formation of oxygen free radicals and hampering lipid peroxidation processes as these are the most important factors in the induction of mutagenesis and carcinogenesis (Wang et al. 2000).

Chemoprevention by synthetic antioxidants

The strategy of prevention is a much more satisfying approach than therapeutic intervention, although the two are not mutually exclusive. Chemoprevention is defined as the introduction of selected natural as well as synthetic substances into the diet for the purpose of reducing cancer incidence, though natural supplements are superior to synthetic forms (Malone 1991; Burton et al. 1998).

In recent years, the preference to antioxidants from natural sources increased as compared to synthetic ones including butylated hydroxylanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (THBQ), etc. as reports of toxicological studies linked some synthetic antioxidants to liver damage, cancer and other diseases which have forced regulatory agencies to impose severe restrictions on their use in human foods (Loliger 1991; Parke and Lewis 1992; Williams et al. 1999). In addition, these naturally occurring antioxidants can be formulated to give nutraceuticals that could help to prevent the occurrence of oxidative damage in the body (Knight 2000; Tsuda et al. 2004). Epidemiological

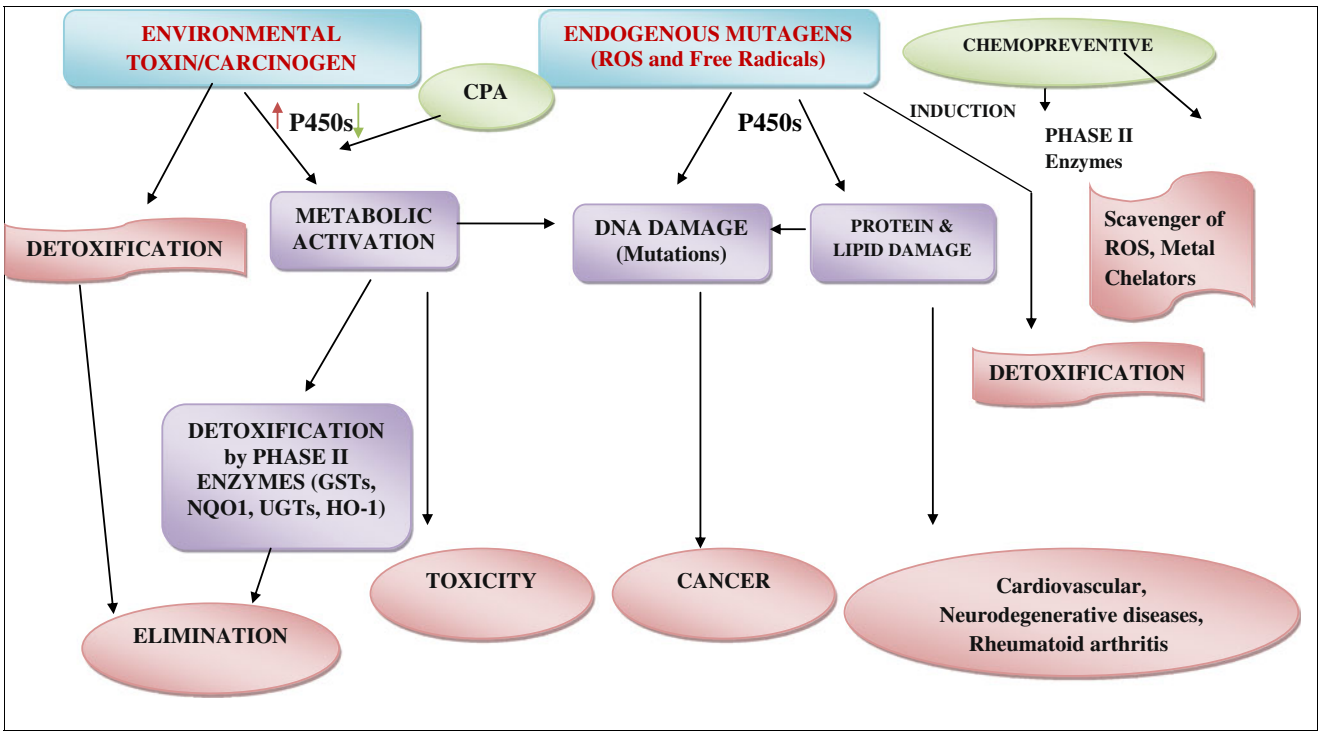

Fig. 2 Pathways of chemical toxicity and protective role of chemopreventive agents. *NQO1* NADPH quinine oxidoreductase, *HO-1* hemoxygenase-1, *GSTs* glutathione-S-transferase, *UGTs* glucouronyl transferases, *CPA* chemopreventive agents (Wolf 2001). *Red upward arrow* represents induction of P450s in the presence of environment toxin and carcinogen leading to metabolic activation of several inactivated carcinogens. *Light green downward arrow* represents downregulation of P450s in the presence of chemopreventive agents

studies have shown that a diet rich in fruits and vegetables is associated with a decreased risk of cardiovascular diseases and certain cancers (Block et al. 1992; Bazzano et al. 2002; Matés et al. 2011). It has been reported worldwide that plants showed strong antioxidant activity and thus acted as powerful scavengers of free radicals (Katalynic et al. 2006; Kumaran and Karunakaran 2007; Atmani et al. 2009; Matés et al. 2009).

Chemoprevention by secondary metabolites

Plants are an eminent source in the introduction of new therapeutic agents, and their medicinal use is based on the premise that they are rich in natural substances that help alleviate human ailments and promote health. The noteworthy preventive and protective properties of these natural substances are related to their strong antioxidative, antimutagenic and anticarcinogenic potentials (Ko et al. 2003; Schwab et al. 2000; Pica et al. 2012). Polyphenols, terpenoids and alkaloids are important secondary metabolites that are known to possess curative properties and are of prime importance for humankind as they prevent the onset of different degenerative diseases by scavenging free radicals and thus preventing chain reaction-mediated damage or binding with catalysts of oxidative reactions, such as some metal ions (Bazzano et al. 2002; Block et al. 1992; Slemmer et al. 2008). According to an estimate by Schwab et al. (2000), over the last 30 years, approximately 160 reports have been published on dietary compounds that protect us from the mutagenic and carcinogenic effects of free radicals generated by mutagens/carcinogens. When people are aware of the sources of natural antimutagens and antioxidants, they are more likely to make selections of food or drink containing substantial amounts of active compounds thereby enhancing their health status (Ko et al. 2003).

Mechanism of actions for secondary metabolites/phytochemicals

Epidemiological studies have indicated an inverse relationship between the intake of protective chemical-enriched fruits and vegetables and the onset of degenerative diseases such as diabetes, cancer, cardiovascular diseases, arthritis, Alzheimer's diseases and osteoporosis (Block et al. 1992; Mahmoud et al. 2000; Bazzano et al. 2002; Park and Pezzuto 2002; Yao et al. 2004; Yoo et al. 2008; Boivin et al. 2009). The protective properties of phytochemicals are the outcome of the multifunctional role associated to their unique chemical nature and their tendency to act as multidentate ligand due to the presence of hydroxyl groups in varying numbers and arrangements around the nuclear structure (Rice-Evans et al. 1996, 1997; Cao et al. 1997). These phytonutrients are important in the physiology of living organisms as they facilitate cell-to-cell communication, repair DNA damage arising from toxic exposure, enhance immune response, cause apoptosis in cancer cells, serve as antioxidants and protect membrane polyunsaturated fatty acids from oxidation that causes biomembrane disruptions of the cell and organelles (Rice-Evans et al. 1997; Ferrari 2004; Lule and Xia 2005; Fresco et al. 2006; Valko et al. 2007). The antioxidative properties of phytochemicals are linked to their ability to scavenge free radicals (HO·, O_2^-, NO·) that because of their short life span and potential to initiate chain reactions put forth damaging effects to biomolecules (Arouma 2003; Soobrattee et al. 2005; Katarina 2007). The scavenging of ROS by dietary antioxidants is supposed to cause the downregulation of transcription factors (AP-1, NF-κB, β-catenin-TcF, p53, TNF-α) that are activated by ROS on interaction with MAPKs (Ma and Kineer 2002; Knassmuller et al. 2008). The secondary metabolites including alkaloids might act as an inhibitor of protein kinases such as cyclin-dependent kinases (CDK), dual specificity tyrosine phosphorylation activated kinase 1A (Dyrk1A), casein kinase 1 (CK1), glycogen synthase kinase-3 (GSK3) and proviral integration site in Moloney murine leukemia virus kinase 1 (PIM-1) that have an important role in promoting apoptotic cell death (Baunbaek et al. 2008). These metabolites also act as antimutagenic agents as they scavenge dietary mutagens through binding or adsorption, hinder the replication process of damaged DNA by acting as bioantimutagenic agents and also act as desmutagens due to indirect activation of mutagens (De Flora 1998; Sangwan et al. 1998; Surh 2003). The plant-derived chemicals are known to detoxify carcinogens through the activation of phase II liver enzymes such as glutathione transferase that tend to detoxify a wide range of carcinogens including isothiocyanates such as benzyl isothiocyanates through the process of hydroxylation and conjugation. The dietary components also act as inhibitors of cytochrome P450-dependent mixed function oxidases and thus suppress the bioactivation of chemical carcinogens to genotoxic agents (Catterall et al. 2000; Ishaq et al. 2003; Soobrattee et al. 2005).

Classification of secondary metabolites

The secondary metabolites, on the basis of their biosynthetic origin, are broadly classified into three important groups such as phenylpropanoids and allied phenolic compounds, alkaloids and terpenoids (Croteau et al. 2000). *Phenolic compounds* are formed by either the shikimic acid pathway or the malonate/acetate pathway, whereas *alkaloids* are primarily synthesized from amino acid and are characterized due to the presence of one or more nitrogen atoms. *Terpenoids* are an important category of secondary metabolites that include both primary and secondary metabolites and are synthesized from the five carbon precursor isopentenyl diphosphate (IPP)—derived from acetate/mevalonate pathway (Croteau et al. 2000). Table 1 describes the important

Table 1 Important classes of secondary metabolites and their effects on living organisms

Class	Subgroups	Example compounds	Some effects and uses	References
Phenolics	Phenols	Catechol, resorcinol	➢ Act as ROS scavengers ➢ Tendency to suppress ROS generating transcription factors ➢ Inactivation of enzymes such as xanthine oxidase (XO), cyclooxygenase type 2 (COX-2), lipoxygenase (LOX) ➢ Detoxification of phase II enzymes interference with cell signalling pathways ➢ Antiviral, antibacterial, antifungal and antiprotozoal effects	Lin and Shih (1994), Fiander and Schneider (2000), Balasundram et al. (2006), Fresco et al. (2006)
	Phenolic acids	Gallic acid, caffeic, synergic acid, ferulic acid		
	Naphthoquinines	K vitamins		
	Xanthones	Mangostin		
	Stilbenes, anthraquinones	Resveratol, alizarin, aloe-emodin, rheinanthrone		
	Flavonoids	Rutin, quercetin, apigenin, hesperitin, catechin		
	Lignans, neolignans	Pinoresinol, podophyllotoxin, steganacin		
	Lignin	Derived from hydroxycinnamyl alcohol monomers		
	Tannins	Tannic acid, gallotannins, ellagitannins		
Alkaloids	Indole alkaloids	Vincristine, vinblastine, strychnine	➢ Anti-inflammatory and cytotoxic agent ➢ Used in the treatment of leukemia, Hodgkin's disease (e.g. vinblastine) ➢ Muscle relaxant (e.g. aporphine alkaloids) and antimicrobial properties ➢ Analgesic and narcotic drug (morphine) ➢ Coniine and strychnine are toxic and may cause paralysis ➢ Inhibitor of protein kinases such as CDK, Dyrk1A, CK1, GSK3 and PIM-1 ➢ Inhibitor of topoisomerase I	Croteau et al. (2000), Cseke et al. (2006), Ziegler and Facchini (2008) Baunbaek et al. (2008), Kabashima et al. (2010)
	Tropane alkaloids	Scopolamine, hyoscyamine, cocaine, atropine		
	Quinoline alkaloids	Quinine camptothecin, chinidin		
	Isoquinoline alkaloids	Morphine, canadine, berberine, ipecac alkaloids		
	Purine alkaloids	Caffeine, theobromine, aminophyline		
	Piperidine alkaloids	Coniine, lobeline		
	Pyridine alkaloids	Nicotine, anabasine, anatabine		
	Imidazole alkaloids	Pilocarpines, lepidilines		
	Pyrrolizidine alkaloids	Senecionine, heliotrine, clivorine		
	Pyrrolidine alkaloids	Hygrine, cuscohygrine		
	Quinolizidine alkaloids	Lupine, lupinine, lupanine		
Terpenoids	Hemiterpene	Isoprene, prenol	➢ Natural flavour additives for food, in perfumery ➢ Traditional and alternative medicines (aromatherapy) ➢ Biochemical processes and also act as immune modulators ➢ Anticancer and antioxidant potential	Croteau et al. (2000), Cseke et al. (2006)
	Monoterpenes	Geraniol, menthol, iridiods		
	Sesquiterpenes	Xanthoxins, gossypol, cedrol, santonnin		
	Diterpenes	Vitamin A, skolin, trisporic acid		
	Sasterpenes	Ophiobolin A, ceroplastol, haslene		
	Triterpenes	Squalene, steroids, sterols, limonoids		
	Tetraterpenoids	Carotenoids		

classes of secondary metabolites along with dietary sources and their effects on living organisms.

In addition to the above-mentioned compounds, there are a number of other chemical groups such as organosulfur compounds such as glucosinolates, organic acids, polysaccharides, lipids, pectins, soluble fibres, phytosterols and amines that are essential for optimal health and longevity (Matés et al. 2012b). Glucosinolates are class of organic compounds that contain sulfur and nitrogen and are derived from glucose and an amino acid and are essential for the proper functioning of phase II enzymes (Nho and Jeffery 2001). The important dietary sources of glucosinolates are members belonging to Brassicaceae family such as *Brassica* spp. These compounds, when ingested, undergo biotransformation to form more active products including indole-3-carbinol, isothiocyanates and thiosulfonates (Nho and Jeffery 2001). The occurrence of thiosulfonates along with organosulfur compounds is reported predominantly from members of Liliaceae family such as *Allium cepa*, *Allium sativum* and *Asparagus* (Wu et al. 2005). The glucosinolates and their biotransformation products are reported to act as inhibitors of platelet aggregation and are also having anti-atherosclerotic, lipid-lowering, antimicrobial, antioxidant, antifungal, antiproliferative and anti-inflammatory properties (Morimitsu et al. 1992; Higdon et al. 2007; Valgimigli and Iori 2009).

Organic acids such as oxalic acid, tartaric acid and salicylic acid form complexes with other phytochemicals to yield compounds with strong health-promoting properties as compared to parent compounds. Phytosterols, pectin and soluble fibres are reported to have cholesterol-lowering properties (Van Bennekum et al. 2005; Ostlund 2007). Phytosterols are important in the way as the sterols, and sterolins isolated from sesame seed are reported to modulate immune functions through thymus hormones and interleukins while those from flaxseeds and olives are reported to have anti-inflammatory effects (Nashed et al. 2005; Aggarwal et al. 2009).

A conclusion can be drawn from the above discussion that oxidative stress is a sole source of a number of complications related to human health due to its damaging effect on all biomolecules including DNA, lipids and proteins. Phytochemicals including phenolics, alkaloids and terpenoids are effective in combating cancer and other oxidative stress-related disorders. These chemical entities, due to their unique chemical structure, act as scavenger of ROS, RNS and modulators of the enzyme system and downregulate the transcription factors involved in cancer progression. The inclusion of these compounds in the diet at recommended doses will be beneficial to prevent several diseases.

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383 – 388

EXPERIMENTAL STUDY

Glycyrrhizic acid attenuated lipid peroxidation induced by titanium dioxide nanoparticles in rat liver

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Abstract

OBJECTIVE: to investigate the hepatoprotective effect of glycyrrhizic acid (GA) against hepatic injury induced by titanium dioxide nanoparticles ($NTiO_2$) in rats.

BACKGROUND: Many recent studies demonstrate that most nanoparticles (NPs) have an adverse or toxic action on liver.

METHODS: NTiO2- intoxicated rats received 300 mg/kg of $NTiO_2$ for 14 days by gavage method. Protection group was pretreated with 10 mg/kg of GA for 7 days before $NTiO_2$ administration. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were detected as biomarkers in the blood to indicate hepatic injury. Product of lipid peroxidation (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were evaluated for oxidative stress in hepatic injury. Light microscopy for histopathological studies was also done.

RESULTS: Administration of $NTiO_2$ induced a significant elevation in plasma AST, ALT and ALP. In the liver, $NTiO_2$ increased the oxidative stress through the increase in lipid peroxidation and decrease in SOD and GPx enzymes. Pretreatment of GA significantly decreased ALT, AST and ALP, attenuated the histopathology of hepatic injury, ameliorated oxidative stress in hepatic tissue, and increased the activities of SOD and GPx.

CONCLUSION: These findings indicate that GA effectively protects against $NTiO_2$-induced hepatotoxicity in rats and might be clinically useful *(Fig. 4, Ref. 47)*. Text in PDF *www.elis.sk.*

KEY WORDS: titanium dioxide nanoparticles, glycyrrhizic acid, hepatotoxicity, oxidative stress, antioxidants.

Introduction

The liver has been considered as the target organ for toxic effects of xenobiotics. The susceptibility of the liver to chemical injury is as much a function of its anatomical proximity to the bloodstream and gastrointestinal tract as to its ability to biotransform and concentrate xenobiotics (1). Previous studies have shown that administration of NPs to rodents result in their accumulation in various tissues including the liver, brain and spleen (2, 3).

Among the various metal nanomaterials, $NTiO_2$ is used in a variety of consumer products such as sunscreens, cosmetics, clothing, electronics, paints, and surface coatings (4, 5). Recent scientific studies show that $NTiO_2$ can be harmful to human and animal health (6–8). It has been reported that $NTiO_2$ can damage liver function and induce oxidative stress and lipid peroxidation in the rodent liver (9–11).

Since NPs, such as $NTiO_2$ affect liver function and its high rate usage it seems essential to find a suitable medication for neutralizing or decreasing their negative side effects. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical disease. More attention has been paid to protective effects of natural antioxidants against chemically induced toxicities (12, 13).

Glycyrrhizic acid (GA) is a natural constituent of liquorice isolated from the dried root of *Glycyrrhiza glabra.* Salts of glycyrrhizic acid are widely used as sweeteners and aromatizers in sweets, drugs, beverages, chewing-gums, chewing tobacco and toothpastes (14). It has been reported that high doses of GA can induce hypertension (15). However, GA possesses numerous pharmacological effects like anti-inflammatory, neuroprotection, anti-viral, antitumor, antioxidant (16–21) and hepatoprotective activities (21–26).

No published data were available about the daily exposure doses of $NTiO_2$ in human. However, $NTiO_2$ increasing use increases the health risk of people exposed to these particles, either occupationally or environmentally. Additionally, it was shown that $NTiO_2$ accumulated mostly in the liver (27, 28). Thus, we used the toxic dose of $NTiO_2$ to evaluate whether GA could prevent hepatotoxicity effects of $NTiO_2$.

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Material and methods

Animals

In this experimental study, 32 healthy and adult male Wistar rats (8–10 weeks old, 180–200 g) were used. The animals were obtained from Ahvaz Jundishapur University of Medical Sciences, Experimental Research Center, and this study was approved by the ethics committee of Jundishapur University and carried out in an ethically proper way by following the guidelines provided. The animals were kept under standard laboratory conditions (12 h-dark and 12 h- light cycles, relative humidity of 50 ± 5 % and 22 ± 3 °C) for at least one week before the experiment and those conditions were preserved until the end of experiment. Animal cages were kept clean, and commercial food (pellet) and water were provided *ad libitum*.

Experimental design

The rats were randomly divided into 4 groups of 8 animals each as follows:

Group 1: Control group; received saline by gavage for 21 days

Group 2: GA group; received 10 mg/kg GA by gavage for 21 days.

Group 3: $NTiO_2$-intoxicated group; 0.2 ml saline was administered for 7 days, and then 300 mg/kg $NTiO_2$ was given for 14 days.

Group4: Protection group; 10 mg/kg GA was administered for 7 days, and then GA (10 mg/kg) plus $NTiO_2$ (300 mg/kg) was given for 14 days.

The doses of $NTiO_2$ (Sigma) were selected according to previous studies that demonstrated significant toxicity in rodents (29). The doses of $NTiO_2$ (Sigma) were selected according to previous studies that demonstrated significant toxicity in rodents (29).

After characterization of $NTiO_2$ (results not shown), the stock solution (2 mg/ml) was prepared in Milli-Q water and dispersed for 10 min by using a sonicator. The stock solution of $NTiO_2$ was kept at 4 °C and used within 1 week for the experiments. Just before use, the stock solution diluted in Milli-Q water and prepared by ultrasonication (Solid State/Ultrasonic FS-14; Fisher Scientific) for 15 min to prevent aggregation. To ensure non-aggregation of $NTiO_2$ before administration, the time interval from preparation to oral gavage was strictly limited in less than 20 min. In addition, 20 min after the preparation, the particle size of $NTiO_2$ was analyzed by atomic force microscopy (AFM).

GA (Sigma) was diluted in saline. The dose of GA was selected based on the results of previous studies (24, 30). One day after the last administration, after blood sampling, the rats were sacrificed by cervical dislocation under ether anesthesia and livers from each animal were removed quickly and weighed. Small pieces of liver were stored separately in a deep freezer (–80 °C) for MDA, SOD and GPx assays. Other pieces were fixed in 10 % buffered formalin.

Biochemical tests

The blood samples were collected in heparinised centrifuge tube and centrifuged. The plasma enzyme levels including plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined spectrophotometrically from plasma samples using commercially available kits (Sigma).

Estimation of lipid peroxidation

The degree of lipid peroxidation in liver tissue homogenate of all the experimental animals was determined in terms of thiobarbituric acid reactive substances (TBARS) formation as previously described (31). A volume of 500 µl of supernatant was mixed with 1.5 ml trichloroacetic acid (10 %) and after centrifugation (4,000× g for 10 min), 1.5 ml of supernatant was added to 2 ml TBA (0.67 %) and heated at 100 °C for 30 min. After cooling, the sample was extracted with 2 ml n-butanol and after centrifugation at 4,000× g for 15 min, the organic phase was collected. The absorbance was read spectrophotometrically at 535 nm. Values were expressed as nmol/mg tissue

Superoxide dismutase (SOD) activity assay

The assay for SOD activity was made according to the method of Suttle (32) using Ransod kit (Randox Labs., Crumlin, UK). This method is based on formation of red formazan from the reaction of 2- (4-iodophenyl)-3- (4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical (produced in the incubation medium from xanthine oxidase reaction), which is assayed in a spectrophotometer at 505 nm. The inhibition of the produced chromogen is proportional to the activity of SOD present in the sample. A 50 % inhibition is defined as 1 unit of SOD, and specific activity is expressed as units per milligram of tissue.

Glutathione peroxidase (GPx) activity assay

GPx activity was determined using the Ransel kit (Randox Labs., Crumlin, UK) according to Paglia and Valentine (1971) (33). GPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized Glutathione (GSSG) was immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance was monitored with a spectrophotometer at 340 nm. One GPx unit is defined as 1 µmol of NADPH consumed per minute, and specific activity is reported as units per milligram of tissue.

Histopathology analyses.

The formalin-fixed samples were embedded in paraffin, sectioned (5 µm) and stained with haematoxylin and eosin (H&E) for histopathology. Six stained microscopy slides per animal were examined microscopically for signs of histopathological features such as necrosis or inflammatory-cell infiltration, hepatocyte vacuolization (fatty deposits) and congestion of red blood cells (RBC).

Statistical analysis

The data were analyzed using one-way ANOVA followed by post hoc LSD test and were presented as mean ± SD while $p < 0.05$ was considered significant.

Results

Nanoparticle characterizations

AFM revealed the size and morphology of the synthesized particles. The complexes appear spherical with a mean size that is inferior to 100 nm as can be seen in Figure 1.

Biochemical tests

GA group showed slightly lower plasma levels of ALT, AST and ALP compared to control group, but the decrease was not significant. Plasma levels of all biochemical tests were significantly increased in $NTiO_2$ group ($p < 0.001$). In GA + $NTiO_2$-treated rats, a significant reduction in the biochemical tests were observed in comparison to $NTiO_2$-intoxicated rats ($p < 0.01$). These findings are depicted in Figure 2.

MDA level, SOD and GPx activities

The administration of $NTiO_2$ significantly increased the hepatic level of MDA when compared with the control animals. This elevation was attenuated by GA ($p < 0.01$). GPx and SOD activities were significantly decreased with $NTiO_2$ compared to the control group ($p < 0.01$). Pre-administration of GA caused a significant increase in SOD and GPx when compared to $NTiO_2$-intoxicated rats. The effects of $NTiO_2$ and GA on MDA, SOD and GPx are reported in Figure 3.

Histological analysis

Under light microscope, liver lobular structures in control and GA groups were clear and regular, and single layer of hepatocytes was arranged around the central vein in a radial pattern (Fig. 4A). In $NTiO_2$-intoxicated rats the normal liver lobular structures were damaged. The hepatocytes showed vacuolization and congestion of RBCs. Infiltration of inflammatory cells was also observed (Fig. 4B). These pathological changes were effectively inhibited by GA (Fig. 4C).

Discussion

Many studies have shown toxic effects of NPs but very little attention has been directed towards the neutralizing or decreasing their toxicity. To our knowledge, this is the first study that demonstrates the protective effect of a natural product against cytotoxicity induced by NPs. In this study, the protective effects of GA against $NTiO_2$-induced hepatotoxicity were investigated. With a histopathological observation, it was possible to determine alterations in liver morphology, such as destruction of lobular structure, vacuolization of hepatocytes (fat deposits), congestion of RBC and infiltration of leukocytes in $NTiO_2$-intoxicated rats.

Hepatocytes fatty deposits might be due to lipid peroxidation that leads to rough endoplasmic damage and detachment of the cytoplasmic lipoprotein. These findings indicate abnormal fat metabolism (34). The abnormal retention of lipids in hepatocytes induced by $NTiO_2$ might indicate toxic injury to the liver in form of hepatocytes liposis by these particles. These results are in agreement with those of the previous investigation describing histologi-

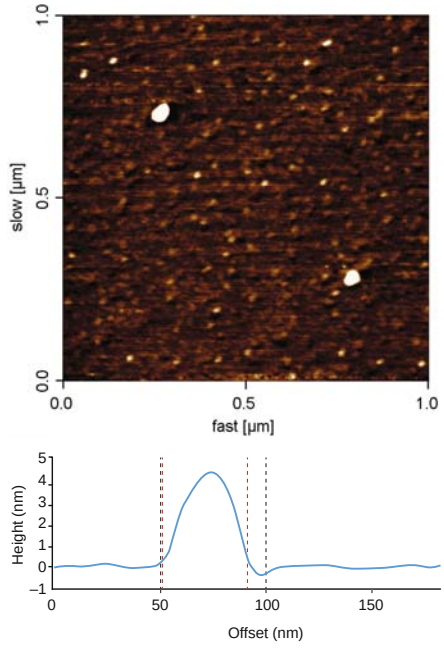

Fig. 1. AFM image of nanoparticles showed distinct spherical particles in size ranging between 50 and 100 nm.

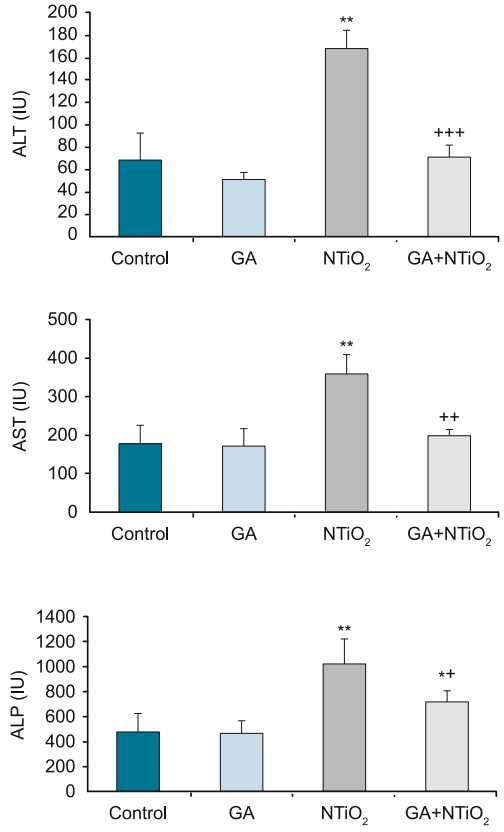

Fig. 2. Biochemical tests in control and experimental groups. Values expressed as mean ± SD for 8 mice. *p < 0.01, **p < 0.001, †p < 0.05, ††p < 0.01, †††p < 0.001; * and † symbols respectively indicate comparison to control and $NTio_2$-intoxicated groups.

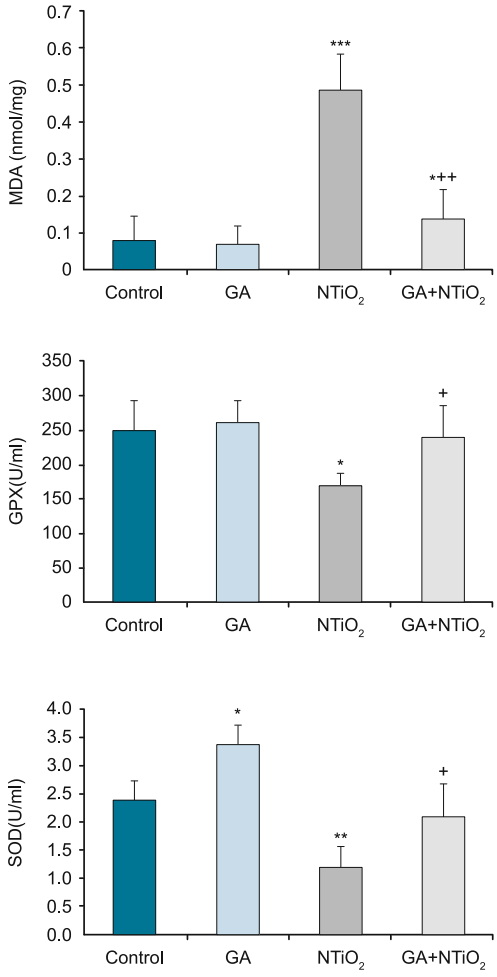

Fig. 3. MDA level, SOD and GPx activities in control and experimental groups. Values expressed as mean ± SD for 8 mice. *p < 0.05, **p < 0.01, ***p < 0.001, †p < 0.01, ††p < 0.001; * and † symbols respectively indicate comparison to control and $NTio_2$-intoxicated groups.

cal alterations in the liver following the administration of $NTiO_2$. Ma et al (2009) show that $NTiO_2$ can induce histopathological changes such as congestion of vascellum, prominent vasodilatation, vacuolization and apoptosis in liver tissue (10). In this study, the histopathological alterations were significantly attenuated by GA. GA improved lobular structure, and decreased vacuolization of hepatocytes (fat deposits), congestion of RBC and infiltration of leukocytes.

$NTiO_2$ significantly elevated the plasma levels of ALT, AST and ALP. The plasma levels of these enzymes are the main indexes which reflect liver injury (35, 36). The rise in plasma AST and ALT has been attributed to the damaged structural integrity of the liver, because these are cytoplasmic in location and are released into circulation after cellular damage. ALP is localized to the bile canalicular pole of hepatocytes. In a diseased liver, this bile duct is often blocked, keeping fluid within the liver. ALP accumulates and eventually escapes into the bloodstream (35).

The reversal of alleviation of plasma enzyme activity in $NTiO_2$-induced hepatic damage by GA could explain the prevention of leakage of intracellular enzymes by its membrane stabilizing activity.

Previous scientific researches demonstrate that $NTiO_2$ induces oxidative stress and lipid peroxidation in the liver of rodents. Shukla et al show that $NTiO_2$ induces oxidative DNA damage and apoptosis in human liver cells (37). The role of oxidative stress in the mechanism of NPs-induced hepatotoxicity has also been reported by Sha et al (38).

In this study, MDA concentration in liver tissue was significantly increased by $NTiO_2$. MDA content is an index of intensified peroxidation process. GA could attenuate the $NTiO_2$- induced increase in the hepatic MDA content. Kiso et al demonstrate that GA can reduce lipid peroxidation (39). Wu et al also showed that 18beta-glycyrrhetinic acid prevents free fatty acid-induced hepatic lipotoxicity (40). As mentioned above histopathological analysis of our study also showed that GA effectively reduced the fatty deposits within the hepatocytes.

Oxidative stress is considered as a major risk factor that contributes to the increase in lipid peroxidation and declines the antioxidants in some degenerative diseases. Oxidative stress has been implicated as one of several mechanisms that have induced toxic effects in different organs due to enhanced production of oxygen free radicals (21).

The body has an effective defense mechanism to prevent and neutralize the free radical-induced damage. This is proficient by a set of endogenous antioxidant enzymes such as SOD and GPx.

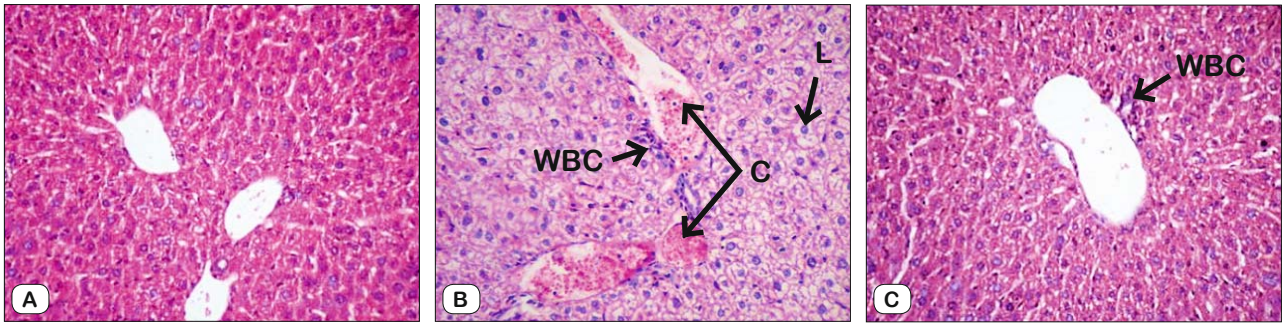

Fig. 4. Light microscopy of cross sections of H&E-stained liver from control and experimental groups. A – Control group; B – $NTio_2$-intoxicated; C – Micro-T group. Magn. x250.

These enzymes constitute a mutually supportive team of defense against reactive oxygen species (ROS) (41).

The observed significantly reduced activities of SOD and GPx point out the hepatic damage in the rats administered with $NTiO_2$ but treatment with GA showed significant increase in the level of these enzymes which indicates the antioxidant activity of the GA.

Kao et al. showed that GA treatment decreased the ROS content by elevating the activities of GPx and catalase in PC12 cells (42). Rahman et al. demonstrate that GA exerts chemopreventive activity against lead acetate-induced hepatic oxidative stress (43).

Numerous scientific studies also suggest that GA has beneficial effects on the liver (22–26). Kiso et al proposed that the antioxidative action of GA plays an important role in its hepatoprotective effects against carbon tetrachloride-induced liver injury (39). Tsai et al. have been shown that GA represses total parenteral nutrition-associated acute liver injury in rats by suppressing the endoplasmic reticulum stress (26). Korenaga et al have been reported that GA-containing preparation reduces hepatic steatosis induced by hepatitis C virus protein and iron in mice (44).

The exact mechanism of GA protection of hepatic injury against $NTiO_2$ is not obtained from this study. However, the reduction in AST, ALT and ALP levels indicates that GA probably reduces necrosis or apoptosis of hepatocytes. Additionally, MDA content which indicates lipid peroxidation was decreased by GA. On the other hand, GA significantly increased the antioxidant activity.

Some researchers have shown that GA induces hypertension and renal damage (15, 45). However, in this study, renal tissues were normal and plasma levels of BUN, Cr and uric acid were not significantly changed in GA (100 mg/kg) treated animals compared to control group (results not shown). Li et al have shown that pretreatment with 200 mg/kg GA improves nephrotic syndrome induced by adriamycin in rats (46). They have also reported that GA reduces the mean arterial blood pressure. Sohn et al have also demonstrated that glycyrrhizin treatment (200 mg/kg) ameliorates renal defects in rats with acute renal failure induced by gentamicin (47).

Conclusion

In conclusion, the biochemical results antioxidant enzyme assessment and histopathological findings found in the present data suggest that GA protects against $NTiO_2$-induced hepatotoxicity. These results suggest that GA may has a potential of clinical application for treating hepatotoxicity induced by metal NPs. However, further studies will be needed to fully understand the exact mechanism of GA on NPs-induced hepatotoxicity.

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Original Article

Factor analysis of the biochemical markers related to liver cirrhosis

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ABSTRACT

Objectives: The purpose of this study was to find the correlations between biochemical study and liver cirrhosis.

Methods: The patients had liver biopsy to check the degree of their liver fibrosis, from August 2013 to August 2014 at the current medical center. In order to find the etiology of hepatitis, a research was done on gender, age, weight, and biochemical study through the investigation of subjects' medical record and medical history. For biochemical study, we examined hemoglobin, platelets, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, gamma-glutamyl transferase (GGT), prothrombin time (PT), and international normalised ratio (INR). We also analyzed the factors that are related to liver cirrhosis.

Results: As a result, the patients at liver cirrhosis F≥2 stage showed 0.973, which is higher than the patients at FO stage with 0.943. F≥2 stage of hemoglobin was 0.544, which is lower than FO stage of hemoglobin with 0.817. Platelet count in F≥2 stage was 0.417, which is higher than FO stage with 0.074. For Albumin, F≥2 stage was 0.155 when F0 stage was 0.135. ATS's F≥2 stage was 0.665, which is 6 times higher than FO stage with 0.100. Moreover, in the case of GGT, F≥2 stage was higher with 0.492 than FO stage with 0.078.

Conclusions: In conclusion, it was confirmed that there is an increase in liver cirrhosis in the following general characteristics and biochemical factors: increase of age, increase of GGT, decrease of albumin, increase of the total bilirubin, and growth of INR (International Normalized Ratio).

KEY WORDS: Correlations, Biochemical study, Liver cirrhosis.

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INTRODUCTION

Liver cirrhosis is a disease when a normal cell is replaced with scar tissue, and the human body becomes disturbed in the performance of essential functions. In liver cirrhosis, liver cells are destroyed without being regenerated, and they become solid and are decreased.[1-4]

Liver cirrhosis is a liver disease and one of the liver cancers. It is also one of the main chronic diseases or the main cause of liver disease in Korea. Compared to the Westerners, Koreans are more likely to get infected by hepatitis, there is a higher repetition rate of liver cirrhosis.[5,6] Since the rate of drinking alcoholic beverages is increasing[7], the occurrence of chronic liver disease is becoming worse. Liver cirrhosis mostly appears when chronic hepatitis due to hepatitis virus is not well treated. Because of the

hepatitis and other factors, normal liver cells are destroyed and they decrease in numbers; this changes the normal structure of liver and disturbs the liver functions.[8,9] There are no specific symptoms of liver cirrhosis at early stages. However, as more liver cells are destroyed, the production of proteins that manage body fluid congestion and blood coagulation, and the metabolism of bilirubin are decreased. For these reasons, there are various complications. Deaths frequently occur due to the serious damage in the liver due to various complications. Therefore, it is very important to prevent varicose vein, seroperitoneum and edema, hepatic encephalopathy, and liver cancer to treat liver cirrhosis.[10]

The methods used for examination and diagnosis of liver cirrhosis are imaging diagnosis and tissue pathology diagnosis; and computed tomography (CT), magnetic resonance imaging (MRI), and Sonography are being widely used.

CT and MRI are costly, and they have side-effects due to the use of contrast media. On the other hand, Sonography is preferred for imaging diagnosis of liver cirrhosis because of its safety, repeatability, and cost.[11] In terms of variables related to the symptoms of liver cirrhosis, sociological variables such as age and gender have been reported.[12] In most of the studies, only the correlation of child-pugh score (sometimes the Child-Turcotte-Pugh score), etiology of liver cirrhosis, and physiological variables have been confirmed.[13,14] However, there has been no clear report about factors related to blood count and biochemical test. Therefore, this study was conducted to find the correlation between biochemical test and liver cirrhosis.

METHODS

A retrospective analysis was conducted on 304 patients who underwent ultrasound test before and after the liver biopsy. They were selected among the patients who received liver biopsy to check the degree of liver fibrosis, from August 2013 to August 2014 at the current hospital. In order to find the etiology of hepatitis, a research was conducted on gender, age, weight, and biochemical study by investigating the subjects' medical record and medical history. In serological study, the patients with chronic hepatitis B virus had positive HBsAg for over-6-month, and the patients with chronic hepatitis C virus had positive anti-HCV and hepatitis C virus (HCV) RNA for over-6-month. The patients with alcoholic hepatitis were also included in this study. Other types of hepatitis caused by the unidentified factors were excluded.

There were a total of 221 subjects which included 144 patients with hepatitis B virus (HBV), 71 patients with HCV, and 6 patients with alcoholic hepatitis. Among all of the subjects, there were 125 men (56.6%) and 96 women (44.3%), and the average age of the subjects was 54.79±11.46. The ultrasound guided percutaneous liver biopsy was performed with a fixed sample with formalin solution. The sample was fixed with formalin solution and treated with paraffin. Then, masson-trichrome staining was done to have a precise understanding of the liver fibrosis and the staining with hematoxylin-eosin was done after sectioning into a thickness of 5μm. According to the guidelines of Korean Society of Pathologies' digestive system of pathology, the system and labeling of hepatitis are categorized into the followings: no fibrosis (F0), portal fibrosis (F1), periportal fibrosis without liver cirrhosis (F2), septal fibrosis (F3), and liver cirrhosis (cirrhosis, F4).[15] In addition, F≥2 stage was analyzed by dividing into significant fibrosis, and F≥3 stage by dividing into advanced fibrosis.

The following 5 items were used for evaluating the individual index: gender, age, height, weight, and degree of obesity. In order to process biochemical index, subjects were instructed to fast for at least 12 hours, and blood and urine tests were given. With the outcomes, we examined 9 factors including the followings: hemoglobin, platelets, albumin, AST, ALT, total bilirubin, GGT, PT, and INR. In order to analyze the factors related to liver cirrhosis, we performed Pearson Correlation coefficient and linear regression analysis. In addition, we conducted logistic regression analysis on the related factors, Pearson's Chi-square test by subject's general characteristic, and Pearson's Chi-square test by hematological index.

RESULTS

The results from the frequency analysis of liver cirrhosis by subjects were as follows: 19 patients in F=0 stage (8.6%), which indicates normal; 29 patients in F≥1 stage (13.1%); 50 patients in F≥2 stage (22.6%); 51 patients in F≥3 stage (23.1%); and 72 patients in F≥4 stage (32.6%) (Table-I). In order

Table-I: The results from the frequency analysis of liver cirrhosis.

Division	*Liver cirrhosis*	*Frequency*	*%*
Biopsy	F=0	19	8.6
	F≥1	29	13.1
	F≥2	50	22.6
	F≥3	51	23.1
	F=4	72	32.6

Table-II: The characteristic correlation between liver cirrhosis and variables.

Variable	*Liver cirrhosis*	*Variable*	*Liver cirrhosis*
Sex	-0.075	[1]AST	0.209
Years	0.260	[2]ALT	0.073
Height	-0.004	Total bilirubin	0.194
Weight	0.000	[3]GGT	0.175
Obesity	0.015	[4]PT	-0.178
Pathogenesis	0.080	[5]INR	0.352
Hemoglobin	-0.152		
Platelets	-0.287		
Albumin	-0.230		

[1]Aspartate aminotransferase, [2]Alanine aminotransferase, [3]Gamma-glutamyl transferase, [4]Prothrombin time, [5]International normalised ratio.

to identify the characteristic correlation between liver cirrhosis and variables, we conducted simple correlation analysis by variables. As a result, we found the correlation in variables as follows: age was positive 0.260, Hemoglobin (g/dL) was negative 0.152, Platelet (10^3/UL) was negative 0.287, AST (IU/L) was positive 0.209, Total bilirubin (mg/dL) was positive 0.194, GGT (IU/L) was positive 0.175, prothrombin time(%) was negative 0.178, and INR was positive 0.352 ($p<0.05$) (Table-II). By conducting linear regression analysis on liver cirrhosis related factors, we were able to compute constant, standard error, and significant probability of each variable and liver biopsy result. There was statistical significance between the result from liver biopsy of liver cirrhosis and the value of age, platelet (10^3/UL), INR in 0.000, Hemoglobin (g/dL) in 0.024, Total bilirubin (mg/dL) in 0.004, and prothrombin time(%) in 0.008 ($p<0.05$) (Table-III). For the precise correlation analysis, simple correlation analysis was performed, and then the multivariate analysis was conducted through logistic regression analysis by using related variables to obtain the odds ratio [Exp(B)]. The odds ratio [Exp(B)] in age for liver cirrhosis patients in F≥2 stage was 0.973 which is higher than the patients in FO stage that was 0.943. For Hemoglobin, F≥2 stage was 0.544 which is lower than F0 that was 0.817. For Platelet count, F≥2 stage was 0.417 which is higher than FO stage that was 0.074. Also, Albumin's F≥2 stage was 0.155 which is higher than F0 stage that was 0.135. AST's F≥2 stage was 0.665 which is over 6 times higher than FO stage that was 0.100. Gamma GT's F≥2 stage was 0.492 which is higher than FO stage that was 0.078 (Table-IV).

Table-III: Linear regression analysis on liver cirrhosis related factors.

Variable	*B*	*SE*
Age	2.299	0.577
Hemoglobin	-0.230	0.101
Platelets	-19.271	4.348
Albumin	-0.173	0.039
[1]AST	6.540	2.073
total bilirubin	0.414	0.141
[2]GGT	10.423	3.963
[3]PT	-5.263	1.967
[4]INR	0.063	0.011

[1]Aspartate aminotransferase, [2]Gamma-glutamyl transferase, [3]Prothrombin time, [4]International normalised ratio

DISCUSSION

In this study, we analyzed the correlations between liver cirrhosis and general characteristic, as well as liver cirrhosis and biochemical examination of blood. Some factors showed a negative correlation.

Table-IV: Logistic regression analysis on liver cirrhosis related factors.

Variable	*Biopsy Classification*	*B*	*SE*	*Exp(B)*
Age	0	-0.059	0.023	0.943
	1	-0.069	0.020	0.933
	2	-0.027	0.016	0.973
	3	-0.023	0.016	0.977
Hemoglobin	0	-0.203	0.532	0.817
	1	-1.496	0.589	0.224
	2	-0.608	0.395	0.544
	3	-1.075	0.426	0.341
Platelets	0	-2.606	0.678	0.074
	1	-1.582	0.464	0.206
	2	-0.876	0.376	0.417
	3	-0.132	0.382	0.876
Albumin	0	-2.003	1.060	0.135
	1	-2.445	1.050	0.087
	2	-1.864	0.649	0.155
	3	-1.332	0.538	0.264
AST	0	-2.307	0.784	0.100
	1	-0.966	0.466	0.381
	2	-0.408	0.370	0.665
	3	-0.364	0.368	0.695
Total bilirubin	0	-2.857	1.066	0.467
	1	-0.807	0.601	0.446
	2	-1.171	0.542	0.310
	3	-0.385	0.443	0.680
GGT	0	-2.554	1.055	0.078
	1	-0.629	0.479	0.533
	2	-0.709	0.401	0.492
	3	-1.204	0.438	0.300
PT	0	-0.693	0.573	0.500
	1	-0.462	0.467	0.630
	2	-0.153	0.377	0.858
	3	-0.102	0.373	0.903

[1]Aspartate aminotransferase, [2]Gamma-glutamyl transferase, [3]Prothrombin time, [4]International normalised ratio

Coco and others have reported that the counts of ALT and AST were elevated in the patients with chronic viral hepatitis.[16,17] According to other studies, the changes of total bilirubin count had an impact on liver cirrhosis.[18] In our study, we looked for other factors that affect liver stiffness from these patients with chronic liver disease. As a result of analyzing how individual characteristic index and biochemical index of blood are related to liver cirrhosis, the followings were shown: growth of elderly, increase of GGT, decrease of albumin, increase of total bilirubin, and decrease of Gamma GT. Although there was an increase of total bilirubin in other studies, there was no report on the changes of ALT value, which differentiate this study from other studies.

A decrease of albumin can cause tissue edema, which results in more serious liver disease and liver cirrhosis. On the basis of the result of this study, overall clinical factors should be considered through medical history, serological examination, and imaging examination of the patients with chronic liver disease, who have no signs of acute exacerbation or superinfection of other acute hepatitis. Especially, when there are growth of elderly, increase of GGT, decrease of albumin and increase of total bilirubin, we can measure liver cirrhosis because these are the signs of increased liver cirrhosis. If there were growth of elderly, increase of gamma GT, decrease of albumin, increase of total bilirubin and increase of INR, it indicates that the liver cirrhosis is being enhanced. Through this study, we assumed that there has to be a massive retrospective study about the direct cause of liver cirrhosis, since it has not been discovered yet.

CONCLUSIONS

In conclusion, it was confirmed that there is an increase in liver cirrhosis in the following general characteristics and biochemical factors: increase of age, increase of GGT, decrease of albumin, increase of the total bilirubin, and growth of INR (International Normalized Ratio).

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Authors' Contribution:

Hyun-Jin Kim and Hae-Kag Lee: Conducted the study and prepared the manuscript.

Jae-Hwan Cho: Helped in conducting the study.

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TOPIC HIGHLIGHT

2016 Alcoholic Liver Disease: Global view

Optimal management for alcoholic liver disease: Conventional medications, natural therapy or combination?

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Abstract

Alcohol consumption is the principal factor in the pathogenesis of chronic liver diseases. Alcoholic liver disease (ALD) is defined by histological lesions on the liver that can range from simple hepatic steatosis to more advanced stages such as alcoholic steatohepatitis, cirrhosis, hepatocellular carcinoma and liver failure. As one of the oldest forms of liver injury known to humans, ALD is still a leading cause of liver-related morbidity and mortality and the burden is exerting on medical systems with hospitalization and management costs rising constantly worldwide. Although the biological mechanisms, including increasing of acetaldehyde, oxidative stress with induction of cytochrome p450 2E1, inflammatory cytokine release, abnormal lipid metabolism and induction of hepatocyte apoptosis, by which chronic alcohol consumption triggers serious complex progression of ALD is well established, there is no universally accepted therapy to prevent or reverse. In this article, we have briefly reviewed the pathogenesis of ALD and the molecular targets for development of novel therapies. This review is focused on current therapeutic strategies for ALD, including lifestyle modification with nutrition supplements, available pharmacological drugs and new agents that are under development, liver transplantation, application of complementary medicines, and their combination. The relevant molecular mechanisms of each conventional medication and natural agent have been reviewed according to current available knowledge in the literature. We also summarized efficacy *vs* safety on conventional and herbal medicines which are specifically used for the prevention and treatment of ALD. Through a system review, this article highlighted that the combination of pharmaceutical drugs with naturally occurring agents may offer an optimal management for ALD and its complications. It is worthwhile to conduct large-scale, multiple centre clinical trials to further prove the safety and benefits for the integrative therapy on ALD.

Key words: Alcoholic liver disease; Alcohol hepatitis;

Conventional medicines; Natural medicines; Hepatic lipid metabolism; Hepatic inflammation; Combination therapy

Core tip: The aim of this article is to review the impairment of hepatocellular dysfunction in alcoholic liver diseases and their prospective managements. Specifically, we focused on the natural therapies with their efficacies and safeties. Moreover, we summarized molecular mechanisms of herbal therapy to treat alcoholic liver disease (ALD). With evidence-based natural therapy, this article highlighted that the combination of pharmaceutical drugs with naturally occurring agents may offer an optimal management for this complex liver disease. It is worthwhile to conduct large-scale, multiple centre clinical trials further to prove the safety and benefits for the integrative therapy on ALD.

Kim MS, Ong M, Qu X. Optimal management for alcoholic liver disease: Conventional medications, natural therapy or combination? *World J Gastroenterol* 2016; 22(1): 8-23 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i1/8.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i1.8

INTRODUCTION

Alcohol is a psychoactive substance and has been widely used in many cultures for centuries. Alcoholic abuse causes a large range of diseases and is also a social and economic burden in world societies. Reduction of alcohol consumption is now becoming the global strategy, with attempts to define "harmful use" of alcohol by the World Health Organization (WHO) to reduce the associated morbidity and mortality. Alcohol intake is a principal etiological factor in chronic liver diseases. Alcoholic liver disease (ALD), which initially manifests as hepatic fat accumulation, followed by an inflammatory response to induce final stage liver failure, is now a deleterious health problem[1]. Alcohol abuse accelerates various types of liver diseases, such as alcoholic fatty liver disease (AFLD), alcoholic steatohepatitis, alcoholic hepatitis (AH), progressive fibrosis, liver cirrhosis and liver failure[2]. Patients with ALD, especially with alcoholic cirrhosis, are associated with increased risk of hepatocellular carcinoma (HCC)[3,4].

The prevalence of ALD has increased in the last years, parallel with the increasing alcohol consumption in the western world as well as in Asian counties[5]. According to the WHO report in 2011, chronic alcohol consumption results in approximately 2.5 million deaths each year with much of the burden related to ALD[6]. Though ALD significantly contributes to the rising morbidity and mortality statistics and related health expenses, there is a lack of effective treatments for ALD, especially cirrhosis and HCC. Abstention from alcohol may reverse the early stage of ALD to a normal condition. The treatment for ALD with conventional medicines, mainly pharmaceutical medications, has limited success with side-effects. Recently, natural medicines, which mainly apply herb-derived agents, are emphasized as alternative therapies to manage the various alcohol-related liver diseases. It is the aim of this article to provide an overview of understanding the mechanisms of ALD, which could generate therapeutic interventions with conventional medicines, natural therapies and their combinations to reverse or retard the progression of ALD.

Pathogenesis of ALD is multifactorial. Strategically, the liver is the most important organ to target as about 90% of alcohol intake is metabolized by the liver[7]. Alcohol consumption can cause fatty liver by disrupting hepatic lipids and glucose metabolism. Furthermore, ethanol and its oxidative and non-oxidative metabolites have direct toxic effects on the liver[8]. Histologically, fatty liver, or HS, is a malfunctioned fat accumulation in the parenchymal cells of the liver. HS is a reversible lesion. Fibrosis (or scar formation) is the subsequent result if liver injury persists. Fibrosis determined by biopsy increases the likelihood of progression to cirrhosis and end-stage liver disease. Biochemically, alcohol abuse contributes to a serious complex phenomenon involving different molecular and biological mechanism to induce ALD. Many hypotheses have been investigated and established to explain the pathogenic mechanisms of ALD. These include: (1) activation of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) to cause the over generation of acetate[9]; (2) induction of cytochrome P450 2E1 (CYP2E1) for alcoholic oxidative stress and hepatotoxicity[10]; (3) abnormal lipid metabolism by increasing fatty acid (FA) synthesis and decreasing of FA oxidation[10]; (4) hepatic inflammation indicated by an increase of tumour necrosis factor-α (TNF-α) and cytokine release[11]; (5) induction of hepatocyte apoptosis and subsequent activation of Kupffer cells; (6) increased hepatic levels of cellular fibronectin and tissue growth factor-β (TGF-β) related to the activation of hepatic stellate cells[12]; and (7) ethanol-induction of gut endotoxins[13,14]. Proposed mechanisms impacting hepatocellular dysfunction by alcohol exposure within ALD pathogenesis are summarized in Figure 1. To further improve current strategies for managing ALD, a deeper understanding of the pathomechanisms of the disease is needed. Various curative approaches based on these mechanisms could prevent the progression of ALD and its downstream sequelae.

CURRENT MANAGEMENT FOR ALD

General management of ALD should initially be

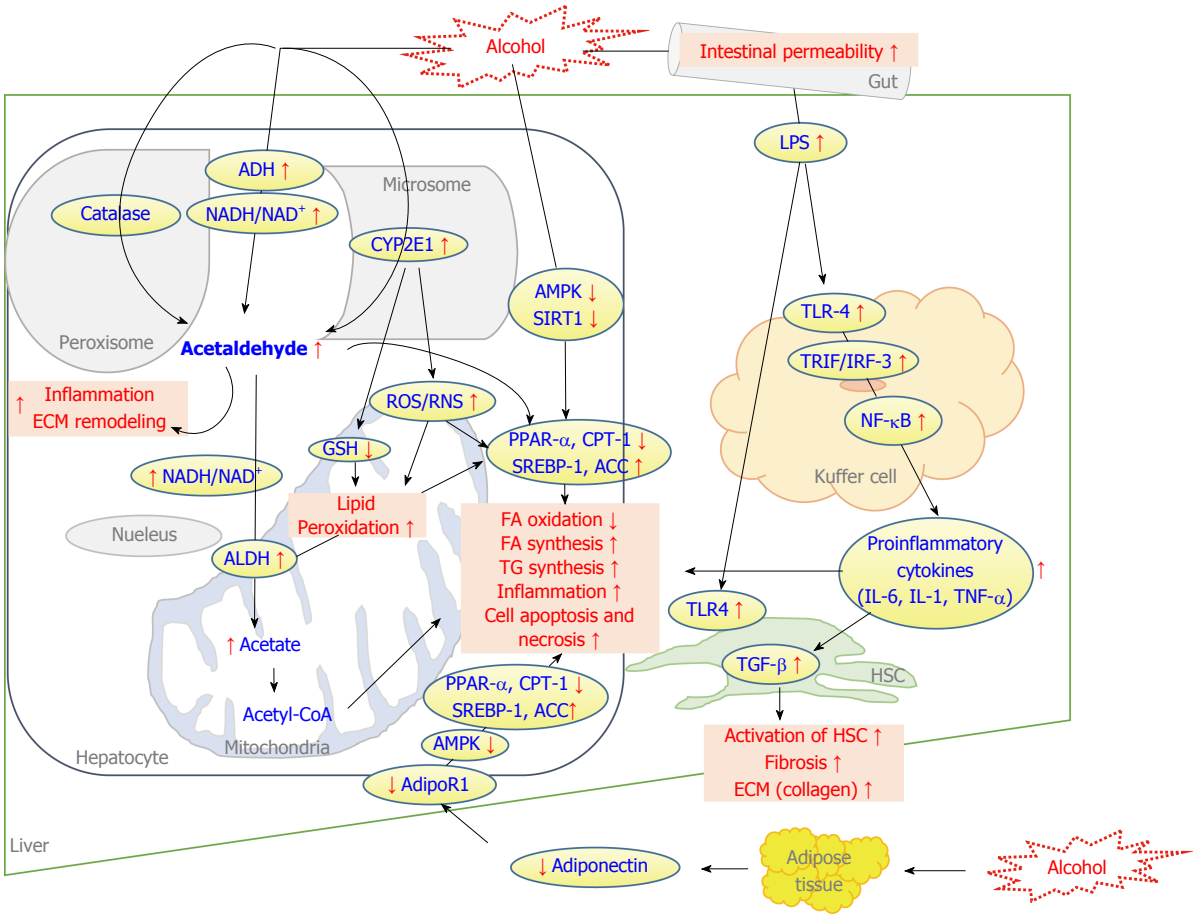

Figure 1 The molecular mechanisms of alcohol-related liver pathogenesis and therapeutic targets. Adapted from references[8,13,159]. When alcohol intake is chronic and heavy, alcohol oxidation occurs *via* cytochrome P450s, resulting in increased levels of CYP2E1, which in turn causes oxidative stress through the generation of ROS which are responsible for lipid peroxidation and alcoholic liver injury. ROS also negatively regulates the activation AMPK and leads to overexpression of SREBP-1, resulting in an increase of *de novo* lipogenesis. GSH also has been reported to its depletion by CYP2E1 followed by the development of oxidative stress. Alcohol consumption negatively affects adiponectin secretion from adipocytes then causes inactive AMPK pathway, leading to elevated *de novo* lipogenesis and inflammatory process in the liver, while simultaneously decreasing fatty acid β-oxidation and contributing to hepatocyte necrosis. The red font indicates the end-point of pathology in the liver. ACC: Acetyl-CoA carboxylase; ADH: Alcohol dehydrogenase; AdipoR1: Adiponectin 1; ALDH: Acetaldehyde dehydrogenase; AMPK: 5' adenosine monophosphate-activated protein kinase; CYP2E1: Cytochrome P450 2E1; CPT-1: Carnitine palmitoyl-transferase-1; ECM: Extracellular matrix; FA: Fatty acid; GSH: Glutathione; HSC: Hepatic stellate cells; IRF-3: Interferon regulatory factor 3; IL-6/IL-1: Interleukin 6 and 1; LPS: Lipopolysaccharide; NADH/NAD+: Nicotinamide adenine dinucleotide hydrogen, nicotinamide adenine dinucleotide; NF-κB: Nuclear factor kappa; ROS: Reactive oxygen species; RNS: Reactive nitrogen species; PPAR-α: Peroxisome proliferator-activated receptor-α; SIRT1: Sirtuin 1; SREBP1: Sterol regulator element binding protein 1; TG: Trigylcerides; TLR-4: Toll-like receptor 4; TNF-α: Tumour necrosis factor-α; TGF-β: Tissue growth factor-β.

abstinence from alcohol[2]. This becomes increasingly vital as the condition progresses. However, this is largely dependent on patient willingness and compliance. Currently, there is no universally accepted therapy to prevent or reverse the progression of ALD. Successful managements include lifestyle modification with the correction of nutritional deficiencies, conventional drugs, liver transplantation, application of complementary medicines, and their combinations[15,16].

Lifestyle intervention

Lifestyle intervention initially entails the abstinence from alcohol. Abstinence limits the development of hepatic steatosis (HS) and prevents further ongoing liver injury, fibrosis and the possibility of HCC. It was reported that abstaining from alcohol improves the prognosis of ALD patients and prevents advancement to hepatic cirrhosis by reducing portal pressure and histological lesions[17,18]. However, long-term alcohol abstinence heavily relies on patient compliance and willingness and therefore may need to be accompanied with psychological therapy or social support. Cognitive behavioral therapy, one of the psychotherapies, reduces the risk of alcoholism and motivates patients to take responsibility and avoid relapse[19]. Pharmacotherapy in combination with psychosocial interventions also encourages patients to sustain abstinence from alcohol. Naltrexone and Acamprosate have been used to manage alcohol abuse in chronic heavy drinkers[20]. Disulfiram has also been approved by the FDA for the management of alcoholism and is broadly utilized despite unclear results from clinical trials[21]. Disulfiram is an acetaldehyde dehydrogenase inhibitor and may cause an increase of serum acetaldehyde by changing

the alcohol metabolism. Disulfiram, therefore, needs to have supervised administration[21]. Furthermore, reducing alcohol consumption, but not completely stopping has also displayed favourable results for the survival of patients with ALD[22]. Other important lifestyle changes can include smoking cessation, adopting a balanced diet and weight control if relevant. Smoking is an independent risk factor for increasing the severity of ALD and its progression to fibrosis and HCC[23,24]. Obesity is another risk factor that can accelerate the progression of ALD as it alone is linked to fatty liver disease and cirrhosis[25].

Nutritional supplements

Malnutrition is another major complication of ALD as poor dietary intake from anorexia, vomiting, mal-digestion, iatrogenic causes, metabolic disturbance, hypermetabolic state, impaired protein synthesis, or mal-absorption can lead to malnutrition and contribute to the disease[26,27]. Nutritional supplementation may address calorie, protein, and nutrient intake to support hepatocyte regeneration[28]. Protein energy malnutrition can be detected with symptoms of muscle wasting, edema and loss of subcutaneous fat. It is recommended that patients with ALD have a daily intake of 1.5 g/kg and 35-40 kcal/kg per day of protein[29]. Supplementation with vitamins such as folate and thiamine should be considered if their deficiencies are detected[16,30]. A discussion concerning micronutrient supplementation is necessary beyond the scope of this article. Nevertheless, an example of micronutrient deficiency related to ALD is seen in zinc deficiency as zinc supplementation has shown to improved ALD by enhancing the activity of ADH and suppressing CYP2E1 in animal models[31,32]. In summary, nutritional status should be balanced in individual cases of malnutrition.

Established conventional therapies for ALD

Although patients living with ALD are mostly treated with strategies to encourage abstinence from alcohol, some patients may need to be accompanied with pharmacological treatment approaches. There are several drugs that have been widely used and reviewed with efficacy, molecular mechanisms (Figure 1), and safety. Table 1 summarizes the effects and mechanism of conventional medicines.

Corticosteroids were one of the first pharmacologic therapies investigated for the treatment of AH and many researchers have extensively studied their use for patients with ALD[33]. The underlying mechanism of action is thought to be the ability of corticosteroids to ameliorate the characteristic inflammatory response by decreasing TNF-α, IL-6, and IL-8[34,35] and also to suppress the formation of acetaldehyde adduct metabolites with inhibition of collagen[36]. Prednisolone, belonging to the corticosteroid family, with a dose of 40 mg/d for 28 d followed by tapering over 2-4 wk is recommended to treat severe alcoholic hepatitis (AH). Another recent published clinical trial randomized 101 acute AH patients to corticosteroids (*e.g.,* prednisone 30 mg/d or methylprednisone 24 mg/d intravenously) and at 30 d, survival was 70% in corticosteroid group when compared to the control (37/53)[37]. Although, corticosteroids have been established as a common treatment for ALD, the data on treatment duration and efficacy of corticosteroids in ALD are conflicting. Long-term usage of corticosteroid seems to enhance the efficacy while others have showed no benefit of corticosteroid therapy after more than 6 mo[38]. Glucocorticoid has been demonstrated to decrease serum bilirubin levels, which is shown to be a clinically useful indicator[39,40]. Using corticosteroids in severe AH, however, carries contraindication including sepsis, hepatitis B, hepatorenal syndrome and gastrointestinal (GI) bleeding[37,41].

Pentoxifylline (PTX), has been thought of as an appropriate substitute to replace corticosteroid treatment in patients with severe AH when there is a contraindication to steroids and is suitable for short-term use[42,43]. The exact mechanism of PTX is not entirely clear. Anti-TNF actions may explain part of its protective effect for ALD. Interesting results have proved that 100 to 1000 μg/mL of PTX inhibited TNF expression by murine peritoneal exudate cells treated with endotoxin 1 μg/mL and PTX treatment significantly reduced serum TNF-α levels in murine *in vivo*[44]. Despite these positive efficacies, treatment with PTX still remains controversial. It has been reported that no significant differences between PTX and corticosteroid treatment groups, or their combination were observed[45]. Meta-analysis also argued that PTX decreased the hepatic-related mortality, but trial sequential analysis did not support this result[46]. Nevertheless, the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases have both recommended that PTX should be considered when AH patients have contraindications to corticosteroid treatment[47].

S-adenosyl-L-methionine (SAM) operates as a methyl donor and contributes in the synthesis of the major cellular antioxidant, glutathione (GSH). In patients with AH and cirrhosis, decreased hepatic SAM, cysteine, and GSH levels caused by abnormality of hepatic gene expression in methionine and GSH metabolism is observed[48]. SAM deficiency arises in early stages of ALD. SAM supplementation can however increase reduced SAM concentrations and reverse liver injury and mitochondrial damage[49]. SAM works to reduce the severity of oxidative stress and hepatic stellate cell (HSC) activation by inhibiting hepatic TGF-α signalling pathway in an ethanol-lipopolysaccharide-induced fibrotic rat model[50]. SAM supplementation has also shown its benefit in 220 patients with chronic hepatitis and cirrhosis in a prospective, double-blind, placebo-controlled trial. Other studies have contradicted these findings, as one demonstrated that there was no significant difference between SAM and placebo in liver

Table 1 The information of effects of current conventional medicines on the treatment of alcoholic liver disease

Medicine/ compound	Type of model	Dosage/duration	Effects	Mechanisms	Ref.
Corticosteroid	RCT in severe AH	Prednisone (30 mg/d, 30 d)	Lower mortality AH group compared to antioxidant treated group		[37]
	RMT and double blind trials in severe AH	Methylprednisolone (32 mg within 7 d) and 28 d treatment and then tapered over 2 wk and discontinued	Decrease short-term mortality of patients with AH		[160]
Pentoxifylline	A double-blind, placebo-controlled trial	400 mg orally 3 times daily for 4 wk	Decrease in the risk of Progressing hepatorenal syndrome; Improve short-term survival in patients with AH		[42]
	A RCT followed by open study in severe AH patients	400 mg/d, orally for 4 wk and then tapered by 5 mg/wk for 7 wk	Better reduced mortality compared to prednisolone in AH		[161]
SAM	Ethanol-LPS-induced fibrotic rats	10 mg/kg bw, ip	Attenuate oxidative stress and HSC activation	Inhibition of TGF-β signaling pathway	[50]
	Double-blind, placebo-controlled trial in 220 patients with AH and cirrhosis	1600 mg/d orally	Decreased serum markers of cholestasis; Improved subjective symptoms such as pruritus, fatigue, and feeling of being unwell		[162]
PPAR-α agonists	C57BL/6J mice with ethanol (27.5% of total calories)	0.1% Wy-14643 for 4 wk	Decreased serum and hepatic fat accumulation	Enhancement of hepatic FA oxidation pathway; Increased PPAR-α mRNA expression by 5-fold	[163]
	C57BL/6J mice with 4 % ethanol (Ethanol feeding for 12 wk)	Wy-14643 (50 mg/kg per day) for last 2 wk	Decreased serum AST and ALT Dropped on score of hepatic steatosis and hepatocyte ballooning	Repress PI3K and COX-2 expression; Enhance adiponectin and HO-1; Increase SIRT1 protein expression	[164]
	C57BL/6J mice with ethanol containing diet (29% of total calories)	Rosiglitazone (3 mg/kg bw/d) for 2 wk	Reduced hepatic triglyceride content; Reduced AST and ALT	Upregulated level of adiponectin and its receptors; Enhance SIRT1-AMPK signaling pathway	[55]
Metformin	C57BL/6 mice TNF knockout mice PAI-1 knockout with ethanol (6 g/kg ig)	200 mg/kg ip for 4 d prior to ethanol administration	Reduced ALT; Prevented hepatic fat accumulation	Prevention of the upregulation of PAI-1	[57]
Metadoxine	HepG2, CFSC-2G and HSC treated with ethanol (50 mM) and acetaldehyde (175 μM)	10μg/mL for 24 h	Reduced peroxidation and oxidized GSH content	Decrease collagen secretion and IL-6, IL-8 and TNF-α secretion	[69]
Carvedilol	Male Wistar rats with ethanol (5%) fed	10 mg/kg per day for last 7 d of total 49 d	Reduced hepatic TG level and the accumulation of fatty droplets within hepatocytes; Inhibited ethanol-induced the thickening of zone 3 vessel walls	Down-regulated FAS and SREBP-1, and up-regulated PPAR-α; Reduced the activation of HSCs with decrease induction of TGF-β1 and collagen	[56]
Propylthiouracil	Male Sprague-Dawley rat with ethanol consumption (36%)	50 mg/kg per day of Wy-14643 for last 2 wk for the last 5 d of 4-6 wk diets with alcohol	Increased hepatic blood flow; Reduces oxidative stress		[71]
Colchicine	RCT in 74 patients with cirrhosis	1 mg/d for 4.4 yr	Reduced serum N-terminal peptide of type Ⅲ procollagen levels		[70]

AH: Alcoholic hepatitis; AMPK: 5′ adenosine monophosphate-activated protein kinase; ALT: Alanine transaminase; AST: Aspartate aminotransferase; bw: Body weight; COX-2: Cyclooxygenase-2, FA: Fatty acid; FAS: Fatty acid synthase; GSH: Glutathione; HepG2: Hepatoma G2 cell-line; HO-1: Heme oxygenase-1; HSC: Hepatic stellate cells; IL-6: Interleukin 6; IL-8: Interleukin-8; LPS: Lipopolysaccharide; mRNA: Messenger ribonucleic acid; P13K: Phosphoinositide 3-kinase; PAI-1: Plasminogen activator inhibiter-1; PPAR-α: Peroxisome proliferator-activated receptor-α; RCT: Randomized controlled trial; RMT: Respiratory muscle training; SIRT1: Sirtuin 1; SREBP1: Sterol regulator element binding protein 1; TG: Triglycerides; TGF-β: Tissue growth factor-β; TNF: Tumour necrosis factor; TNF-α: Tumour necrosis factor-α.

biopsies[51] and no improvement to liver histopathology scores or steatosis, inflammation, fibrosis in ALD patients[52]. Large and high-quality clinical trials are needed to further prove clinical benefits of SAM in ALD.

Peroxisome proliferator-activated receptor-alpha (PPAR-α), a member of the nuclear receptor superfamily and mainly expressed in liver, participates in the regulation of the transcription of genes involved in fatty acid (FA) oxidation, FA transportation, and export of free fatty acid. Alcohol intake inhibits FA oxidation *via* the suppression of PPAR-α in hepatocytes[53]. PPAR-α agonists, are therefore a potential therapy to reverse fat accumulation in AFLD. Many PPAR-α agonists have been assessed as a therapeutic approach to patients with ALD owing their positive efficacies to their anti-inflammatory and hypolipidaemic effects, as well as the induction of FA oxidation. Adiponectin and its receptors, recently, are a new prospective target to manage ALD through PPAR-α, TNF-α and sirtuin 1 (SIRT-1)-related pathways[54]. Thereby, drug discovery of adiponectin agonists may be a potential strategy to manage ALD. PPAR-α plays a pivotal role in the regulation of adiponectin level and rosiglitazone, one of the PPAR-α, agonists has shown to increase the circulating level of adiponectin and enhance hepatic adiponectin receptors (AdipoRs) in ethanol-fed mice[55]. These increments are correlated with the activation of the SIRT-1-AMPK signalling system. Carvedilol, a beta-blocker, has been recently reported to attenuate the development of AH by decreasing hepatic trigylceride (TG) levels and lipid droplets within hepatocytes through down-regulation of fatty acid synthase (FAS) and sterol regulator element binding protein 1 (SREBP-1), and up-regulated PPAR-α[56]. Carvedilol also prevented ethanol-induced thickening of zone 3 vessel walls and reduced HSC activation, decreased induction of TGF-β1 and collagen[56]. Metformin, an antidiabetic drug, has recently been discovered to improve HS by lowering fat accumulation and liver damage in mouse models of acute and chronic alcohol stimulation. This was through the prevention of the up-regulation of plasminogen activator inhibitor-1 and improvement of insulin resistance and liver injury by increasing PPAR-γ and adiponectin levels[57,58].

Although CYP2E1 inhibitors prevent alcohol-induced liver damage, compounds, which have been available in commercial markets, have been too toxic for clinical use. Polyenylphosphatidylcholine (PPC), an innocuous mixture of polyunsaturated lecithins, extracted from soybeans, was recently showed to reduce CYP2E1 activity and also attenuate hepatic oxidative stress and fibrosis[59]. However, one randomized controlled trial (RCT) did not confirm the clear association with the progression of liver fibrosis[60]. Further studies into the effect of PPC on ALD should be encouraged. Clomethiazole, another potential CYP2E1 inhibitor, has also shown to protect the liver from injury in ethanol-fed rats and humans[61,62].

The liver and GI tract shares a reciprocal relationship and therefore establishing healthy gut microbiota has been put forward as a strategy in the treatment of ALD. Lipopolysaccharide (LPS) binds to CD14 in Kupffer cells which reacts with toll like receptor-4 (TLR-4) to activate and release pro-inflammatory cytokines genes[63]. LPS signaling and gut microbiota can be adjusted by probiotics, prebiotics and TLR-4 antagonists to treat ALD[64]. In animal experiments, treating with antibiotics or lactobacillius improved ALD by reducing the gut microflora which suggests that gut-derived endotoxin/LPS may be important in the study of ALD[65]. The modified probiotic, *Escherichia coli* Nissle 1917 was shown to secrete pyrroloquinoline quinone (PQQ), which decreased lipid peroxidation and increased GSH to have an anti-inflammatory, anti-oxidant and anti-hyperlipidaemia effect in acute alcohol exposure in rats[66]. LPS antibody may also benefit injury from LPS induced by exposure to microorganisms in the gut. The outcome of experimental studies has provoked the need to compare the effects of LPS antibodies when combined with corticosteroids for translation into clinical trials for AH[67].

As mentioned, oxidative stress is regarded as a key player in the pathogenesis of ALD with increasing evidence of its importance, such as products of lipid peroxidation being detected in both heavy drinkers and ALD patients[68]. It, therefore, has been anticipated that anti-oxidant therapy would benefit the outcomes for ALD patients. In an animal study, metadoxine had preventative effects of lipid peroxidation and GSH depletion in human hepatocyte (HepG2), exposed with ethanol and acetaldehyde[68]. In HSC, metadoxin also prevents the increase of collagen production and TNF-α secretion[69].

In addition, some drugs have been used for alcoholic liver cirrhosis or fibrosis. Colchicine when administered (1 mg/d) to patients with chronic liver cirrhosis over a three year period displayed anti-fibrotic effects[70]. Propylthiouracil (PTU), typically used as an anti-thyroid medication may also ameliorate liver fibrosis. PTU has been suggested as a therapeutic drug to treat alcohol-induced liver cirrhosis as it increases hepatic blood flow and also reduces oxidative stress in rats[71].

Approach to late stage ALD

Liver transplantation is an effective therapy and is the next viable option for ALD patients with alcoholic cirrhosis who have not responded to abstaining from alcohol. Liver transplantation proposes to be a rescue option for patients with decompensated liver injury and no response from pharmaceutical therapy[72]. Liver transplantation is regarded as a final treatment option for end-stage ALD. However, many reviews have reported that relapse incidences following liver transplantation are common and generally occurs in approximately 10%-52%[73,74] of patients. Moreover, patients who have been treated with liver

transplantation related to ALD display an increased likelihood of *de novo* cancer in other organs, especially, a high rate of cardiovascular complications[75-77]. In addition, the transplantation should be followed after six months of abstinence to avoid histological damage[78,79].

Safety issues with drug treatment

Chronic alcohol uses and subsequent injury to the liver involves various changes to cells and molecules that can compromise the metabolic capabilities of the liver. Therefore, medications that rely mainly on the liver for their metabolism and clearance should be used with caution as there is subsequently less elimination and higher plasma concentrations[80]. Impaired drug metabolism may also relate to changes of the enzyme, CYP2E1[81] which is effected by chronic alcohol use. Additionally, in a cirrhotic liver, drug administration may also be compromised with an increased risk for adverse drug reactions. Furthermore, adverse reactions in the treatment of ALD may often occur due to individual differences such as food intake, genetic differences, age, gender and environmental factors including obesity and diabetes. Although it was found that there was no significantly increased risk of hepatotoxicity in patients with liver disease[80], there still should be considerable attention to dosage when medicating ALD patients to minimise the risk of adverse drug reactions and hepatotoxicity. Caution, for example, should also be taken when using corticosteroids and anti-TNF medications[82] in the hospital setting due to an increased risk of immune-related events including pneumonia, staphylococcus septicaemia, candida septicaemia and GI bleeding[83]. This outcome was seen in a previous study investigating the effects of infliximab and prednisolone which was terminated prematurely due to a significantly higher occurrence of severe infections in the treatment group when compared to placebo and prednisolone[83]. Corticosteroids also increase the risk of coincidental bacterial infections, gastrointestinal bleeding, or renal failure indicating the need for more clinical evidence to confirm this safety issue. Metformin and alcohol combination may produce severe consequences as a side effect of lactic acidosis. This condition can generate when the blood has insufficient oxygen, which is required to transport the glucose throughout the body. Metformin also should be prudent in patients with cirrhosis due to arterial hypoxemia[84]. Other drugs lack evidence in human models beyond preliminary trials due to toxicity or no significant treatment effect. This could perhaps be influenced by the high morbidity rates and short-term mortality associated with end stage ALD, especially acute AH. This highlights the need for complementary and alternative medicine (CAM) where side effects and potential for toxicity are significantly reduced.

Natural medicines as a potential alternative treatment of ALD

CAM encompasses a wide range of approaches with many advantages as it is readily available, has fewer side effects and is a natural way of healing with lasting effects when compared to conventional medicines[85]. With these benefits, natural therapy and herbal medicines have attracted a lot of attention as a potential therapy for the treatment of ALD. It was found that 41% of outpatients with liver disease had utilized some form of CAM[86]. In the US survey, Silymarin and garlic were reported as the most used herbs for liver disease with 12% and 8% respectively. Ginseng (6%), green tea (5%), gingko (5%), echinacea (5%), and St. John's wort (4%) have also been reported. Other than silymarin, the only herb used specifically for liver diseases was licorice root (1%)[87]. Herbal medicines and formulations are commonly grouped according to country. This includes Traditional Chinese Medicine (TCM), Japanese Herbal Medicine, Ayurvedic Medicine (Indian Subcontinent), Traditional African Medicine, Traditional Medicines of the Amazonian Basin in South America, and Arab Traditional Medicine. In general, the markers to display the benefits of herbal products rely on serum/plasma enzymes such as alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Other characteristics, including lipid lowering effects, anti-oxidant, anti- inflammation and anti-fibrotic effects are also used to show the hepatoprotective effects[87]. This review may describe the potential role and effects of natural products in chronic ALD including biological properties and their beneficial effects. A summary list of natural compounds is shown in Table 2.

Green tea polyphenols

Green tea is derived from the leaves and buds of *Camellia sinensis*. Green tea contains rich polyphenolic compounds and has pleiotropic effects including anti-oxidant, hypolipidaemic and anti-inflammatory actions. Green tea polyphenols (GTP) significantly ameliorated ethanol-induced hepatic necrosis. Furthermore, while ethanol significantly increased the accumulation of 4-hydroxynonenal, a product of lipid peroxidation and an index of oxidative stress, green tea extract down regulated this effect. This indicated that GTP protects alcohol-induced liver injury through preventing oxidative stress[88]. The major tea catechins include epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) with EGCG being the major constituent of the catechins with numerous biological functions[89-91]. Our previous study showed total GTP and EGCG enhance glycogen synthesis and inhibit lipogenesis in hepatocytes[92]. EGCG treatment also significantly decreased elevated serum ALT in alcohol-induced rats[88] and normalised the activities of enzymatic anti- oxidants[93]. Another study has reported that EGCG ameliorated plasma endotoxin levels in alcohol-induced female rats with endotoxemia, displaying anti- inflammatory effects through the inhibition of TNF-α, COX-2, and iNOS mRNA expression[94]. EGCG also protected against liver impairment through the enhancement of FA oxidation

Table 2 The information of effects of current natural medicines on the treatment of alcoholic liver disease

Medicine/ compound	Type of model	Dosage/duration	Effects	Mechanisms	Ref.
Vitamin E and C	Malnourished rats with ethanol	Vitamin E (15 mg/kg) Vitamin C (10 mg/kg); single and combined treatments	Decreased ethanol induced hepatic glutathione peroxidase activity and hepatic fibrosis; Attenuated the development of hepatomegaly and hepatic necroinflammation		[165]
Epigallocatechin-3-gallate (EGCG)	Male albino Wistar rats with Ethanol (6 g/kg per day) for 60 d	EGCG (100 mg/kg per day) for the last of 30 d of ethanol administration	Normalization of activities of enzymatic antioxidants; Reduction of lipid peroxidation		[93]
	Female Sprague-Dawley rats with ethanol (56%)	EGCG (100 mg/bw)	Partly blocked the gut leakiness; Reduced endotoxemia and lipid peroxidation	Blunted the elevated expressions of CD14, TNF-α, COX-2 and iNOS	[94]
	Male Wistar rats with ethanol for 5 wk	EGCG contained diet (3 g/L) for 2 wk and then ethanol-EGCG diet for 5 wk	Reduced serum AST and ALT	Enhancement of FA oxidation through increasing of CTP-1 and p-ACC expression	[95]
Silymarin (*Silybum marianum*)	RCT, double-blind, 170 patients with cirrhosis	140 mg/d for 3 times orally	Reduced the rate of mortality No side effect		[33]
	Alcohol induced baboons (50% of calories)	0.84 mg/calorie for 36 mo	Improve histologic stage of fibrosis	Decrease collagen I and (I) procollagen	[100]
	Alcohol and high fat induced rats	100 mg/kg per day, 150 mg/kg per day, 200 mg/kg per day for 6 wk	200 mg/kg per day decrease serum ALT and AST and hepatic fat contents	Attenuated NF-κB p65, ICAM-1 and IL-6 were found in silymarin groups (150 mg/kg, 200 mg)	[101]
Betaine	Ethanol diet Wistar rats	1% (w/v) in diet	Decrease hepatic ballooning and fat contents	Attenuate NOS production; Decrease CYP2E1 protein and activity	[106]
	High fat containing ethanol diet rats 6 g/kg for 8 more wk	200 and 400 mg/kg per day for 4 wk	Decrease ALT and AST	Inhibition of TLR-4 expression; Decrease serum endotoxin, TNF-α, IFN-γ and IL-18	[107]
Glycyrrhizin (*Glycyrrhiza glabra*)	Ethanol-CCl_4 induced male SD rats	Intraperitoneal injections of potenlin (acquired from Hai Ning Pharmaceutical Co., Zhe Jiang, China)	Decreasing serum ALT levels	Normalized NF-κB binding activity	[114]
Ginsenosides	Ethanol-feeding mice Ethanol feeding hepatocytes (AML12 cell lines)	Red ginseng extract containing abundant ginsenosides (Rb1, Rb2, and Rd) (250 mg/kg or 500 mg/kg) for 4 wk in mice	Improves chronic alcohol-induced histopathological changes; Decreases in hepatic triglyceride content	Inhibition of lipogenesis pathway; Attenuated EtOH-induced cytochrome; P450 2E1, 4-hydroxynonenal, and nitrotyrosine levels; Activation of AMPK-SIRT1	[166]
	Alcohol consumption with high fat diet mice	Red ginseng (200 mg/kg per day) for last 2 wk	Lower ALT levels and no different AST	Reduced level of TNF-α and IL-1 and increase IL-10	[124]
Fenugreek seed polyphenol	EtOH (30 mM) induced Chang liver cells	20, 40, 60 mg/mL	Increased GSH/GSSG ratio Reduced EtOH-induced LDH leakage	Inhibition of NF-κB	[129]
	Ethanol induced rats (6 g/kg per day) for 30 d	200 mg/kg per day for 30 d	Improved lipid profile and reduced collagen content, crosslinking, aldehyde content and peroxidation		[132]
Curcumin	Alcohol (100 mM) induced rat primary hepatocytes	0-50 μmol/L	Ameliorated MDA and AST	Improved GSH and heme oxygenase-1 (HO-1) induction	[133]
	Alcohol-induced female Sprague-Dawley rats	400 mg/kg bw	Improved liver pathology, decreasing elevation of hepatic MDA	Suppressing of NF-κB activation	[134]
LIV-52	Ethanol-induced HepG2 cells (100 and 200 mM)	1% or 2% Liv.52 for 24 h		Normalized suppressed PPAR-α and induced TNF-α	[148]

ACC: Acetyl-CoA carboxylase; AML12: Alpha mouse liver 12; AMPK: 5′ adenosine monophosphate-activated protein kinase; ALT: Alanine transaminase; AST: Aspartate aminotransferase; bw: Body weight; CCL4: Chemokine ligand 4; CD14: Cluster of differentiation 14 gene; COX-2: Cyclooxygenase-2; CPT-1: Carnitine palmitoyl-transferase 1; CYP2E1: Cytochrome P450 2E1; EGCG: Epigallocatechin-3-gallate; etOH: Ethanol; FA: Fatty acid; GSH: Glutathione; GSSG: Glutathione disulfide; HepG2: Hepatoma G2 cell-line; HO-1: Heme oxygenase-1; ICAM-1: Intercellular adhesion molecule-1; IFN-γ: Interferon-γ; IL-1: Interleukin 1; IL-6: Interleukin 6; IL-10: Interleukin-10; IL-18: Interleukin-18; iNOS: Inducible nitric oxide synthase; LDH: Lactate dehydrogenase; MDA: Malondialdehyde; NF-κB: Nuclear factor kappa; NOS: Inducible nitric oxide synthase; RCT: Randomized controlled trial; SIRT1: Sirtuin-1; TLR-4: Toll-like receptor-4; TNF-α: Tumour necrosis factor-α.

with increased carnitine palmitoyl transferase 1 (CPT-1) levels[95]. Despite much evidence supporting the effects of EGCG on ALD, clinical studies are still limited and large-scale human studies may be needed to confirm this effect. GTP, catechins may also increase HO-1 activity to reduce oxidative stress in human liver tissue (hHeps). Administering polyphenols prior to ethanol exposure reduced the diminishing of GSH to display a protective effect on liver cells by regulating the immune system and pro-inflammatory mediators such as TGF-β1[96].

Herbal medicines

Silymarin (Milk thistle), which is one of the active compounds extracted from *Silybum marianum* (Asteraceae) is the most common type of CAM therapy for treatment of liver diseases[85]. Silymarin is a complex mixture of polyphenolic molecules including 7 flavonolignans (silybinA, B, isosilybin A, B, silychristin, isosilychristin, silydianin) and 1 flavonoid (taxifolin)[97]. Silibinin, which is a semi-purified fraction of silymarin, has been shown to be a radical scavenger[98] and a RCT with silymarin also improved survival from patients with alcoholic and non-alcoholic cirrhosis without any side effect[33]. Another RCT was also conducted with the prospective effect of silymarin (140 mg/d) in AH[99]. Mechanisms by which silymarin extracts ameliorate liver disease include anti- inflammatory, anti-oxidative, antifibrotic, and immune-modulating activities[100]. Silymarin has retarded the development of alcohol-induced hepatic fibrosis in baboons by preventing collagen type Ⅰ and decreasing histological progression to fibrosis[100]. Silymarin also reduces the elevated serum AST and ALT levels, and decreases hepatic lipid contents in alcohol-induced fatty liver in rats. These effects may be through down-regulating the expression of NF-κB p65, ICAM-1 and IL-6 in liver tissue[101]. However, Cochrane Database reviews have been unsuccessful in demonstrating the beneficial effect of silymarin on ALD. Further studies are needed to provide the appropriate data concerning efficacy.

Betaine is another natural dietary compound, synthesized *in vivo* from choline and converts homocysteine to methionine[102]. Betaine (trimethylglycine), a pivotal nutrient for humans, can be acquired from a wide range of foods and nutritional supplements[103]. It attenuates AH by restoring phosphatidylcholine generation *via* the phosphatidylethanolamine methyltransferase pathway[104]. Betaine also increases SAM, normalizing hepatocellular SAM:S-adenosyl-*L*-homocysteine ratio[105], resulting in the attenuation of fatty liver[103]. The study of mitochondrial dysfunction in the progression of ALD has also been enforced by many research areas. Betaine gives a beneficial effect against loss of oxidative phosphorylation system proteins by alcohol, by preventing NOS_2 induction and NO generation in male Wister rats with exposed to ethanol[106]. The mechanism of betaine in liver is also involved in the suppression of endotoxin/TLR-4 signaling pathways. Betaine treatment decreased the levels of serum ALT, AST, endotoxin, TNF-α, IFN-γ, IL-18, and TLR-4, and improved the degree of HS and inflammation in liver tissues in rats with alcohol-induced liver injury[107].

Glycyrrhiza glabra (Leguminosae), commonly named licorice root, is a perennial herb cultivated in temperate and subtropical regions of the world as well as central and South-Western Asia[108]. Glycyrrhizin, as a main constituent in aqueous extract, is a conjugate of two molecules of glucuronic acid and one of 18β-glycyrrhetic acid[109]. Glycyrrhizin is hydrolysed by intestinal bacterial into 18β-glycyrrhetic acid, which is reabsorbed into the bloodstream. Glycyrrhizin suppressed serum AST and ALT levels and histologically inhibited the spread of deteriorating areas in hepatocytes in an animal model of concanavalin A-induced liver injury[110]. Moreover, the effects of Glycyrrhizin has extended to the attenuation of inflammation response by regulation of NF-κB and MAPK pathway[111] and inhibition of TNF-α, ROS, and proinflammatory interleukins such as IL-6 and IL-1β[112,113]. Another beneficial effect of glycyrrhizin, from Potenlini compound, was shown in ethanol-CCl_4 induced liver cirrhosis rats by decreasing serum ALT levels and normalising NF-κB binding activity[114,115]. In clinical trials, glycyrrhizin is mostly used to treat hepatitis C and interferon treatment[16]. One clinical trial showed to lower level of ALT with 200 mg of glycyrrhizin for 5 d per week for approximately 10 years in hepatitis C patients[116]. Glycyrrhizin has potential effects to cure various chronic liver diseases but large-scale, rigorously designed clinical trials for ALD is warranted.

Ginseng, an ancient medicinal herb, has been popular as a tonic for the treatment of various diseases including diabetes and hepatic diseases[117]. Ginsenosides, the main active constituent groups of all ginseng species, has been used as a supplementary medicine and is mostly responsible for the beneficial pharmacological effects in metabolic disorders and various cancer *via* its ability to boost immunity and its anti- inflammation function[118,119]. Ginsenoside Rb1, as the most abundant ginsenoside in *P. ginseng* has improved hepatic fibrosis induced by CCl_4 by down regulation of hepatic prostaglandin E2 and TIMP-1[120]. Ginsenoisde Rg1 showed to inhibit TNF-α-mediated NF-κB transcriptional activity in HepG2 cells with IC50 of 28.14 μmol/L and gene expression of iNOS and COX-2 inducible inflammatory enzymes[121]. It was also reported that ginsenoside Rc (40 mg/kg) attenuates AFLD in alcoholic-fed ICR mice through the regulation of AMPK and MAPK pathways[122]. Red ginsengs, which contain an abundance of ginsenosides, showed to improve chronic alcohol-induced histopathological changes and hepatic TG content through inhibition of the lipogenesis pathway and AMPK-SIRT1 activation in alcohol-fed mice[123]. Red ginseng also reduced levels of TNF-α and IL-1β which were increased by alcohol consumption with a high fat diet in mice[124]. Recently,

compound K which is a metabolite form of ginsenoside Rb1 and Rc has been identified as an inducer to increase AMPK expression, resulting in reduction of lipid droplets and hepatic TG accumulation in high FA exposed human hepatocytes (Huh7). This compound had better efficacy to treat HS compared to metformin[125]. With the hepatoprotective effects of many types of ginsenosides, these herbal components should be potential targets to improve liver damage caused by alcohol abuse.

Fenugreek (*Trigonella foenum graecum*) seeds have been reported to have beneficial effects on enhancing the antioxidant apparatus[126]. Fenugreek seeds also have been used in remedies for diabetes and high cholesterol in various traditional medicines and proven experimentally in diabetic humans[127]. The polyphenolic extract of the seeds is the main constituent beneficial for the treatment of ALD according to many articles. In general, fenugreek seed polyphenols contain five different polyphenolic flavonoids, vitexin, tricin, naringenin, quercetin, and tricin-7-O-b-D-glucopyranoside[128]. These polyphenols prevent the alcohol-induced toxic effect in human Chang liver cells through improvement of GSH/GSSG ratio[129]. Interestingly, quercetin ameliorated ethanol-stimulated mitochondrial dysfunction by induced permeability transition though suppressing GSH depletion. This indicates a promising preventive strategy for ALD[130,131]. Fenugreek seed polyphenols also have demonstrated improvement to lipid profiles and collagen content in alcohol-fed rats[132]. Compared to other herbal medicines, however, the clinical studies of fenugreek seed polyphenols are lacking evidence in metabolic disorders, especially in relation to ALD, suggesting the need for accurate standardization of polyphenols and their individual efficacy for the treatment of ALD.

Curcumin, commonly called turmeric yellow and compound of *curcuma longa*, is a low-molecular weight polyphenol derived agent. It is commonly used in Ayurvedic medicine and has various pharmacological effects including anti-oxidative, anti- inflammatory, and hepatoprotective activities[105]. This historical use of curcumin has prompted investigation into the hepatoprotective effects against liver damage induced by alcohol abuse and the molecular mechanisms, however the research in this area is still lacking. Curcumin has ameliorated malondialdehyde (MDA) and AST and improved GSH and heme oxygenase-1 (HO-1) induction in alcohol (100 mM) induced rat primary hepatocytes[133]. Curcumin (400 mg/kg bw) has also been examined in alcohol-induced female Sprague-Dawley rats to show improvement by reducing the elevation of hepatic MDA, and the suppressing of NF-κB activation[134]. Another similar result confirmed the inhibition of the expression of NF-κB-dependent genes by the supplementation of curcumin (75 mg/kg per day) for 4 wk in rats[135]. Recently, low dosage of curcumin prevented HS compared with the alcohol control group with inhibition of dehydrogenase, ALDH2 as well as CYP2E1 activation[136]. Curcumin also proved its anti-oxidant effect against ethanol-exposed mice by decreasing ROS generation[137]. Despite the beneficial effects of curcumin in ALD, it is unfavourable to apply as a medical strategy due to its decreased bioavailability and rapid metabolism with systemic elimination in animals and humans, suggesting an investigation of more sophisticated pharmacokinetic improvements of curcumin such as curcumin phospholipid complex or curcumin nanoparticles[105,138].

Herbal formulas to treat ALD

Historically, some herbal blends have been commonly used for hepatic disorders around the world. Clinical trials to evaluate their efficacy are, however, challenging to demonstrate due to heterogeneous formulations and dosages. Moreover, these blends have not been regulated by the Food and Drug Administration. In this review, a few blends are evaluated in ALD. Herbal Medicine861 (HM 861) consists of 10 herbs, based on TCM including *Salvia miltiorrhiza*, *Astragalus membranaceus* and *Spatholobus suberectus*[139]. HM861 has shown the inhibition of HSC proliferation and induction of HSC apoptosis in patients with chronic hepatitis[140].

Moreover, the compound also suppressed tissue inhibitor metalloprotease 1 (TIMP-1) mRNA expression in HSC-T6 cells[141]. In clinical trials, this herbal blend was tested for anti-fibrotic activity encompassing 107 patients with hepatitis B resulting in the drop of ALT levels to the normal range in 73% of patients[142]. In a clinical trial, HM861 showed to improved fibrosis and early cirrhosis in 52 patients with HBV-related fibrotic patients with the hepatic inflammatory scores also decreased[143]. More Cochrane reviews, however, are needed to confirm the efficacy of the blend in the treatment of ALD. TJ-9, also referred to as Shosaiko-to in Japan or xiao-chai-hu- tang in China, containing 7 herbal constituents including: bupleurum root, pinellia tuber, scutellaria toot, jujube fruit, ginger rhizome, ginseng root, and glycyrrhiza root. The formula has been traditionally used for the treatment of liver diseases. Usually, the preparation contains 7.5 g of TJ-9 with the active components, baicalin and baicalein, responsible for the anti-oxidant activity[144]. Although the exact of mechanism of TJ-9 is unknown, one anticipated mechanism may be exposed in the observation of stellate cells which has shown an inhibition of α-smooth muscle actin, type Ⅰ collagen production, and cell spreading, indicating the suppression of HSC activation[144]. TJ-9 has been associated with several cases of interstitial pneumonitis, especially when used with interferon for the treatment of chronic hepatitis[145]. Liv-52, which is an Indian Ayurvedic medicine that has been used for the treatment of liver diseases, is a herbal preparation including *Capparis spinosa* (Himsara), *Cichorium intybus* (Kasani), *Mandur bhasma, Solanum nigrum* (Kakamachi), *Terminalia arjuna* (Arjuna),

Cassia occidentalis (Kasamarda), *Achillea millefolium* (Biranjasipha), *and Tamarix gallica* (Jhavaka)[146]. It was originally used to treat ALD, but a recent RCT from Europe demonstrated a detrimental effect on advanced alcohol-induced cirrhosis[147]. Contrastingly, an *in vitro* study, reported that Liv.52 improved PPAR-γ suppression and TNF-α expression in HepG2 cells[148]. Another clinical study also showed the efficacy of LIV.52 in 26 cirrhotic patients for 6 mo with normalized serum ALT and AST levels compared to the placebo group[149]. Although there are many positive effects of LIV.52 for the improvement of ALD, underlying molecular mechanisms are still necessary to support its potential function. In summary, many compounds are available on the open market to treat ALD, but their efficacy data is still not supported by clinical trials. Comprehension of herbal interaction with pharmacological medicines is also necessary to improve the treatment of ALD.

The combination therapies of drugs and natural agents

More readily available on the market and popular than ever, CAM and herbal therapy are increasingly attractive treatments for chronic liver diseases with many advantages, such as low side effect and toxicity. Augmentation of herb-drug interaction has consequently followed stipulating dialogue between pharmaceutical experts and practician. Although there is strong evidence, supporting the use of herbal medicine for the treatment of ALD, combination therapy should be speculated for any safety issues. Magnesium isoglycyrrhizinate injection is one example of a drug containing glycyrrhizin and possesses effective and safe treatment for chronic liver diseases[150] and down regulates the progress of pulmonary fibrosis[151]. Glycyrrhizin also has been combined with matrine to improve CCl_4-induced liver fibrosis through lowering levels of collagen and less HSC proliferation[152], suggesting the positive effect of their combination to protect against liver fibrosis. Recently, a combination of silymarin and SAM was evaluated in ALD markets with much promise[153]. There have been some cases of adverse events and hepatotoxicity caused by herbal medicines[154]. *Xiao Chaihu Tang*, alone or in combination with interferon, may induce acute interstitial pneumonia in chronic hepatitis patients[155]. Despite the emerging of CAM on the markets, documented herb-drug interactions are still sparse, especially in relation to ALD with a reliance only on individual case reports. Although some combinations of CAMs and conventional drugs contribute many beneficial efficacies, some herb-drug interaction also may be associated with toxicity in certain situations such as the combination of silymarin with indinavir therapy in AIDS patients[156].

FUTURE OUTLOOK AND PERSPECTIVE

It is undeniable that chronic alcohol abuse leads to deleterious implications for the liver regardless of age, gender or other factors. The more advanced the stage of alcoholic liver disease, the more morbid and life-threatening the complications become. Various therapies should, therefore, aim to reverse these complications but are often only palliative. As an initial therapy of ALD, abstinence from alcohol accompanied with basic lifestyle modifications when appropriate. When the injury becomes decompensated, pharmacological therapy should be accompanied to reverse the more severe stages of ALD. The present studies are still inconclusive with humble methodological quality and design, high heterogeneous patient populations, and poorly defined point of progression of ALD. These flaws may partly explain the conflicting reports in the literature to support the effectiveness of treatment for ALD for many conventional medications, such as Corticosteroids, PTX, and SAM. Mechanistic studies are still insufficient to prove their pharmaceutical potency and future studies will be thirsty to investigate the molecular mechanisms related in the complex relationships between ethanol metabolism, oxidative stress, immune response, HSC activation and extracellular matrix remodelling. Larger RCTs with a longer follow-up period are required for further evaluation.

Increasing attention has been paid to herbal medicines as a newly emerging treatment strategy for ALD. In this review, we summarized functionalities of CAM for the prevention and treatment of ALD. Most single herbs and formulae have resulted in improvement of ALD-related conditions with multiple and diverse mechanisms of actions in spite of their mild side effects. To date, only a few active compounds from herbal extracts have been identified as candidates to treat ALD and alcohol induced liver injury. Although many herbal constituents have shown promising potential for the treatment of ALD with multi-targets, the underlying molecular mechanisms, especially that of single herbal compounds, have not been completely elucidated. Moreover, clinical trials to adjust concurrent systematic review are still required in the area of ALD research. More profound studies underlying the prospective effect of herbal medicines should be further investigated. In addition, some unique therapies are now increasing to develop the treatment of ALD. Epigenetic regulation underlying alcohol metabolism, such as histone modification may be a new trend to advance effective treatment strategies for ALD[157]. Acetate may affect histone modification by up-regulating acetyl-CoA and enhance the inflammation in ethanol-exposed macrophages by interfering histone deacetylase activity[158]. With many natural products potentially possessing this ability of epigenetic modification, it would be a particularly beneficial breakthrough for ALD patients.

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Oxidative Damage of DNA Induced by the Cytochrome *c* and Hydrogen Peroxide System

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To elaborate the peroxidase activity of cytochrome *c* in the generation of free radicals from H_2O_2, the mechanism of DNA cleavage mediated by the cytochrome *c*/H_2O_2 system was investigated. When plasmid DNA was incubated with cytochrome *c* and H_2O_2, the cleavage of DNA was proportional to the cytochrome *c* and H_2O_2 concentrations. Radical scavengers, such as azide, mannitol, and ethanol, significantly inhibited the cytochrome *c*/H_2O_2 system-mediated DNA cleavage. These results indicated that free radicals might participate in the DNA cleavage by the cytochrome *c* and H_2O_2 system. Incubation of cytochrome *c* with H_2O_2 resulted in a time-dependent release of iron ions from the cytochrome *c* molecule. During the incubation of deoxyribose with cytochrome *c* and H_2O_2, the damage to deoxyribose increased in a time-dependent manner, suggesting that the released iron ions may participate in a Fenton-like reaction to produce ·OH radicals that may cause the DNA cleavage. Evidence that the iron-specific chelator, desferoxamine (DFX), prevented the DNA cleavage induced by the cytochrome *c*/H_2O_2 system supports this mechanism. Thus we suggest that DNA cleavage is mediated via the generation of ·OH by a combination of the peroxidase reaction of cytochrome *c* and the Fenton-like reaction of free iron ions released from oxidatively damaged cytochrome *c* in the cytochrome *c*/H_2O_2 system.

Keywords: Cytochrome *c*, DNA, Fenton Reaction, Peroxidase

Introduction

Cytochrome *c* has been studied extensively because of its central role in electron transfer in living organisms. The protein is localized on the intermembrane-space side of the inner mitochondrial membrane and participates in mitochondrial electron transport (Wilkstrom and Saraste, 1984). Recent reports implicate cytochrome *c* in oxidative stress, which results from the run-away production of reactive oxygen species. In addition, cytochrome *c* acts as a mediator of apoptotic cell death signals (Cai *et al*., 1998). The oxidative stress-mediated cellular damage and apoptotic cell death have been associated with neurodegenerative disorders, Parkinson's disease (PD) (Hashimoto *et al*., 1999), and cancer (Park and Kim, 2005).

During exposure to hydrogen peroxide, many proteins with metal binding sites are susceptible to oxidative damage, and free metal ions could be released (Kang and Kim, 1997). It has been reported that the reaction of hydrogen peroxide with heme proteins, such as cytochrome *c*, produces highly reactive ferryl-heme species that are capable of oxidizing biomolecules and initiating lipid peroxidation (Radi *et al*., 1991a; 1993b). Recently, it was reported that hydrogen peroxide oxidized cytochrome *c* to a peroxidase compound I-type intermediate, in which one oxidizing equivalent is present as an oxoferryl heme species and the other is present as the protein tyrosyl radical (Lawrence *et al*., 2003). The reaction of cytochrome *c* with H_2O_2 may lead to release of iron ions from cytochrome *c*. Therefore, the transition metal, iron, may react with H_2O_2 to produce ·OH through a Fenton-like reaction. ·OH is the most powerful oxidizing species among several reactive oxygen radicals, and is able to oxidize most macromolecules including DNA, protein, lipid, and carbohydrate (Frank *et al*., 1989; Breen and Murphy, 1995; Dean *et al*., 1997). Oxidative DNA damage from reactive oxygen species (ROS) has been hypothesized to play a critical role in several diverse biological processes including mutagenesis, aging, carcinogenesis, radiation effects, and cancer chemotherapy (Ames, 1983; Cerruti, 1984; von Sonntage, 1987).

In this study, we examined the DNA cleavage caused by cytochrome *c* and H_2O_2. Our results suggest that the DNA cleavage induced by cytochrome *c* and H_2O_2 is due to the oxidative damage resulting from ·OH generated by a

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combination of the peroxidase activity of cytochrome *c* and the Fenton-like reaction of free iron ions released from oxidatively damaged cytochrome *c*.

Materials and Methods

Materials. pUC19 plasmid DNA was prepared and purified from *E. coli* cultures using QIAGEN plasmid kits (Santa Clarita). Bovine cytochrome *c*, catalase, 2-deoxy-D-ribose, thiobarbituric acid, bathophenanthroline sulfonate and deferoxamine (DFX) were purchased from Sigma. Chelex 100 resin (sodium form) was obtained from Bio-Rad. All solutions were treated with Chelex 100 resin to remove traces of transition metal ions.

Analysis of DNA cleavage. Supercoiled plasmid pUC19 DNA (1 μg) in 10 mM potassium phosphate buffer (pH 7.4) was incubated for 2 h at 37°C with different cytochrome *c* and H_2O_2 concentrations in a total volume of 20 μl. The reactions were stopped by freezing at −80°C. 5 μl of loading buffer (0.25% bromophenolblue, 40% sucrose) was added and the samples were analyzed by electrophoresis in 0.8% agarose in TBE buffer (2 mM EDTA, 89 mM boric acid and 89 mM Tris at pH 8.3). The resulting gel was stained with ethidium bromide. Bands of DNA were detected and photographed under UV light in a dark room.

Measurement of damage to deoxyribose. Detection of damage to deoxyribose was determined by measuring thiobarbituric acid-reactive 2-deoxy-D-ribose oxidation products (Kang, 2004). The assay mixture contained 10 mM potassium phosphate buffer (pH 7.4), 10 mM 2-deoxy-D-ribose, 1 mM H_2O_2 and 100 μM protein in a total volume of 100 μl. The reaction was initiated by addition of H_2O_2 and incubated for 1 h at 37°C. The reaction was terminated by addition of 2.8% trichloroacetic acid (200 μl), PBS (200 μl), and 1% thiobarbituric acid (200 μl), then boiled at 100°C for 15 min, after which the samples were cooled and centrifuged at 15,000 rpm for 10 min. Results were read by uv/vis spectrophotometer (Shimadzu, UV-1601) at 532 nm.

Determination of free iron ion concentration. The concentration of iron ions released from oxidatively damaged cytochrome *c* was measured using bathophenanthroline sulfonate by the method described previously (Pieroni *et al*., 1994). The reaction mixture contained 100 μM cytochrome *c*, 1 mM H_2O_2 and 10 mM potassium phosphate buffer (pH 7.4) in a total volume of 0.5 ml. The reaction was initiated by addition of H_2O_2 and incubated for 1 h at 37°C. After the incubation, the mixture was then placed into Ultrafree-MC filter and centrifuged at 13,000 rpm for 1 h. The colorimetic reagent was added for analysis by a uv/vis spectrophotometer at 535 nm. The final concentrations in the color reagent were 1% ascorbate, 0.02% bathophenanthroline sulfonate and 1% acetic acid-acetate buffer (pH 4.5).

Results and Discussion

Untreated DNA showed a major band corresponding to the supercoiled form (form I) and a minor band corresponding to nicked circular form (form II) (Fig. 1, lane 1). Plasmid DNA remained intact after incubation with 50 μM cytochrome *c* (Fig. 1, lane 2) or 0.3 mM H_2O_2 (Fig. 1, lane 3) alone. However, when DNA was incubated in a mixture of cytochrome *c* and H_2O_2, the DNA damage occurred (Fig. 1, lane 4). This indicates that both cytochrome *c* and H_2O_2 were required to cause strand breaks in the DNA. The effect of the concentrations of cytochrome *c* and H_2O_2 on the production of DNA strand breaks was then studied. The DNA cleavage increased dose-dependently with doses of up to 100 μM cytochrome *c* (Fig. 2A) and 1 mM H_2O_2 (Fig. 2B), respectively. Previous studies have shown that the reaction of cytochrome *c* with H_2O_2 generates a free radical that oxidizes amino acid residues at or near the cation-binding site, which then introduces carbonyl groups. Such an oxidative modification is an indicator of oxidative stress and may be significant in several physiological and pathological processes (Davies, 1986; Oliver, *et al*., 1987).

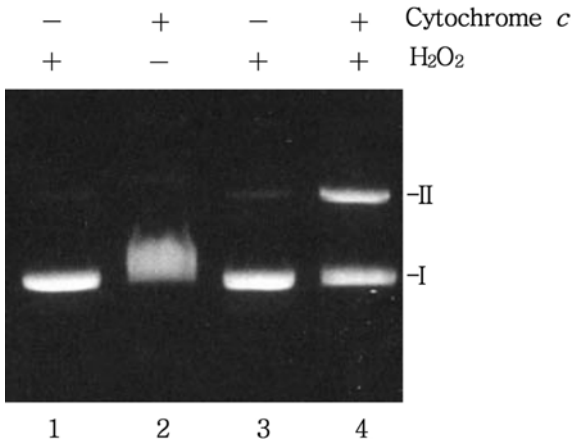

Fig. 1. DNA strand breakage after incubation with cytochrome *c* and H_2O_2. pUC 19 DNA (1 μg) was incubated at 37°C for 2 h: Lane 1, pUC 19 DNA control; lane 2, DNA + 50 μM cytochrome *c*; lane 3, DNA + 0.3 mM H_2O_2; lane 4, DNA + 50 μM cytochrome *c* + 0.3 mM H_2O_2. The reactions were stopped by freezing at −80°C. Loading buffer was added and the samples were analyzed by electrophoresis on 0.8% agarose gel. I and II indicate the positions of the supercoiled and nicked circular DNA plasmid forms, respectively.

The participation of free radicals in the DNA damage by the cytochrome c/H_2O_2 system was studied by examining the protective effect of radical scavengers. When plasmid DNA was incubated with cytochrome *c* and H_2O_2 in the presence of azide, mannitol, or ethanol at 37°C for 2 h, all scavengers significantly prevented the DNA cleavage (Fig. 3). The ability of radical scavengers to protect DNA from damage indicates that free radicals may participate in the mechanism of strand breaks produced by the cytochrome *c* and H_2O_2 system.

Cellular metabolism has been shown to generate the reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and superoxide radical (Cerruti, 1984). Trace metals

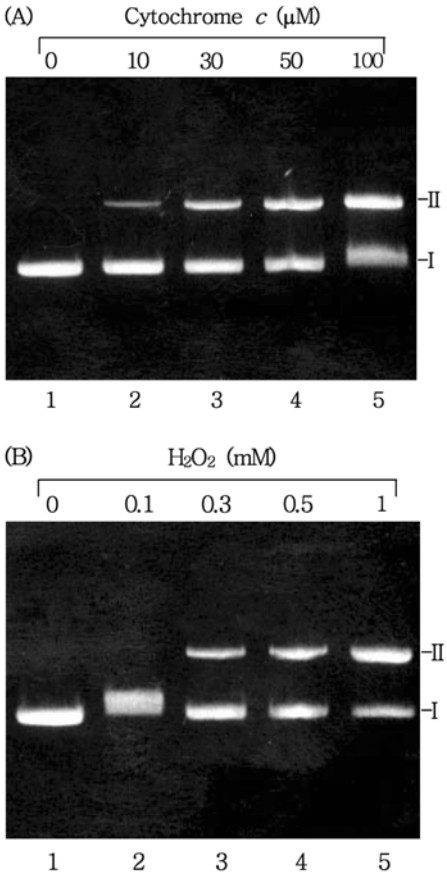

Fig. 2. Effects of cytochrome *c* and H_2O_2 concentrations on DNA strand breakage. (A) pUC 19 DNA (1 μg) was incubated with increasing doses (0-100 μM) of cytochrome *c* and 0.3 mM H_2O_2 at 37°C for 2 h. (B) pUC 19 DNA (1 μg) was incubated with 50 μM cytochrome *c* and increasing doses (0-1 mM) of H_2O_2 at 37°C for 2 h.

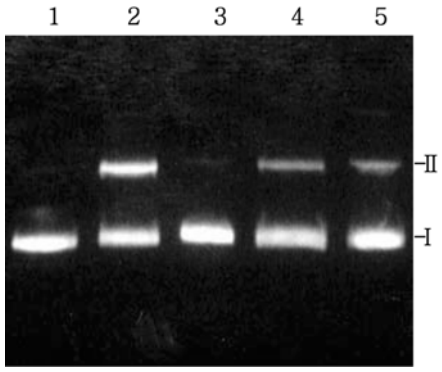

Fig. 3. Effect of radical scavengers on DNA strand breakage induced by cytochrome *c* and H_2O_2. pUC 19 DNA was incubated with 50 μM cytochrome *c* + 0.3 mM H_2O_2 in the presence of a radical scavenger. Lane 1, DNA alone; lane 2, no addition of effector; lane 3, 200 mM azide; lane 4, 200 mM mannitol; lane 5, 500 mM ethanol.

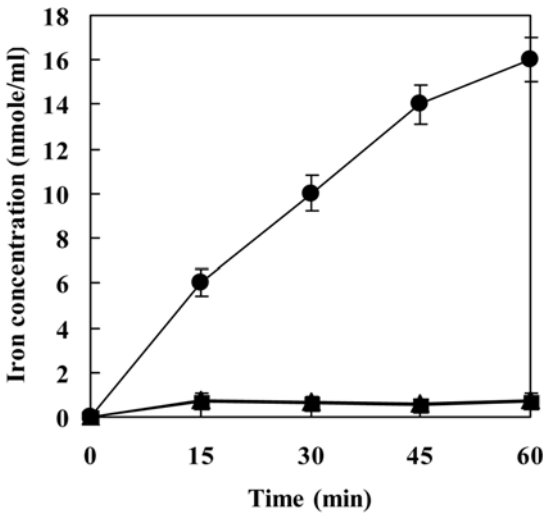

Fig. 4. Iron release in the cytochrome *c* and H_2O_2 system. The samples were incubated in 10 mM potassium phosphate buffer (pH 7.4) at 37°C for various incubation periods with the following: 100 μM cytochrome *c* alone (■); 1 mM H_2O_2 alone (▲); 100 μM cytochrome *c* plus 1 mM H_2O_2 (●). Free iron ion concentrations were determined by a colorimetic reagent utilizing bathophenanthroline sulfonate.

such as copper and iron that are present in biological systems may interact with active oxygen species, ionizing radiation, or microwaves to damage macromolecules (Prutz, 1984; Samuni *et al.*, 1984; Goldstein and Czapski, 1986; Sagripanti *et al.*, 1987; von Sonntage, 1987; Imlay *et al.*, 1988). The cleavage of metalloproteins by oxidative damage may lead to increases in the levels of metal ions in biological cells. We investigated the release of iron in the reaction of cytochrome *c* with H_2O_2. During incubation of 100 μM cytochrome *c* with 1 mM H_2O_2, free iron ions gradually increased as a function of time (Fig. 4). It has been reported that iron ions could stimulate the Fenton-like reaction to produce ·OH, which mediates DNA strand breakage (Tachon, 1989). Attack of ·OH on the sugar, 2-deoxyribose, produces a huge variety of products, some of which are mutagenic in bacterial systems. Some of the fragmentation products can be detected by adding thiobarbituric acid (TBA) to the reaction mixture, resulting in formation of a pink $(TBA)_2$-MDA chromogen (Halliwell and Gutteridge, 1981). This can be used to detect ·OH production, although it is unclear whether or not some other ROS can also degrade deoxyribose. Our results showed that damage to deoxyribose was induced by the cytochrome *c* and H_2O_2 system (Fig. 5). The result suggests that ·OH may participate in the cytochrome *c*/ H_2O_2-mediated DNA cleavage. It has been reported that berberine inhibited the cytochrome *c*/H_2O_2-mediated DNA strand breakage through the scavenging of superoxide anion (Choi *et al.*, 2001). In this study, however, superoxide dismutase could not inhibit the cytochrome *c*/ H_2O_2-mediated DNA strand breakage (data not shown). Therefore, we assumed that the cytochrome *c*/H_2O_2-mediated DNA strand breakage might occur through a Fenton-like

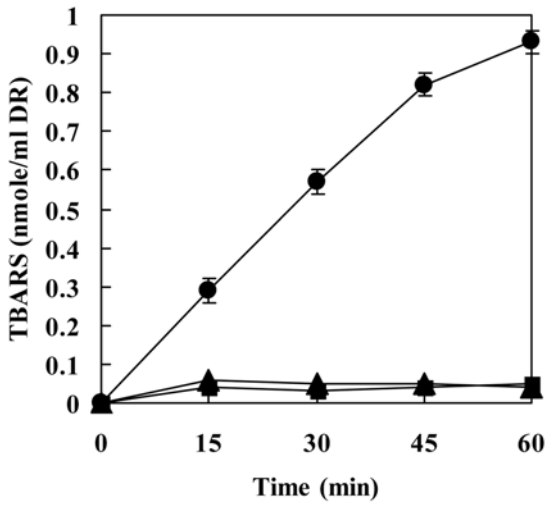

Fig. 5. 2-Deoxy-D-ribose degradation in the cytochrome *c* and H_2O_2 system. 10 mM 2-deoxy-D-ribose was incubated in 10 mM potassium phosphate buffer (pH 7.4) at 37°C for various incubation periods with the following: 100 μM cytochrome *c* alone (■); 1 mM H_2O_2 alone (▲); 100 μM cytochrome *c* plus 1 mM H_2O_2 (●).

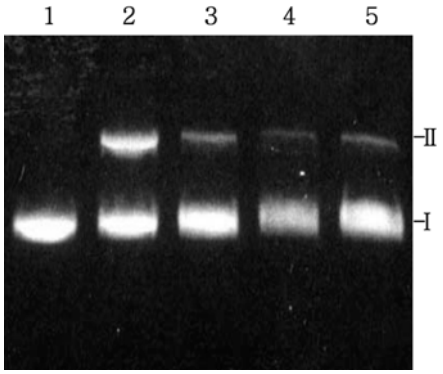

Fig. 6. Effect of DFX on DNA strand breakage induced by the cytochrome *c* and O_2 system. pUC 19 DNA was incubated with 50 μM cytochrome *c* and 0.3 mM H_2O_2 in the presence of various concentrations of DFX. Lane 1, DNA alone; lane 2, no addition of effector; lane 3, 1 mM DFX; lane 4, 5 mM DFX; lane 5, 10 mM DFX.

reaction. The participation of iron ions in the production of DNA strand breaks was studied by examining the protective effect of the iron chelator, DFX. The DNA cleavage was effectively inhibited by 1 mM DFX (Fig. 6). The results indicate that free iron ions are involved in DNA cleavage by the cytochrome c/H_2O_2 system.

In conclusion, the present results indicated that DNA cleavage was induced by the reaction of cytochrome *c* with H_2O_2, involving ·OH generation from H_2O_2. The ·OH radicals were generated through a combination of the peroxidase reaction of cytochrome *c* and the Fenton-like reaction of free iron ions released from oxidatively damaged cytochrome *c*. DNA damage caused by the cytochrome c/H_2O_2 system could be relevant in diseases where mitochondrial dysfunction is elevated, such as Parkinson's disease (Schapira, 1994). It has been reported that at least 0.1 mM/min H_2O_2 will be produced continuously under physiological conditions; this rate increases in adverse conditions such as hyperoxia or ischemia and reperfusion (Boveries *et al*., 1972; Britton *et al*., 1987). Hence, in these abnormal conditions, the modification of human cytochrome *c* using H_2O_2 as a substrate could be potentiated, resulting in DNA damage.

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In vivo glycyrrhizin accelerates liver regeneration and rapidly lowers serum transaminase activities in 70% partially hepatectomized rats

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Abstract

The *in vivo* effects of glycyrrhizin on restoration of liver mass and recovery of liver function were compared with those of epidermal growth factor (EGF), ibuprofen and dexamethasone in 70% partially hepatectomized rats. Hepatic regenerative activity was assessed based on the ratio of liver weight to 100 g body weight, and 5-bromo-2′-deoxyuridine (BrdU) incorporation into hepatocyte DNA in the remnant liver. Glycyrrhizin (50 mg/kg/day, i.p.)- or EGF (1.0 μg/kg/day, i.p.)-treated rats showed an approx. 1.4-fold increase in liver weight/100 g body weight ratio over saline-treated control rats on days 2 and 3 after 70% partial hepatectomy. BrdU labeling index in the remnant regenerating liver was significantly higher in glycyrrhizin- or EGF-treated rats when compared with saline-treated control rats on days 0.5 and 1. Ibuprofen (100 mg/kg/day, i.p.) and dexamethasone (0.1 mg/kg/day, i.p.) did not significantly increase either liver weight/100 g body weight ratio or BrdU labeling index. Serum activity of liver-related transaminases, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), elevated rapidly on day 1 and decreased to near pre-operative levels on day 5 after 70% partial hepatectomy in saline-treated control rats. Injection of glycyrrhizin or EGF significantly decreased the elevated serum ALT and AST activities on days 2 and 3 after hepatectomy when compared with saline-treated control rats. The transaminase-lowering effects of glycyrrhizin or EGF were smaller than those of ibuprofen and dexamethasone. These results demonstrate that injection of glycyrrhizin or EGF significantly enhances regeneration of liver mass and function, as well as recovery from the liver damage induced by surgical resection.

Keywords: Liver regeneration; Serum transaminase; Glycyrrhizin; Epidermal growth factor; Ibuprofen; Dexamethasone

1. Introduction

Liver regeneration is a process in which the liver recovers mass and function after events such as hepatectomy, viral infection or chemical toxicity. In rats, 70% partial hepatectomy induces a rapid regenerative process in the remaining liver tissue, and within 2 weeks, the liver returns to its original size and regeneration is terminated (Higgins and Anderson, 1931; Steer, 1995; Fausto et al., 1995). During the last 10 years, new information has become available on the events that initiate and terminate liver regeneration (Michalopoulos and DeFrances, 1997). Such regeneration invokes a series of complex processes that probably involve cell proliferation and cell growth induced by the actions of various circulatory factors. The circulatory factors required for these processes may be largely categorized into three networks: growth factors, cytokines and metabolic agents (Gallucci et al., 2000; Argast et al., 2004; Kimura and Ogihara, 1997a,b, 2005; Kimura et al., 2007). There is much redundancy within each network, and complex interactions exist between them. To date, no single mediator has been identified as being responsible for triggering hepatocytes to enter the cell cycle. For instance, epidermal growth factor (EGF) is thought to promote DNA synthesis and proliferation in rat hepatocytes and regenerating liver after 70% partial hepatectomy (Francavilla et al., 1986; Johansson and Anderson, 1990; Jones et al., 1995; Kimura and Ogihara, 1997a).

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Glycyrrhizin is a triterpene glycoside extracted from licorice root. Glycyrrhizin and its analogues have various pharmacological effects, including anti-inflammatory, antitumorgenic and antihepatotoxic activity (Inoue et al., 1996). Stronger Neo-Minophagen C (SNMC), the active ingredient of which is glycyrrhizin, has been widely used in Japan to treat chronic hepatitis for over 25 years. Intravenous administration of SNMC markedly decreases elevated serum alanine aminotransferase (ALT) levels in patients with chronic hepatitis and improves liver function with occasional complete recovery from hepatitis (Arase et al., 1997). SNMC is recognized by many clinicians as a safe and effective agent for chronic hepatitis. In randomized controlled trials, glycyrrhizin induced a significant reduction in serum alanine aminotransferase and an improvement in liver histology when compared to placebo in European patients (van Rossum et al., 1998, 1999). The hepatoprotective effects produced by glycyrrhizin are largely thought to be related to its potent anti-inflammatory action.

On the other hand, few studies have investigated the specific effects of glycyrrhizin on hepatocyte growth. We previously demonstrated that glycyrrhizin stimulates DNA synthesis and proliferation directly via the EGF receptor *in vitro* in primary cultures of adult rat hepatocytes (Kimura and Ogihara, 2001). These findings indicate that glycyrrhizin is a natural ligand for the EGF receptor. Glycyrrhizin, a potent mitogen for mature hepatocytes *in vitro*, appears to function as a hepatotrophic factor for liver regeneration *in vivo*; however, there is no direct evidence that glycyrrhizin stimulates DNA synthesis and proliferation of hepatocytes to accelerate liver regeneration *in vivo*. Partial hepatectomy is the most often used stimulus to study liver regeneration, because in contrast to methods that use hepatic toxins (i.e., carbon tetrachloride), it is not associated with severe inflammation and tissue necrosis, and initiation of the regenerative stimulus is precisely defined (i.e., loss of hepatic tissue) (Michalopoulos and DeFrances, 1997; Stolz et al., 1999). Furthermore, *in vivo* investigation of the effects of glycyrrhizin on hepatic growth response is of particular importance, as glycyrrhizin may improve liver function by stimulating liver regeneration.

The purpose of the present study was to evaluate whether exogenous administration of glycyrrhizin is able to stimulate liver regeneration *in vivo* in 70% partially hepatectomized rats. To examine whether glycyrrhizin protects liver integrity after partially hepatectomy, we measured the serum levels of liver cytosolic enzymes, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In addition, to elucidate the mechanisms of action of glycyrrhizin, the effects of glycyrrhizin on the liver regeneration and liver function in rats were compared with those of EGF, the non-steroidal anti-inflammatory drug ibuprofen, and the anti-inflammatory steroid dexamethasone during recovery from 70% partial hepatectomy. Our results demonstrate that administration of glycyrrhizin or EGF stimulates hepatocyte DNA synthesis following liver regeneration after 70% partial resection of liver, and more rapidly normalizes serum ALT and AST levels than in saline-treated controls. Neither ibuprofen nor dexamethasone enhanced regeneration of liver mass, but they both significantly normalized serum ALT and AST activities when compared with saline-treated controls. Taken together, our findings indicate that glycyrrhizin is useful in accelerating liver regeneration and in protecting the integrity of hepatocytes against liver damage.

2. Materials and methods

2.1. Animals

Seven-week-old male Wistar rats weighing 200 g obtained from Tokyo Experimental Animal Co. (Tokyo, Japan) were used. Rats were kept at a controlled temperature (24 °C) under a 12-h light–dark cycle and fed standard laboratory chow and water *ad libitum*. Rats were handled in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. 70% partial hepatectomy and drug administration

Under ether anesthesia, 70% (two-thirds) partial hepatectomy was performed according to a minor modification of the Higgins and Anderson method. The procedure involves the ligation and resection of the left lateral and median hepatic lobes, which constitute about 70% of the total liver weight, as described previously (Higgins and Anderson, 1931). Sham-operated controls were similarly anesthetized, and their livers were briefly brought outside the peritoneal cavity, but were not tied or excised.

The rats were randomly divided into 5 groups that received saline, glycyrrhizin, EGF, ibuprofen or dexamethasone; intraperitoneal injections of glycyrrhizin (50 mg/kg), ibuprofen (100 mg/kg), dexamethasone (0.1 mg/kg) or EGF (1.0 μg/kg) were administered once daily (at 10 A.M.) for 1 to 12 days unless otherwise indicated.

2.3. Determination of liver regeneration

All rats were killed at the indicated time points after 70% partial hepatectomy under ether anesthesia. The regenerating liver was excised, and liver weight after regeneration and body weight was measured. The ratio of total liver weight after regeneration normalized against 100 g body weight was used as an indicator of liver regeneration (Assy and Mink, 1997).

2.4. 5-Bromo-2′-deoxyuridine (BrdU) incorporation

Liver regeneration was also monitored by mitotic index, as determined by nuclear BrdU incorporation into hepatocyte DNA after 70% partial hepatectomy, according to a previously described method (Gratzner, 1982; Ishiki et al., 1992). This parameter is known to be a useful indicator of the effects of growth factors on cell proliferation in a variety of experimental systems. BrdU is a thymidine analogue that is incorporated into hepatocyte nuclei during DNA synthesis (S phase in cell cycle). BrdU (50 mg/kg, i.p.) was injected into rats, and after 2 h, rats were anesthetized by ether and the livers were removed. Liver specimens were fixed in cold 10% neutralized formalin solution.

To measure the labeling index, liver cells undergoing DNA synthesis were immunohistochemically identified using monoclonal anti-BrdU antibodies. Briefly, livers were fixed in 10% neutralized formalin and embedded in paraffin. Paraffin-embedded tissue sections (5-μm thick) were deparaffinized and stained with hematoxylin. For assay of labeling index, deparaffinized tissue sections of liver, were incubated in 2 N HCl for 30 min, washed several times with phosphate-buffered saline (pH 7.4), and stained with anti-BrdU monoclonal antibody. BrdU was detected using avidin DH-biotinylated horseradish peroxidase complex according to manufacturer's instructions (Exalpha, Biologicals, Inc., Maynard, USA). Labeling index was determined by counting the number of labeled nuclei per 1000 nuclei in randomly selected fields under a light microscope.

Histological changes in livers of rats not given or given saline (0.25 mL/kg/day, i.p.), glycyrrhizin (50 mg/kg/day, i.p.), dexamethasone (0.1 mg/kg/day, i.p.) and EGF (1.0 μg/kg, i.p.) on day 3 after 70% partial hepatectomy were examined. Rats were treated according to the schedule described in Materials and methods. Liver specimens were fixed in cold 10% neutralized formalin and embedded in paraffin. Paraffin-embedded tissue sections (5 μm) were deparaffinized and stained with hematoxylin and eosin.

2.5. Determination of serum transaminase activity

In order to determine whether glycyrrhizin affects liver integrity in 70% partially hepatectomized rats, we measured the activity of liver cytosolic enzymes in sera. After remnant livers were removed, blood samples were quickly collected from the inferior vena cava. Enzyme assays were carried out in normal rats with or without glycyrrhizin, EGF, ibuprofen and dexamethasone administration. Serum ALT and AST were assayed using commercially available diagnostic kits in accordance with the manufacturer's instructions (Wako Pure Chemical Industries, LTD., Osaka, Japan). The enzyme reaction was performed by addition of substrate with diaminobenzidine. One international unit of enzyme activity was a change in optical density of 0.000482/min/mL of serum.

2.6. Materials

Glycyrrhizin (ammonium salt) was prepared by Dr. S. Iwata at the Research Laboratory of Minophagen Pharmaceutical Co., Ltd. (Kanagawa, Japan). The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA): EGF (human recombinant); ibuprofen sodium salt; dexamethasone; and BrdU. BrdU was dissolved in 50% dimethylsulfoxide (DMSO) at 120 mg/mL (stock solution). Monoclonal anti-BrdU antibodies were purchased from Exalpha Biologicals, Inc. (BrdU Immunohistochemistry Assay Kit, Maynard, MA, USA). Assay kits for serum ALT and AST were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All other reagents were of analytical grade.

2.7. Statistical analysis

Results are presented as means ± S.E.M. Group comparisons were carried out by ANOVA for unpaired data followed by post-hoc analysis using Dunnett's multiple comparison test. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Dose-dependent effects of glycyrrhizin and EGF on liver regeneration

In the preliminary study, recovery of liver mass in the remnant liver after 70% partial hepatectomy initially peaked after 3 days in glycyrrhizin- or EGF-treated rats. We thus examined the dose–response effects of glycyrrhizin or EGF on the ratio of liver weight/100 g body weight at 3 days after 70% partial hepatectomy as compared with ibuprofen and dexamethasone. As shown in Fig. 1A, a bell-shaped dose–response curve indicates that, at smaller doses, glycyrrhizin (30–70 mg/kg) can stimulate liver regeneration, whereas higher doses (100 mg/kg) are less effective. The maximal effects induced by glycyrrhizin were seen in response to 50 mg/kg. EGF showed a similar biphasic dose–response curve (Fig. 1B). The maximal effect induced by EGF was seen in response to 1.0 μg/kg. Ibuprofen (10–150 mg/kg) and dexamethasone (0.1–1.5 mg/kg) had no effect on liver weight/100 g body weight at any of the doses tested (data not shown).

3.2. Time courses associated with effects of glycyrrhizin, EGF, ibuprofen and dexamethasone on liver regeneration

In order to examine the effects of glycyrrhizin on liver regeneration of remnant liver after 70% partial hepatectomy, glycyrrhizin (50 mg/kg/day) was administered intraperitoneally

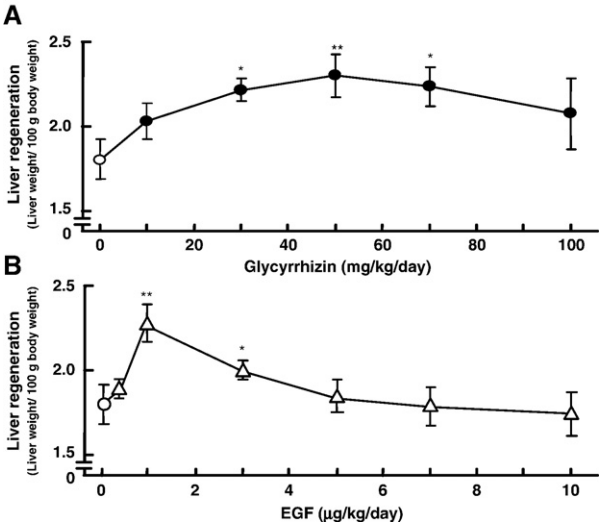

Fig. 1. Dose-dependent effects of glycyrrhizin and EGF on liver regeneration on day 3 following 70% partial hepatectomy. Rats were treated according to the experimental schedule described in Materials and methods. Glycyrrhizin (10–100 mg/kg, i.p.) and EGF (0.5–10 μg/kg, i.p.) were injected once a day (at 10 A.M.) and remnant livers were removed rapidly under ether anesthesia and weighed on day 3 after 70% partial hepatectomy. The ratio of total liver weight after regeneration normalized against 100 g body weight was used as an indicator of liver regeneration. Rats ($n=5$–8) were used in each experimental group. Values are means ± S.E.M. *, $P<0.05$; **, $P<0.01$ compared with controls.

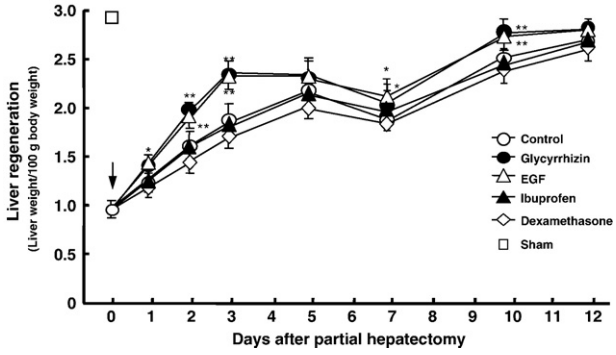

Fig. 2. Time courses associated with effects of glycyrrhizin, EGF, ibuprofen and dexamethasone on liver regeneration following 70% partial hepatectomy. Rats were treated according to the experimental schedule described in Materials and methods. Saline (0.25 mL/kg, i.p.), glycyrrhizin (50 mg/kg, i.p.), EGF (1.0 μg/kg, i.p.), ibuprofen (100 mg/kg, i.p.) or dexamethasone (0.1 mg/kg, i.p.) was injected once a day and remnant livers were removed rapidly under ether anesthesia and weighed at the time points indicated. Rats (n=5–8) were used in each experimental group. The ratio of total liver weight after regeneration normalized against 100 g body weight was used as an indicator of liver regeneration. 2.92: Sham-operated rats. Values are means±S.E.M. *, P<0.05; **, P<0.01 compared with saline-treated controls.

and the effects were compared with those of EGF (1.0 μg/kg/day). After 70% partial hepatectomy, liver weight/100 g body weight increased rapidly and reached an initial peak on day 5 in saline-treated control rats and on day 3 in glycyrrhizin-treated rats (Fig. 2). The glycyrrhizin-treated rats showed an approx. 1.4-fold increase over saline-treated controls on day 3 after 70% partial hepatectomy. The effects of glycyrrhizin were statistically significant when compared with saline-treated controls. Liver weight/100 g body weight peaked again on day 10 and the wet weight of liver was comparable to pre-operative levels. There were no significant differences in body weight between saline-injected controls and the glycyrrhizin-injected group from days 1 to 10. EGF-treated rats (1.0 μg/kg, i.p.) reached a first peak on day 3, and the time course associated with the effects of EGF was very similar to those of glycyrrhizin, as assessed by liver weight/100 g body weight (Fig. 2).

In addition, to determine the mechanisms responsible for the enhanced liver regeneration observed in glycyrrhizin- or EGF-treated rats, we compared the effects of the non-steroidal anti-inflammatory agent ibuprofen (100 mg/kg/day, i.p.) and the anti-inflammatory steroid dexamethasone (0.1 mg/kg/day, i.p.) with those of glycyrrhizin (50 mg/kg/day, i.p.) and EGF (1.0 μg/kg, i.p.). As shown in Fig. 2, liver weight/100 g body weight in rats given ibuprofen or dexamethasone once a day after 70% partial hepatectomy was not significantly greater than those of saline-treated control rats on days 2 and 3 (Fig. 2).

Sections from the regenerating liver were stained with hematoxylin and eosin in order to aid observation of histological changes on day 3 after 70% partial hepatectomy. Histological observation also confirmed almost no abnormal morphologies, such as cell swelling, atypical tissue changes or cell infiltration, in remnant regenerating liver from rats given glycyrrhizin, dexamethasone or EGF, as compared with saline-treated controls (data not shown).

3.3. Time courses associated with the effects of glycyrrhizin and EGF on hepatocyte DNA synthesis

Fig. 3 shows light micrographs of the typical distribution of hepatocytes undergoing DNA synthesis in the remnant liver of

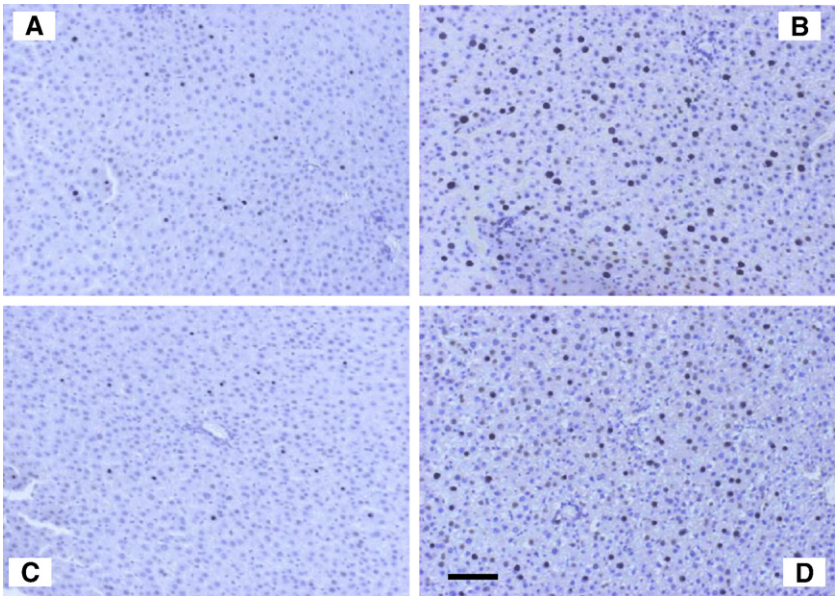

Fig. 3. Liver distribution of hepatocytes undergoing DNA synthesis after 70% partial hepatectomy. Light micrographs showing the typical distribution of hepatocytes undergoing DNA synthesis in the remnant liver of rats treated without or with glycyrrhizin, dexamethasone and EGF on day 3 after 70% partial hepatectomy. Livers were pulsed with 5-bromo-2′-deoxyuridine (BrdU) *in vivo*. Liver sections (5 μm) were stained with hematoxylin following immunochemical staining with monoclonal antibody to BrdU, as described in Materials and methods. Dark spots indicate hepatocytes undergoing DNA synthesis. Bars represent 50 μm. A: saline, 0.25 mL/kg/day, i.p.; B: glycyrrhizin, 50 mg/kg/day, i.p.; C: dexamethasone, 0.1 mg/kg/day, i.p.; D: EGF, 1.0 μg/kg, i.p.

rats treated without (A) or with glycyrrhizin (B), dexamethasone (C) and EGF (D) on day 1 after 70% partial hepatectomy. Hepatocytes were pulsed with 5-bromo-2′-deoxyuridine (BrdU) for 2 h *in vivo* and were visualized by immunochemical staining. Dark spots indicate hepatocytes undergoing DNA synthesis. Light micrographs confirmed that glycyrrhizin and EGF, but not dexamethasone, stimulate hepatocyte DNA synthesis.

Fig. 4 shows the time course associated with the effects of glycyrrhizin and EGF on BrdU labeling index in hepatic parenchymal cells. Although the BrdU labeling index of untreated rat liver sections before 70% partial hepatectomy was less than 0.1%, it increased moderately from the first day after 70% partial hepatectomy in saline-treated control rats. Thereafter, it decreased to pre-operative levels at day 3 after 70% partial hepatectomy. In contrast, the BrdU labeling index in 50 mg/kg/day glycyrrhizin-injected rats increased significantly from 1.0 to 9.0% on day 1 and then declined to 3.0% on day 2. In both groups, BrdU labeling index returned to pre-operative levels on days 3 and 4 after 70% partial hepatectomy. These results indicate that glycyrrhizin significantly extended mitogenic activity of hepatocytes following 70% partial hepatectomy, as compared with controls. Hepatocytes undergoing DNA synthesis in both saline- or glycyrrhizin-treated animals were randomly distributed in hepatic lobes. Thus, exogenous glycyrrhizin markedly stimulates DNA synthesis in hepatocytes and thereafter accelerates liver regeneration after 70% partial hepatectomy *in vivo*. Injection of EGF (1.0 μg/kg, i.p.) once daily also significantly enhanced the BrdU labeling index of liver cells (from 0.1 to 8.9%) on day 1, followed by a decrease to 1.1% on day 3 after 70% partial hepatectomy (Fig. 4). In the labeling index study, about 9% of total nuclei were labeled with BrdU after EGF administration in normal rat liver *in vivo*. On the other hand, 100 mg/kg i.p. ibuprofen-treated rats and 0.1 mg/kg i.p. dexamethasone-treated rats exhibited moderate induction of DNA synthesis on days 1 and 2 after 70% partial hepatectomy when compared with the sham-operated group (data not shown), but

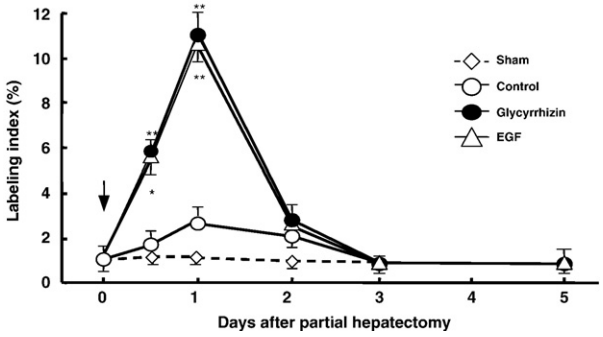

Fig. 4. Time courses associated with effects of glycyrrhizin and EGF on 5-bromo-2′-deoxyuridine (BrdU) labeling indexes in the livers of rats injected with glycyrrhizin following 70% partial hepatectomy. BrdU-mitotic indexes were determined based on BrdU incorporation into DNA and following immunohistochemical staining using monoclonal antibodies for BrdU on days 0–5 after partial hepatectomy, as described in the legend of Fig. 3. BrdU labeling index in control and glycyrrhizin-treated rats indicates the percentage of BrdU-positive hepatocyte nuclei out of 1000 nuclei in randomly selected fields under a light microscope. Values are means±S.E.M of five-different rats. *, $P<0.05$; **, $P<0.01$ compared with sham-operated controls.

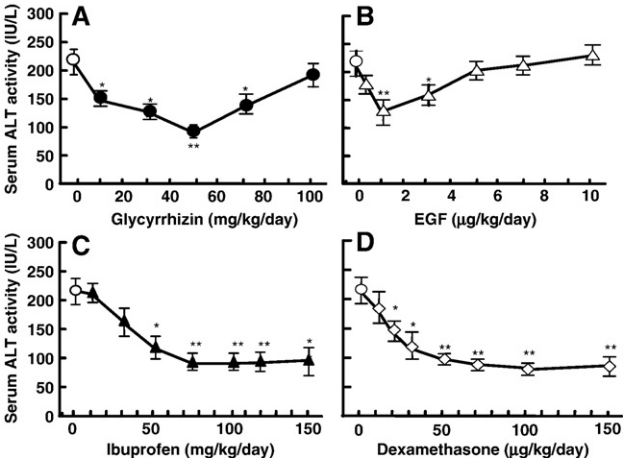

Fig. 5. Dose-dependent effects of glycyrrhizin, EGF, ibuprofen, and dexamethasone on liver-specific cytosolic alanine aminotransferase (ALT) activity in serum on day 1 after 70% partial hepatectomy. Rats were treated according to the experimental schedule described in Materials and methods. After remnant livers were removed on day 1 after 70% partial hepatectomy, blood samples were quickly collected from the inferior vena cava and serum ALT activity was determined using a commercially available diagnostic kit. Each experimental group comprised 5–8 rats. Values are means±S.E.M. *, $P<0.05$; **, $P<0.01$ compared with controls.

DNA synthesis decreased to pre-operative levels on day 5. The effects of ibuprofen and dexamethasone were not statistically significant when compared with saline-treated control rats.

3.4. Dose-dependent effects of glycyrrhizin, EGF, ibuprofen and dexamethasone on serum ALT activity

Of the known liver-related enzymes, transaminases (ALT and AST) in serum are good markers of liver injury or damage. To examine whether glycyrrhizin and EGF affect liver integrity in 70% partially hepatectomized rats, we measured serum ALT and AST activity. First, we examined the dose–response effects of glycyrrhizin and EGF on liver-specific transaminase ALT activity in serum on day 3 after 70% partial hepatectomy, as restoration of liver mass peaked on day 3 (Fig. 2). As shown in Fig. 5, a biphasic dose–response curve was obtained. Glycyrrhizin in smaller doses significantly reduced ALT activity, with a maximal effect at 50 mg/kg/day, while higher doses were less effective (Fig. 5A). In addition, the dose–response effects of EGF on serum ALT activity showed a similar biphasic-shaped dose–response curve with a maximal effect at 1.0 μg/kg/day i.p. (Fig. 5B). The mechanisms behind these results are unknown at present.

Because glycyrrhizin and EGF were found to reduce the elevated serum ALT activity induced by 70% partially hepatectomized rats, the dose–response effects of ibuprofen and dexamethasone on serum ALT activity were also examined on 1 day after 70% partial hepatectomy. The dose–response curve showed that both ibuprofen and dexamethasone reduced the increase in serum ALT activity in a dose-dependent manner (Fig. 5C and D). The maximal ALT-lowering effects of ibuprofen and dexamethasone were observed at doses of 75 mg/kg/day and 50 μg/kg/day, respectively (Fig. 5C and D).

3.5. Time courses associated with effects of glycyrrhizin, EGF, ibuprofen and dexamethasone on serum transaminase activity

As shown in Fig. 6A and B, serum activity of ALT and AST was elevated in rats after 70% partial hepatectomy. Serum levels of AST and ALT rapidly increased, peaking on day 1 after 70% partial hepatectomy, thus confirming that liver damage occurred. Transaminase activity returned more rapidly to the pre-hepatectomy levels in animals treated with glycyrrhizin (50 mg/kg, i.p.) or EGF (1.0 μg/kg, i.p.) than in saline-treated controls. The transaminase-lowering effects of glycyrrhizin were significant when compared with saline-treated control groups on days 1 and 2 after 70% partial hepatectomy. Serum ALT and AST were elevated in sera from 70% partial hepatectomized rats. ALT and AST levels were significantly higher on days 1 and 2, respectively, after hepatectomy in both control and glycyrrhizin-treated rats. These results suggest that injection of glycyrrhizin had a significant protective effect against acute liver injury. Fig. 6 shows that injection of 1.0 μg/kg EGF also suppressed the elevated ALT and AST levels induced by 70% partial hepatectomy on days 2 and 3; therefore, acute liver damage was also ameliorated by EGF.

In order to determine the mechanisms responsible for the serum transaminase-lowering effects, we compared the effects of non-steroidal and steroidal anti-inflammatory agents (ibuprofen and dexamethasone, respectively) on serum ALT and AST levels with those of glycyrrhizin and EGF in the early stages after 70% partial hepatectomy. As shown in Fig. 6A and B, injection of both ibuprofen and dexamethasone markedly suppressed the increase in the serum ALT and AST activity induced by 70% partial hepatectomy, possibly as a result of their anti-inflammatory effects. However, administration of dexamethasone was more effective in reducing serum ALT and AST levels on days 1–3 after partial hepatectomy than saline or ibuprofen.

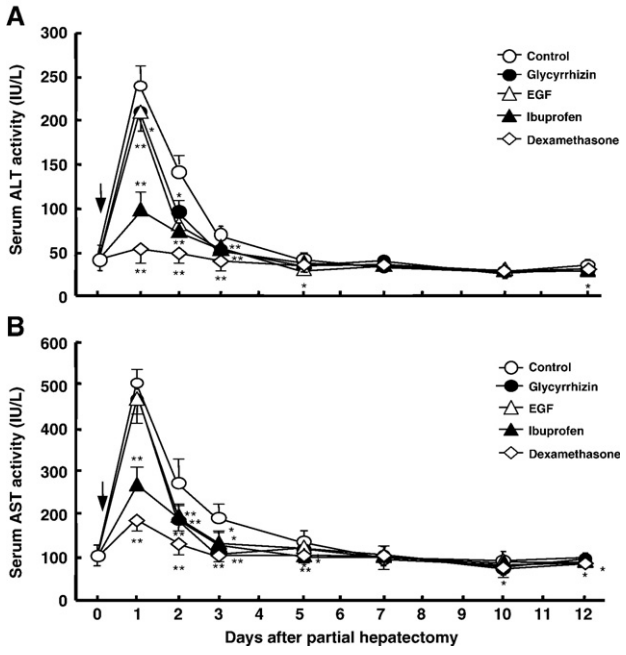

Fig. 6. Time courses associated with the effects of glycyrrhizin, EGF, ibuprofen, and dexamethasone on liver-related cytosolic transaminase (ALT and AST) activity in serum after 70% partial hepatectomy. Time courses associated with the effects of glycyrrhizin (50 mg/kg, i.p.), EGF (1.0 μg/kg, i.p.), ibuprofen (100 mg/kg, i.p.), and dexamethasone (0.1 mg/kg, i.p.) on serum ALT (A) and AST (B) activity were assessed on days 0–12 after 70% partial hepatectomy, as described in legend of Fig. 5. Data from control, glycyrrhizin-, EGF-, ibuprofen-, and dexamethasone-treated rats are shown as means ± S.E.M. (n = 7–10). *, P < 0.05; **, P < 0.01 compared with saline-treated controls.

4. Discussion

Liver regeneration after the loss of hepatic tissue is a fundamental parameter of the liver response to injury. Numerous tissue- and serum-based methods are presently used in experimental and clinical studies to assess liver regeneration in animals and humans. However, a gold standard has yet to be identified (Assy and Mink, 1997). It has thus been recommended that wherever possible, researchers should incorporate at least two assays into their studies of hepatic regeneration. In this study, we employed three tests, i.e., regeneration ratio, BrdU incorporation and serum transaminase activity, to assess the *in vivo* effects of glycyrrhizin on hepatic regeneration in 70% partially hepatectomized rats.

As shown in Figs. 1 and 2, it is clear that with regard to liver weight/100 g body weight, restoration is almost completed by the 12th day in saline-injected control rats. These data are consistent with those of Higgins and Anderson (1931). In the present study, administration of glycyrrhizin or EGF was found to accelerate remnant liver regeneration after 70% partial hepatectomy when compared with saline-treated control rats. Many growth factors and cytokines are hepatocyte mitogens *in vitro* and *in vivo*, but their relative importance *in vivo* remains unclear (Simpson et al., 1997). During the last 10 years, it was shown that hepatocyte DNA synthesis after partial hepatectomy is impaired in interleukin-6-deficient (IL-6 (−/−)) mice, which results in significantly delayed, but eventual, recovery of normal liver weight, compared with the IL-6 (+/+) control (Sakamoto et al., 1999). Yamada et al. reported that hepatic regeneration following partial hepatectomy is severely impaired in tumor necrosis factor (TNF) receptor-1 knock-out mice (Yamada et al., 1997). These results suggest that pro-inflammatory mediators such as IL-6 and TNF-α play a pivotal role in liver regeneration after partial hepatectomy in control rats. In addition, we found that both glycyrrhizin and EGF potentiated IL-6- or TNF-α-induced DNA synthesis and proliferation in primary cultured hepatocytes (unpublished data). Although intrahepatic modulations of these pro-inflammatory factors after partial hepatectomy by treatment with glycyrrhizin or EGF are unknown, glycyrrhizin and EGF may act by activating parenchymal hepatocyte EGF receptors in a synergistic manner with endogenous IL-6 and TNF-α.

To determine the mechanisms by which glycyrrhizin stimulates liver regeneration, we pharmacologically compared the effects of glycyrrhizin and EGF with those of the anti-inflammatory drugs ibuprofen and dexamethasone on liver weight/100 g body weight in 70% partially hepatectomized rats. Anti-

inflammatory doses of ibuprofen and dexamethasone did not significantly enhance liver regeneration when compared with saline-treated control rats at the selected time points (Fig. 2). These results suggest that the anti-inflammatory action of glycyrrhizin or EGF (Inoue et al., 1996) is not markedly involved in enhancing liver regeneration.

In the study of BrdU labeling index, administration of glycyrrhizin or EGF significantly stimulated BrdU labeling index at 0.5 and 1 days after 70% partial hepatectomy (9% of total nuclei, $n=5$) when compared with saline-injected control rats (3.0% of total nuclei, $n=5$), while treatment with ibuprofen or dexamethasone only slightly increased BrdU labeling index (3.0% of total nuclei, $n=5$). To our knowledge, this is the first report of glycyrrhizin stimulating hepatocyte DNA synthesis *in vivo* during liver regeneration in 70% partially hepatectomized rats (Figs. 3 and 4). These results are consistent with our *in vitro* studies, which indicated that glycyrrhizin and EGF stimulate hepatocyte DNA synthesis and proliferation via EGF receptor signaling in primary cultures of adult rat hepatocytes. The potent mitogenic activity of glycyrrhizin and EGF in parenchymal hepatocytes is probably one of the predominant mechanisms of glycyrrhizin and EGF toward liver regeneration.

It has been reported that although liver regeneration results ultimately in restoration of liver mass and function, partial hepatectomy is primarily a compensatory hyperplasia, and does not involve an inflammatory process (Michalopoulos and DeFrances, 1997). To evaluate the degree of liver injury after 70% partial hepatectomy, the activity of liver-related enzymes in serum, such as ALT and AST, and the effects of anti-inflammatory agents on the transaminase activity in sera were examined. We found that serum ALT and AST activity increased rapidly and significantly after 70% partial hepatectomy on day 1 (Fig. 6) and returned almost to pre-operative levels after 2–3 days in control rats. This is because local inflammatory reactions may occur in response to damage around the ligated area of the liver, resulting in transient increases in serum ALT and AST activity. In contrast, serum ALT and AST activity returned more rapidly to near pre-hepatectomy levels in animals treated with glycyrrhizin or EGF (Fig. 6). These results indicate that glycyrrhizin and EGF significantly suppressed the leakage of cytosolic enzymes from liver cells and prevented the early stages of liver damage caused by 70% partial hepatectomy. These biochemical data thus support the notion that moderate inflammatory responses may occur during the initial stages of hepatic resection. Glycyrrhizin and EGF may act by inhibiting production of inflammatory mediators, such as prostaglandins and leukotriens (Inoue et al., 1996). In support of this hypothesis, the increases in ALT and AST activity were also significantly suppressed by administration of ibuprofen, and, to a greater degree, dexamethasone (Fig. 6). Thus, the notion that the hepatoprotective effects of glycyrrhizin and EGF are associated, at least in part, with their anti-inflammatory action is supported by these pharmacological data (Fig. 6).

Ishiki et al. and other investigators have shown that injection of hepatocyte growth factor (HGF) after 70% hepatectomy produced dose-dependent increases in hepatic DNA synthesis and proliferation, and lowered serum ALT and AST activity (Ishiki et al., 1992; Ishii et al., 1995; Kaibori et al., 1997). These results are comparable to the present data. However, the mechanisms by which HGF improves liver histology and biology are undefined. In addition, increasing evidence indicates that glycyrrhizin or its metabolite 18β-glycyrretic acid protects against several models of oxidant-mediated toxicity (Jeong et al., 2002; Gumpricht et al., 2005), and ischemia–reperfusion injury (Nagai et al., 1991). Inoue et al. evaluated the effects of glycyrrhizin on lipopolysaccharide (LPS)/D-galactosamine (Gal)-induced hepatocyte injury in rats *in vivo* using Tunel assay. They found that administration of glycyrrhizin significantly reduced LPS/Gal-induced apoptosis (manuscript in preparation). Thus, the protective effect of glycyrrhizin against inflammatory liver injury may be also involved in the inhibition of the partial hepatectomy-induced increase in serum ALT and AST activities (Fig. 6). Although several hypotheses have been offered to account for the hepatoprotective effects of glycyrrhizin, the effects of glycyrrhizin on signal transduction pathway of cell injury remain to be elucidated. In the present study using glycyrrhizin, EGF, and anti-inflammatory agents, we propose that exogenous glycyrrhizin and EGF have overall pharmacological actions that accelerate the proliferation of parenchymal hepatocytes and suppress inflammatory reactions, thereby normalizing liver function in 70% partial hepatectomized rats.

Because liver function is markedly impaired in hepatectomized patients and hepatitis, it is important to stimulate both regeneration of liver mass and function of remnant liver after liver damage. The same principle that governs liver regeneration after partial hepatectomy in rats may apply to the growth response of human livers transplanted into new hosts and those of healthy living donors following living liver transplantation. In fact, in phase II/III clinical trials, glycyrrhizin has been reported to reduce serum ALT in patients with hepatitis C (van Rossum et al., 2001). Based on our *in vivo* data, the cytoprotective action of glycyrrhizin and EGF in hepatocytes may be responsible for their effects in damaged and inflamed livers.

In conclusion, we obtained direct evidence that exogenous glycyrrhizin and EGF significantly stimulate both liver regeneration and recovery of function *in vivo* in response to 70% partial hepatectomy, possibly via stimulation of EGF receptor. In addition to the stimulation of hepatic DNA synthesis and proliferation, it should be emphasized that glycyrrhizin and EGF decreased serum ALT and AST activity, which indicates rapid recovery of liver function from 70% partial hepatectomy. Our results should thus provide new insight into treating patients after living liver transplantation or with acute or chronic hepatitis C. However, further experiments are needed to understand whether glycyrrhizin and EGF are able to suppress liver damage induced by toxic chemical agents or in acute and chronic hepatitis models (Matsuda et al., 1995). Moreover, the development of new glycyrrhizin-related agents that enhance liver regeneration more effectively and protect liver function after partial hepatectomy or hepatotoxic agents would be of substantial benefit to numerous patients with acute and chronic liver dysfunction.

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Minireview

DNA damage and neurotoxicity of chronic alcohol abuse

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Abstract

Chronic alcohol abuse results in a variety of pathological effects including damage to the brain. The causes of alcohol-induced brain pathology are presently unclear. Several mechanisms of pathogenicity of chronic alcoholism have been proposed, including accumulation of DNA damage in the absence of repair, resulting in genomic instability and death of neurons. Genomic instability is a unified genetic mechanism leading to a variety of neurodegenerative disorders. Ethanol also likely interacts with various metabolic pathways, including one-carbon metabolism (OCM). OCM is critical for the synthesis of DNA precursors, essential for DNA repair, and as a methyl donor for various methylation events, including DNA methylation. Both DNA repair and DNA methylation are critical for maintaining genomic stability. In this review, we outline the role of DNA damage and DNA repair dysfunction in chronic alcohol-induced neurodegeneration.

Keywords: alcohol, DNA damage, neuron, cell death

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Introduction

Chronic alcohol consumption is associated with an increase in the incidence of a variety of diseases, including central nervous system (CNS) degeneration.[1] The brain is one of the major targets of alcohol actions. In adult human chronic alcoholics, brain damage is characterized by cerebral and cerebellar atrophy, and impaired neuronal function within the hippocampus and frontal cortex.[2,3] Besides specific alcohol-related disorders, such as Wernicke–Korsakoff syndrome, hepatic encephalopathy and pellagra, heavy alcohol consumers exhibit cognitive and motor impairments, cholinergic deficits and dementia. It is estimated that 50–75% of long-term alcoholics show cognitive impairment and structural damage to the brain, making chronic alcoholism the second leading cause of dementia behind Alzheimer's disease.[4–6] These changes may be caused by loss of neurons, shrinkage of neuronal cell bodies, or reduction in the number and extent of dendrites. Careful neuropathological analyses have provided evidence for loss of neurons in certain regions of the brain of alcoholics.[2,7] A direct toxic effect of ethanol on the brain has been suggested as the primary cause of alcohol-related neuronal loss.[8] The effects of alcohol on the brain are well documented. However, the mechanisms are poorly understood. Several mechanisms have been proposed to explain ethanol-related brain damage. The *N*-methyl-D-aspartate (NMDA)-glutamate receptor could contribute to alcohol-induced brain damage. Ethanol, when administered acutely, potently inhibits NMDA receptors, while chronic exposure causes adaptive upregulation in the sensitivity of NMDA receptors and can result in an increased vulnerability for glutamate-induced cytotoxic response (excitotoxicity).[9] Increased calcium influx through NMDA receptors, in turn, is tightly coupled to uptake into mitochondria and causes the production of reactive oxygen species (ROS). Alcohol metabolism is also associated with ROS production[10,11] (Figure 1). Ethanol oxidation by cytochrome P450 2E1 (CYP2E1) and catalase is particularly relevant to ethanol metabolism in the brain.[12] It is well known that ROS cause damage to DNA.[13] Acetaldehyde, the primary metabolite of ethanol, can also damage DNA.[14,15]

Chronic ethanol exposure has been shown to be more harmful than acute exposure.[16,17] Chronic alcoholism is mutagenic and carcinogenic in humans and is also associated with brain dysfunction.[1,6,10,13,18–20] However, the mechanisms by which long-term chronic excessive alcohol consumption leads to a variety of pathologies are unclear.

The sulfur-containing amino acid homocysteine (Hcy) has been suggested to be toxic in alcoholism.[6,21] Chronic alcoholic patients often have sustained hyperhomocysteinemia, a marker of one-carbon metabolism (OCM) impairment and a role for alcohol in disrupting OCM is strongly supported by the literature.[10,21] OCM impairment directly impacts the

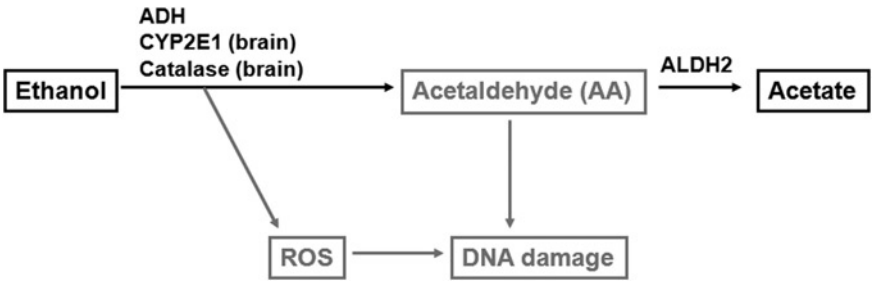

Figure 1 How alcohol may impact DNA. Ethanol is metabolized to acetaldehyde (AA) by alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1) and catalase. AA is metabolized to acetate by aldehyde dehydrogenase 2 (ALDH2). Catalase and CYP2E1 are particularly relevant to ethanol metabolism in brain tissue. The CYP2E1-dependent pathway is a major contributor to ethanol-generated reactive oxygen species (ROS). ROS induce DNA damage

brain in the conditions of ethanol exposure, as evidenced by increased Hcy concentrations in the cerebellum of rats[22] and increased risk of alcohol withdrawal seizures with the increase of Hcy plasma concentrations.[23] Also, plasma Hcy concentrations show the most significant correlation to hippocampal volume reduction in patients with alcoholism.[3] Such effects of OCM impairment can be explained by the critical importance of OCM for the synthesis of DNA nucleotides (dNTPs), precursors for DNA biosynthesis and repair, and for methylation reactions.[24,25] DNA repair and methylation of DNA play essential roles in maintaining genomic stability. Given that ethanol (or its metabolites) is genotoxic[14,17] and OCM is critical for maintaining genomic stability,[26] alcohol-induced OCM impairment may play a significant role in alcohol pathogenicity. In addition, Hcy itself may act as a convulsant through its agonism at NMDA receptors.[27]

Here, we link chronic alcohol-induced neurotoxicity to DNA damage, which suggests far-reaching implications for the understanding of the pathogenesis of alcoholism in humans.

Potentially toxic metabolites of ethanol

As shown in Figure 1, ethanol is metabolized mainly through oxidation catalyzed by alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1) and catalase enzymes.[28–30] Due to the low capacity and the relatively high affinity (K_m = 0.05–0.1 g/L) of ADH, the enzyme gets saturated after only a few drinks. Once formed, acetaldehyde is oxidized by the mitochondrial isoform of aldehyde dehydrogenase (ALDH), ALDH2, to acetate, in an irreversible reaction. ALDH2 has a very low K_m value, which makes the elimination of toxic acetaldehyde highly efficient. The rates of ethanol metabolism by ADH and ALDH2 are critical in determining its toxicity because the alcohol metabolite acetaldehyde is highly toxic.[29,31] ADH and ALDH2 use the co-factor nicotinamide adenine dinucleotide (NAD+), which converts to NADH and significantly changes the ratio NADH/NAD+, resulting in an altered cellular redox and overall energy metabolism states.[32] ALDH2 is one of 19 members of the ALDH gene family in humans that play a crucial role in the oxidation and detoxification of numerous reactive aldehydes including 4-hydroxy-2-nonenal (4-HNE), a well-known, highly toxic by-product of lipid peroxidation, and nitroglycerin.[32–34] Consistent with the critical importance of ALDH2 for detoxification of acetaldehyde, a risk of alcohol-induced toxicity in individuals with mutant ALDH2 is remarkably increased. Approximately 500 million Asians are heterozygous for the ALDH2 gene and, therefore, possess one normal and one mutant copy of the ALDH2 gene, termed ALDH2*2. The mutant copy encodes an inactive isozyme.[35,36]

Although ADH activity is not present in the brain, there is evidence of brain ethanol metabolism through ethanol oxidation into acetaldehyde by catalase[37,38] and CYP2E1[39] (Figure 1). It is estimated that catalase accounts for 60–70% of ethanol-generated acetaldehyde in the brain, while CYP2E1 accounts for 10–20%.[39,40] Acetaldehyde is then readily oxidized into acetate by ALDH.[38] A number of studies based on the analysis of brain homogenates[39,41] and at least one study based on the *in vivo* microdialysis[42] provide evidence for the presence of acetaldehyde in the brain following ethanol intake. The levels of acetaldehyde were significantly increased under conditions of ALDH2 deficiency in ALDH2-knockout mice and were consistent with the dose of ethanol.[42] ALDH2 transgenic overexpression alleviated chronic alcohol-induced cell death in the cerebral cortex of mice.[43] Since ALDH2 is essential for detoxification of acetaldehyde, ALDH2 deficiency can directly contribute to excess acetaldehyde accumulation, while its overexpression can reduce acetaldehyde concentrations (and toxicity). Other indications of acetaldehyde presence in the brain include a reduction in acetaldehyde accumulation under conditions of pharmacological[38] or genetic (via injection of anti-catalase shRNA construct into the CNS)[44] inhibition of catalase. Thus, acetaldehyde can be produced in the brain by metabolic transformation of ethanol, and neurotoxic effects of ethanol may be associated with its toxicity.

Alcohol and DNA damage

Preservation of genetic information is of prime importance to all living systems. However, the integrity of the genome is continuously threatened by environmental agents (e.g. the ultraviolet [UV] component of sunlight, ionizing radiation and genotoxic chemicals) or intrinsic sources of DNA damage (metabolic by-products, spontaneous disintegration of DNA structure). DNA damage can lead to

mutations, a primary step into cancer initiation. DNA damage may also result in cellular malfunction, senescence or death. Together, these changes may cause the progressive loss of tissue homeostasis and organismal decline. Oxidative stress, ionizing radiation, UV light and numerous chemicals induce a wide verity of DNA lesions.[45] To cope with this permanent challenge, cells are equipped with an efficient genome defense mechanism responsible for detecting and removing DNA lesions, as well as eliminating the irreparably damaged cells. Alcohol metabolism generates potentially genotoxic ROS and acetaldehyde, which have been shown to induce DNA damage, including oxidative modifications, acetaldehyde-derived DNA adducts and cross-links.[14,17,18,46–48] One of the most abundant ROS-induced DNA lesions is the oxidative DNA damage, 7,8-dihydro-8-oxo-2′-deoxyguanosine (oxo8dG),[49] which is repaired by the base excision repair (BER) pathway. The primary acetaldehyde-derived DNA adduct is *N*2-ethylidene-deoxyguanosine,[50] which may be converted *in vivo* to *N*2-ethyldeoxyguanosine (*N*2-ethyl-dG). *N*2-ethyl-dG has been found in the DNA of ethanol-treated mice[51] and human alcoholics,[52] suggesting that *N*2-ethyl-dG is a potential biomarker of acetaldehyde-induced DNA damage. This DNA adduct blocks translesion DNA synthesis (a DNA damage tolerance process that allows the DNA replication machinery to replicate past DNA lesions) catalyzed by a variety of DNA polymerases, and induces mutations.[53,54] DNA polymerase η can bypass *N*2-ethyl-dG in an error-free manner.[55] The DNA repair pathway for *N*2-ethyl-dG has not been identified. *N*2-ethyl-dG is not the only acetaldehyde-generated DNA damage. Acetaldehyde can also form DNA–DNA and DNA–protein cross-links.[46,47] The major mechanism responsible for efficient cross-link removal is the Fanconi anemia (FA) pathway. Cross-link repair also involves the coordinated activities of other DNA repair pathways, such as nucleotide excision repair (NER) and homologous recombination (HR).[56] Defects in a cluster of FA proteins leading to inactivation of associated DNA repair pathways are associated with hereditary disease FA that causes developmental defects, sterility, bone-marrow failure and a highly elevated risk of cancer.[15] Langevin *et al.*[15] recently provided strong evidence that metabolically produced acetaldehyde is indeed a DNA-damaging agent normally counteracted by the FA network. They demonstrated that simultaneous knockout of the *Aldh2* gene (which encodes Aldh2, the main detoxifying enzyme of acetaldehyde) and the *Fancd2* gene (a key player in the FA system) in double-mutant ($Aldh2^{-/-}Fancd2^{-/-}$) mice make these mice unusually sensitive to ethanol exposure. Although many studies of alcohol-mediated DNA damage have been conducted in the liver and other proliferating tissues in association with carcinogenesis, evidence exists that alcohol can produce DNA damage in the brain. Brains of mice exposed to ethanol exhibited FA activation, suggesting formation of acetaldehyde-induced DNA damage in the brain, although DNA damage was not determined directly.[14] Importantly, acute alcohol intoxication induces reversible DNA lesions which do not exceed the capacities of the cellular repairing systems, while chronic alcohol exposure is associated with extensive DNA damage[17,18] associated with genomic instability. Genomic instability refers to a set of events capable of causing unscheduled alterations, either of a temporary or permanent nature, within the genome and encompasses diverse genetic changes. Genomic instability usually results from an aberrant cellular response to DNA damage. DNA damage response is an extremely important mechanism of preserving genomic stability which includes DNA repair machinery. Damage to the genome is not only caused by exogenous and endogenous chemical and physical agents but can also occur due to inherited or acquired defects in DNA repair,[57–59] conditions where the rate of DNA damage exceeds DNA repair capacity of the cell. As a result, fundamental changes to the genetic code and gene expression may cause serious defects in cellular function and tissue physiology. This can be a consequence of dysfunctional DNA repair, epigenetic changes leading to disturbed DNA damage response and accumulation of genetic aberrations in cells. These changes can be classified into the two major groups: instability occurring at the chromosomal and at the nucleotide levels. Instability at the nucleotide level occurs due to faulty DNA repair pathways such as BER and NER. The chromosomal instability represents alterations, which result in gains or losses of whole chromosomes as well as inversions, deletions, duplications and translocations of large chromosomal segments.[60] DNA repair processes play critical roles in repairing damaged DNA, and in ensuring accurate transmission of genetic material. Inherited defects of genes in these pathways lead to disorders, most of which significantly increase susceptibility to cancer and neurodegeneration.[26,61] Epigenetic modifications such as methylation and histone modification have also been shown to affect genomic stability.[60] Global hypomethylation and genomic instability are seen in many cancers.[62] The classic model for neurodegeneration due to dysfunctional DNA repair represents the idea that DNA damage accumulates in the absence of repair, resulting in the death of neurons.[5] According to this mechanism, which presently is generally accepted, endogenous DNA damage is constantly being produced in normal conditions but also repaired, resulting in a low-steady-state level of damage which is compatible with normal cellular function. However, under conditions of DNA repair deficiency, endogenous damage is not repaired and therefore accumulates over time, ultimately leading to neuronal death as a result of impaired transcription. Indeed, defects in DNA damage response/DNA repair observed in patients with a variety of hereditary DNA repair diseases are associated with neurological abnormalities.[61] Therefore, alcohol-induced DNA damage, if not repaired, may play a key role in alcohol-induced neurotoxicity.

Alcohol and OCM

Hyperhomocysteinemia, which is an indication of OCM dysfunction, is often observed in patients suffering from chronic alcoholism.[10,21] It has been known for many years that ethanol has an effect on folate metabolism. The etiology of folate deficiency in alcoholism can be caused by any or all

of the following: poor absorption, decreased uptake and retention, and increased urinary excretion.[21] Furthermore, increased Hcy in patients suffering from alcohol dependence may be due to direct interactions of ethanol or its metabolites with OCM.[63] Indeed, ethanol has been shown to directly affect OCM by inhibiting a key OCM enzyme, methionine synthase (MS), by 50% as early as six days of ethanol exposure.[64,65] These findings have been corroborated by other investigators using different animal models of ethanol injury.[21,66,67] Ethanol treatment has been demonstrated to reduce MS activity in the brain.[68] Based on *in vitro* experiments, it has been suggested that acetaldehyde, but not ethanol, causes inhibition of MS activity.[69] In spite of supraphysiological inhibitory levels of acetaldehyde in these experiments, the actual concentrations may be much lower than the reported values because acetaldehyde is highly volatile. Thus, it may be reasonable to assume that the *in vitro* assay could represent an acute treatment for a short time and the *in vitro* data may not reflect the true situation occurring *in vivo*. Given that MS is critical for OCM function, its down-regulation or direct inhibition may play a significant role in alcohol-induced OCM impairment.

OCM reactions (Figure 2) can be viewed as two intertwined cycles, one producing dNTPs, the precursors for DNA biosynthesis and repair, and the other producing and utilizing *S*-adenosyl methionine (SAM), the universal donor in cellular methylation reactions.[24,25] 5,10- methylene-tetrahydrofolate (5,10-methylene-THF) is a critical OCM junction where one-carbon groups can either be used to produce dNTPs for DNA synthesis or serve for the methylation cycle via irreversible synthesis of 5-methyl-THF catalyzed by methylene THF reductase. Following SAM-dependent transmethylation and hydrolysis of the ensuing *S*-adenosyl homocysteine (SAH), the resulting Hcy can be re-methylated back to methionine by the folate-mediated MS reaction. OCM dysfunction causes alteration in the *de novo* synthesis of DNA precursors, purines and thymidylate, negatively affecting the intracellular dNTP pool and, consequently, DNA synthesis (and cell proliferation) and repair (and genomic stability).[70] The dNTP supply via the salvage pathway, whose purpose is to provide the cell with a low supply of dNTPs, cannot compensate DNA precursor pool imbalance from aberrant *de novo* dNTP synthesis. As a result, the dNTP synthesis pathway is the predominant mechanism for supplying dNTPs in the brain.[71,72] The DNA repair pathway for removal of an incorrect DNA base, such as one of the most abundant alcohol-induced DNA base lesions oxo8dG, is BER.[73] Acetaldehyde-derived cross-links are repaired by the FA-linked pathway, coordinated with classical NER, applicable to non-replicating cells such as neurons.[56,74] Both DNA excision repair pathways, BER and NER, involved in the repair of alcohol-generated DNA damage, operate through the removal of damaged bases and their substitution with correct ones by the pathway-specific polymerases. The availability of dNTPs from OCM is important for BER and NER because DNA polymerases involved in these pathways are not functional when nucleotides are depleted. This leads to compromised DNA repair and accumulation of DNA lesions.[75] Thus, OCM dysfunction is associated with compromised DNA excision repair pathways due to a decrease of synthesis of dNTPs and ensuing dNTP pool imbalance. Particularly, chronic alcohol administration has been reported to significantly reduce BER.[76] Dysfunctional DNA repair results in accumulation of DNA damage and death of neurons.[5]

OCM is essential for DNA methylation and its disturbance can result in global DNA hypomethylation.[77] DNA methylation is a major epigenetic mechanism central to regulation of chromatin structure, genomic stability, gene transcription, and ultimately cell function and development. Functionally, proper DNA methylation can restrain the inappropriate expression of genes, thereby shaping cellular fate and function. In higher organisms, a methyl moiety is preferentially targeted to the DNA base cytosine in the context of a CpG dinucleotide, with the exception being X-inactivation. DNA

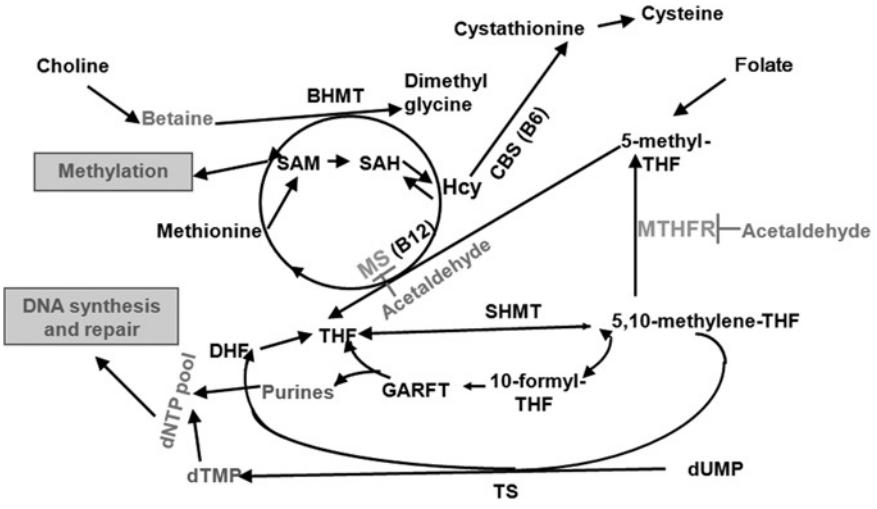

Figure 2 One-carbon metabolism as a target for ethanol. BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; DHF, dihydrofolate; dNTP, deoxyribonucleotide triphosphate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; GARFT, glycinamide ribonucleotide formyltransferase; Hcy, homocysteine; MS, methionine synthase; MTHFR, methylene THF reductase; SAH, *S*-adenosyl homocysteine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase

is highly methylated in CpG-rich sequences (>50%), which has been suggested to represent a defense mechanism for silencing parasitic DNA elements such as transposons and retrotransposons.[78] DNA methylation leads to a condensed structure and transcriptional repression. Aberrant methylation is associated with increased genomic instability[79] and carcinogenesis,[80] as well as brain disorders such as ischemia.[81] At least three hereditary diseases with aberrant methylation, immunodeficiency/centromeric instability/facial anomalies (ICF), Rett and fragile X syndromes,[82] are characterized by mental impairment, indicating the importance of methylation for brain function. DNA methylation depends on OCM as a source of methyl groups and dNTP methyltransferases which methylate cytosine residues at CpG sites in DNA. For this reason, hypomethylation can be caused by OCM impairment or mutations/inactivation of methyltransferases. Indeed, DNA hypomethylation has been demonstrated experimentally as a result of dietary folate deficiency,[83] or mutations of enzymes, important for DNA methylation.[83,84] Given that alcohol interferes with OCM,[63,85,86] alcohol-induced aberrant DNA methylation[87] is likely mediated by OCM impairment. Interestingly, elevated Hcy (OCM impairment) is considered an independent risk factor for numerous neurodegenerative diseases.[26,78]

In summary, alcohol affects OCM function. OCM is critical for synthesis of DNA precursors and methylation reactions (including DNA methylation). OCM impairment may cause DNA precursor imbalance and ensuing disturbance of DNA synthesis, which is important for cell proliferation and DNA repair. Dysfunctional DNA repair leads to genomic instability, and is a unified genetic mechanism causing a variety of neurological and neurodegenerative disorders.[88–90] There has been compelling evidence accumulated that disorders such as Alzheimer's, Parkinson's and Huntington's diseases and Down's syndrome result from dysfunctional DNA repair and increased DNA damage.[90] Different brain regions are affected in these neurodegenerative diseases, as well as hereditary diseases with DNA repair deficiencies: cerebellar Purkinje neurons in ataxia telangiectasia, dopaminergic neurons in the substantia nigra in Parkinson's disease, neuronal loss in the striatum and cerebral cortex in Huntington's disease, and loss of neurons in the cerebral cortex in Alzheimer's disease.[90] It is unclear, however, why different brain regions are affected in these disorders. Analysis of the types of neurons lost from the frontal cortex of alcoholics revealed that this population of neurons is also more vulnerable in both Alzheimer's disease[91] and normal aging[92] in which DNA repair disturbance plays an important role.[26] Atrophy of the cerebellum is commonly associated with alcoholism. Torvik *et al*.[93] reported that 26.8% of alcoholics with Wernicke–Korsakoff syndrome had cerebellar atrophy with a loss of Purkinje cells that correlated with clinical ataxia/unsteadiness.[94] This is especially interesting given the ataxia and death of Purkinje neurons in patients with ataxia telangiectasia, a hereditary disease with impaired DNA damage response.[90] Understanding the causes and time-courses of DNA damage in the brain generally and in specific regions following chronic ethanol exposure will help to understand how DNA repair dysfunction and ensuing DNA damage may cause the damage of particular brain regions.

The phenotype of OCM impairment includes reduced tolerance to DNA-damaging agents, and genomic instability.[70,75,95,96] Genomic instability results in mutagenesis and carcinogenesis.[97] At the same time, it is associated with neuronal cell death and neurodegeneration.[88–90] Alcohol abuse is known to increase the risk for various types of cancer,[10,20,97–99] and contributes to neurodegeneration.[88–90] The contribution of genomic instability to both carcinogenesis and neurodegeneration is illustrated by the fact that DNA repair defects in various human syndromes are usually characterized by both cancer predisposition and neurological abnormalities.[90] Since OCM impairment is associated with DNA repair dysfunction and aberrant methylation, both linked to genomic instability, chronic alcohol-induced genomic instability and associated neurodegeneration are likely mediated, at least in part, by alcohol's impact on OCM.

Conclusion

Throughout this paper, we have emphasized the role of genomic instability in the adverse effects of chronic alcohol abuse on the brain. Ethanol metabolism, including those in brain, elicits the formation of genotoxic ROS and acetaldehyde.[5,14,42] If not efficiently detoxified, these metabolites induce DNA damage, which is reversible in conditions of an acute alcohol exposure.[14,17,18,48] However, chronic alcohol abuse is characterized by extensive DNA damage and genomic instability,[14,17,18] which is a risk factor for different types of cancer[10,20,98,99] and neurodegeneration.[88–90] Alcoholism is known to increase the risk for cognitive impairment and dementia and is characterized by structural damage to the brain.[4–6] The present review describes the current knowledge concerning alcohol-induced genomic instability with alcohol-induced OCM impairment. Increased Hcy concentrations, a marker of OCM disturbance is common in alcoholics,[21,100] and at the same time is a risk factor for various neurodegenerative diseases.[101,102] OCM is vital for cellular homeostasis, providing cells with DNA precursors, essential for cell proliferation and DNA repair, as well as methyl groups for DNA methylation. Thus, OCM dysfunction can lead to a shortage of DNA precursors, resulting in impaired DNA repair, as well as aberrant DNA methylation. Both DNA repair dysfunction and aberrant DNA methylation cause genomic instabilty.[79,97] OCM impairment is among the factors that promote genomic instability.[67,95] Different factors can affect the sensitivity of brain to alcohol in this context. Two examples are age and gender. Accumulated DNA damage is thought to be an important factor underlying aging.[103] Defective DNA repair causes an accelerated aging-like phenotype of the brain.[104] Hcy concentrations (and OCM impairment) increase with age,[101] and age-related brain atrophy in healthy elderly people correlates with plasma Hcy concentrations.[105,106] Among the factors that influence vulnerability of the brain to alcohol is gender. Women show a greater susceptibility to alcohol-related

diseases, including an increased risk for brain damage with significantly lower alcohol exposure compared with men.[107] The mechanism involving increased DNA damage and impaired DNA repair may contribute to the gender-related differences in vulnerability to alcohol. It is known, for example, that poly (ADP-ribose) polymerase-1 (PARP-1) deletion reduces ischemic brain injury in men but exacerbates injury in women.[108] Since PARP-1 is a factor that plays an important role in the DNA damage response and DNA damage-induced cell death signaling,[109] this difference suggests that the DNA damage response is different in men and women, and that the gender-related differences in vulnerability to alcohol may be caused by different responses to DNA damage. Further investigation of the cellular and molecular mechanisms involved in the genotoxicity of chronic alcohol, its interference with OCM, and the impact on DNA repair and DNA methylation will help to understand the mechanisms of ethanol-induced brain damage and will likely contribute to the development of treatments and/or therapeutic agents that could reduce or eliminate the deleterious effects of alcohol on the brain.

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Long-Term Treatment of Chronic Hepatitis C with Glycyrrhizin [Stronger Neo-Minophagen C (SNMC)] for Preventing Liver Cirrhosis and Hepatocellular Carcinoma

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Key Words

Hepatitis C virus · Hepatitis, chronic · Liver cirrhosis · Hepatocellular carcinoma · Glycyrrhizin · Stronger Neo-Minophagen C (SNMC®)

Abstract

In Japan, hepatitis C virus (HCV) is the single most frequent cause of hepatocellular carcinoma (HCC), resulting in yearly deaths of over 30,000. Although the mechanism of how HCV induces HCC is not clear, persistent HCV infection and necro-inflammatory changes in chronic hepatitis C accelerate the development of liver cirrhosis and can eventuate in HCC. Hence, means of eradicating HCV as well as suppressing inflammation in the liver, even if patients stay infected with HCV, would decrease the incidence of HCC with chronic hepatitis C. For more than 40 years, a preparation of glycyrrhizin [Stronger Neo-Minophagen C (SNMC)] has been used for the treatment of 'allergic' hepatitis in Japan. In 1977, intravenous injection with SNMC was started in patients with chronic hepatitis or liver cirrhosis, most of whom have turned out to be infected with hepatitis viruses. In a multicenter double-blind study, alanine aminotransferase (ALT) levels decreased in the patients who received 40 ml/day of SNMC for 4 weeks at a rate significantly higher ($p < 0.001$) than controls receiving placebo. Furthermore, SNMC 100 ml/day for 8 weeks improved liver histology in 40 patients with chronic hepatitis, in correlation with improved ALT levels in serum. Liver cirrhosis occurred less frequently in 178 patients on long-term SNMC than in 100 controls (28 vs. 40% at year 13, $p < 0.002$). Finally, HCC developed less frequently in the 84 patients on long-term SNMC than in the 109 controls (13 vs. 25% at year 15, $p < 0.002$). Combined, these results indicate that a long-term treatment with SNMC prevents the development of HCC in the patients with chronic hepatitis. SNMC is particularly helpful in the patients with chronic hepatitis C who fail to respond to interferon and in those who cannot be treated with it for various reasons.

The mechanism of how hepatocellular carcinoma (HCC) develops in persistent hepatitis virus infections is not clear as yet. It is not even known for HCC arising in carriers of hepatitis B virus (HBV) that can integrate into the host genome. With regard to HCC associated with hepatitis C virus (HCV) infection, it is much harder to sort out how it contributes toward the development of HCC. Being an RNA virus that is not reverse-transcribed into DNA, HCV cannot integrate into host DNA for activating proto-oncogenes or inactivating tumor suppressor genes.

Many lines of clinical and epidemiological evidence indicate that HCC develops against the background of liver cirrhosis that is characterized by accumulation of

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fibers in the liver. When hepatocytes are damaged for any reason (or any cells constituting the human body for that matter), a healing process sets in accompanied by accumulation of fibers (fibrosis). When it occurs in the liver, it takes the form of cirrhosis. Hence, cirrhosis is a natural response of the host to heal inflammation in the liver; it would be difficult to prevent it in the first place.

The ideal means of preventing HCC is to get rid of offending agents. Hence, the primary goal is to chase away HCV or HBV. Unlike HBV infection in adulthood that terminates by itself in most cases, HCV infection hardly resolves spontaneously. It is the general consensus that once the host contracts HCV infection, it becomes chronic in ~70% of the cases. Although the natural course of chronic HCV infection has not been fully understood, chronic hepatitis C can be elicited that evolves to liver cirrhosis and eventuates in HCC.

Interferon is the only available drug effective in terminating HCV infection for the prevention of HCV-associated HCC. Combination therapy with interferon and ribavirin increases the efficacy of antiviral activity substantially. Even with the combination therapy, however, a sustained virological response with loss of HCV RNA in serum can be achieved in at most ~50% of the treated patients [1]. So, we are left with the other half of the patients with chronic hepatitis C who fail to respond to interferon/ribavirin. There is a pressing need, therefore, to treat nonresponders with sophisticated therapies.

Inasmuch as chronic inflammation in the liver triggers the development of HCC through chronic hepatitis and cirrhosis, agents capable of suppressing inflammation are expected to prevent or postpone it. Here I relate my experience with glycyrrhizin over 20 years in the treatment of patients with chronic hepatitis C.

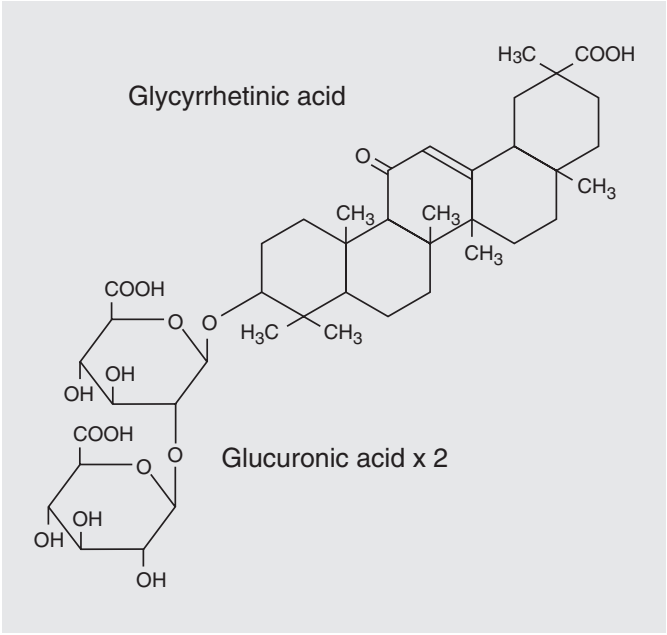

Fig. 1. Chemical structure of glycyrrhizin.

Table 1. Mechanisms for the pharmacological activity of glycyrrhizin (SNMC)

Mechanism	Reference
Decreases cell membrane permeability	Shiki et al. [2]
Interferes with complement pathways	Fujisawa et al. [3]
Inhibits phospholipase A_2	Okimasu et al. [4]
Scavenges radicals	Kiso et al. [5]
Induces γ-interferon	Abe et al. [6]
Augments NK activity	Itoh and Kumagai [7]
Modulates T cells	Kimura et al. [8]
Inactivates viruses	Baba and Shigeta [9]
Inhibits proliferation of viruses	Ito et al. [10]
Prevents liver cancer in mice	Shiota et al. [11]

Glycyrrhizin

Glycyrrhizin is composed of one molecule of glycyrrhetinic acid coupled with two molecules of glucuronic acid (fig. 1). It has been commercialized by Minophagen Pharmaceutical Co. (Tokyo, Japan) under the trade name Stronger Neo-Minophagen C (SNMC®) and marketed for more than 60 years. SNMC is an intravenous drug, and one ampoule (20 ml) contains 40 mg of glycyrrhizin as the active ingredient along with 400 mg of glycine and 20 mg of cysteine. Two amino acids are added to reduce the side effects of glycyrrhizin.

At the outset, glycyrrhizin was indicated primarily to treat dermatitis with the aim of suppressing allergic or inflammatory process. Understandably, hepatitis was considered an 'allergic' disease, before the discovery of HBV and HCV. Hence, SNMC was tried out initially in patients with 'allergic' hepatitis in Japan, and it worked [12].

SNMC is known to have many pharmacological activities. Table 1 lists mechanisms proposed for the activity of SNMC [2–11]. As far as suppression of necroinflammatory reactions in the liver is concerned, I am inclined to believe that the cytoprotective potential attributed to SNMC will play a major role in treating patients with chronic hepatitis. Whether such effects are achieved by direct action of SNMC on cell membranes or mediated by its interaction with the complement pathway awaits further studies. After intravenous injection with

SNMC, serum levels of ALT start to decrease within 15 min, peak at 30 min and then gradually decrease; they remain detectable 24 h after injection with levels dependent on the dose [13].

Glycyrrhizin is the active principle of SNMC, and extracted from the roots of *Glycyrrhiza glabra*. A big advantage of SNMC is its long track record for safety. I have been very comfortable with it, especially after I saw the severe side effects of interferon. As pseudoaldosteronism, hypokalemia and elevated blood pressure are reported as side effects of SNMC, albeit at low incidence rates, it is necessary to take the blood pressure and determine serum levels of potassium regularly in the patients who receive it.

Cumulative ALT Score as a Measure for Estimating the Net Burden with Stimuli for Fibrogenesis and Hepatocarcinogenesis in Patients with Chronic Hepatitis C

Liver cirrhosis is a process for healing hepatocytes damaged by inflammation. HCC is considered to develop as a result of continuous stimuli for carcinogenesis occurring in the liver undergoing persistent necro-inflammation and regeneration. Agents that stimulate fibrogenesis and carcinogenesis accumulate in the liver during chronic hepatitis C, and when they exceed a certain threshold level, manifest themselves in liver cirrhosis and HCC in the long run. Since ALT in serum is the most reliable and sensitive indicator of hepatocytolysis in chronic hepatitis, the net burden imposed by offending agents can be estimated by the increment of ALT over normal levels during a certain period of time (fig. 2). Thus, the ALT index during a given year is defined as the mean ALT level in serum samples examined at regular intervals divided by the upper limit of normal (ULN: 50 IU/l). The cumulative ALT score is the sum of the yearly ALT index during years of follow-up. Admittedly, this is a rough clinical estimate; fluctuating ALT levels can be misleading at regular check-ups and inflammation may go on even in the patients with ALT levels below 50 IU/l. Still, the cumulative ALT score is clinically and pragmatically relevant.

Figure 3 illustrates the relationship between cumulative ALT score and progression in the stage of fibrosis in patients with chronic hepatitis C. Two things are apparent on figure 3. First, there is a linear correlation between the cumulative ALT score and an increase in the stage of fibrosis, irrespective of the stage of fibrosis found in the first biopsy.

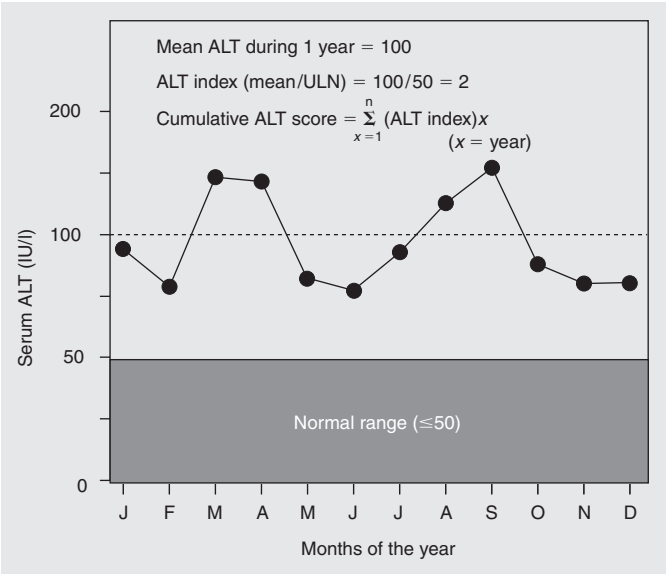

Fig. 2. Calculation of cumulative ALT score.

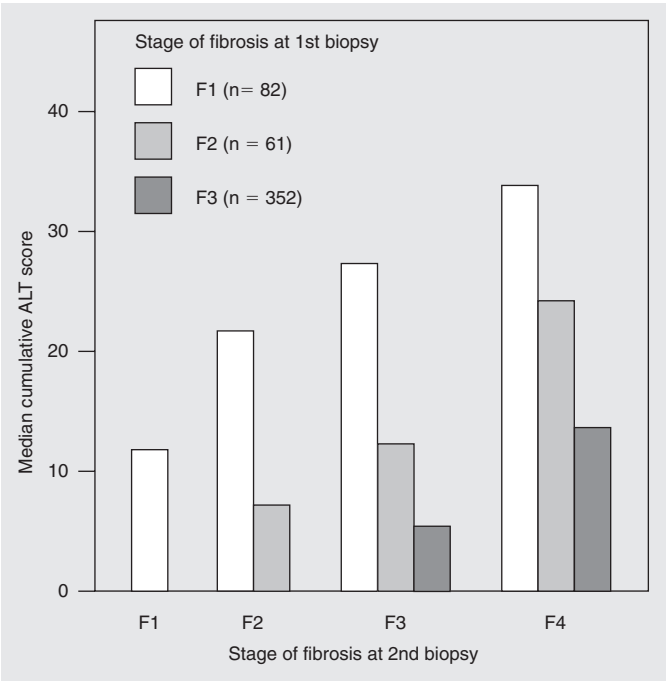

Fig. 3. Distinct rates of progression in the stage of fibrosis in patients with chronic hepatitis C who were found to have stages F1–F3 at first biopsy.

Second, the higher the stage of fibrosis in the first biopsy, the lower the cumulative ALT score required for the progression to the next stage of fibrosis. In order to prevent the progression of fibrosis, therefore, the

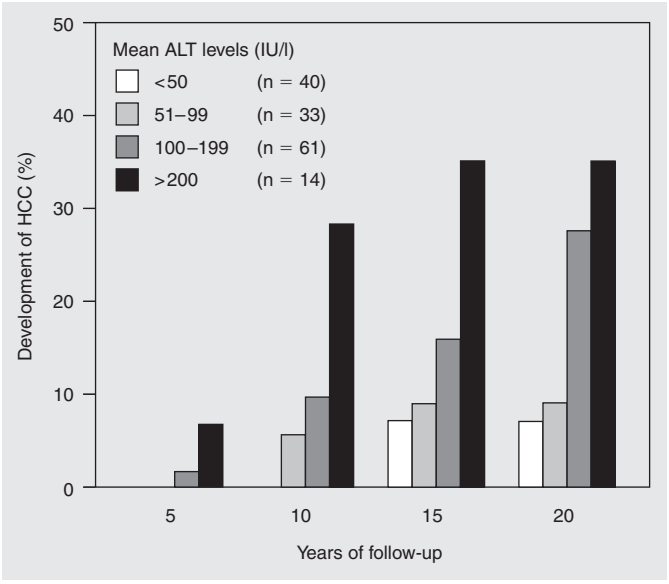

Fig. 4. Development of HCC as a function of mean ALT levels.

cumulative ALT score needs to be kept increasingly lower, as the stage of fibrosis increases from F1 through F2 to F3 in the first biopsy.

It is reasonably presumed that the cumulative ALT score would also be reflected in the development of HCC. Figure 4 illustrates the development of HCC in 148 patients with chronic hepatitis C during 5–20 years, stratified by mean ALT levels which serve as estimates for cumulative ALT scores. Obviously, the incidence of HCC increases in parallel with the mean ALT level. From what is seen in figure 4, it is imperative to keep ALT levels <100 IU/l (twice the upper limit of normal), desirably within normal limits (<50 IU/l).

Long-Term Treatment with SNMC for Preventing Liver Cirrhosis and HCC in Patients with Chronic Hepatitis C

When I started out as a clinical and research hepatologist in 1977, I was amazed at the large number and the misery of patients with chronic hepatitis at the Gastroenterology Department of the Toranomon Hospital. The great majority of them were without ongoing infection with HBV, and were classified as non-A, non-B hepatitis. Almost all of them turned out to be infected with HCV when their stored serum samples were tested for antibody to HCV (anti-HCV) and HCV RNA when these tests became available. Deeply concerned about my patients, I felt obliged to find a way to take care of them. I was aware that SNMC can lower serum levels of ALT.

Table 2. Comparison between the patients who did or did not receive SNMC

Features	SNMC	
	with	without
Number	181	221
Age[a], years	48 (23–74)	47 (20–75)
Male[b]	135 (75)	166 (79)
Transfusion[b]	147 (81)	84 (38)
Histology[b]		
F1	82 (45)	101 (46)
F2	66 (36)	79 (36)
F3	33 (18)	41 (19)
Platelets[a] $\times 10^4$/ml	17.8 (7.8–28.9)	18.3 (8.1–29.3)
ALT[a,c], IU/l	240 (72–1, 120)	132 (52–840)
ICG_{15}%[a]	16 (6–28)	16 (6–31)
HCV genotype[b]		
1b	105 (58)	91 (41)
2a/2b	34 (19)	26 (12)

Median values are shown.
[a] Values in parentheses represent range.
[b] Values in parentheses represent percentage.
[c] $p < 0.0001$.

Professor Emeritus Hiroshi Suzuki (former President of Yamanashi Medical University, Yamanashi, Japan) and his colleagues were the first to evaluate the efficacy of SNMC in decreasing serum transaminase levels by a randomized, double-blind placebo-controlled clinical trial [14]. In this trial, 2 ampoules of SNMC (glycyrrhizin 80 mg) were given intravenously to 67 patients and placebo to 66 patients during 4 weeks. When codes were broken, serum levels of ALT and aspartate aminotransferase decreased significantly in the patients who received SNMC compared to those receiving placebo ($p < 0.001$). Levels of γ-glutamyl transpeptidase also decreased after SNMC therapy ($p < 0.05$).

Inspired by their results, I started to treat patients with chronic hepatitis with 2–5 ampoules of SNMC (glycyrrhizin 80–200 mg). Initially, we gave it to patients every day of the week. Table 2 compares various demographic, clinical, pathological and virological features between patients with chronic hepatitis C who did and did not receive SNMC. Although they were not randomized, features are reasonably comparable between the two groups. The pretreatment median ALT level was significantly higher in the 181 patients who received SNMC than in the 221 who did not ($p < 0.0001$), but this would

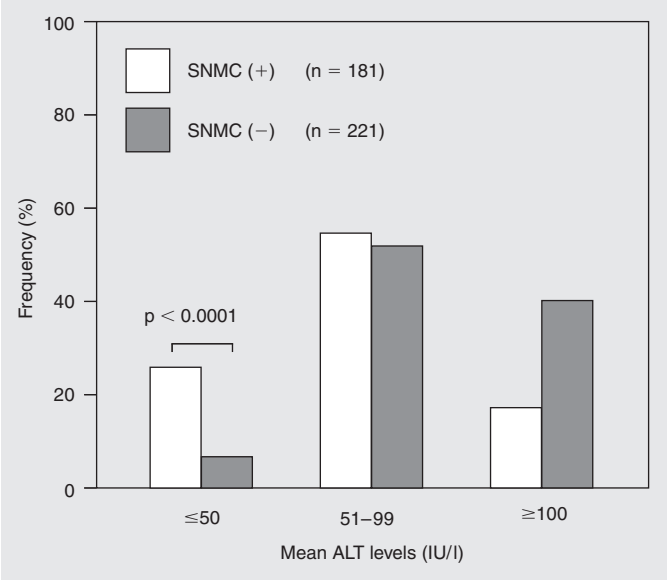

Fig. 5. ALT levels in patients with chronic hepatitis C who did or did not receive SNMC.

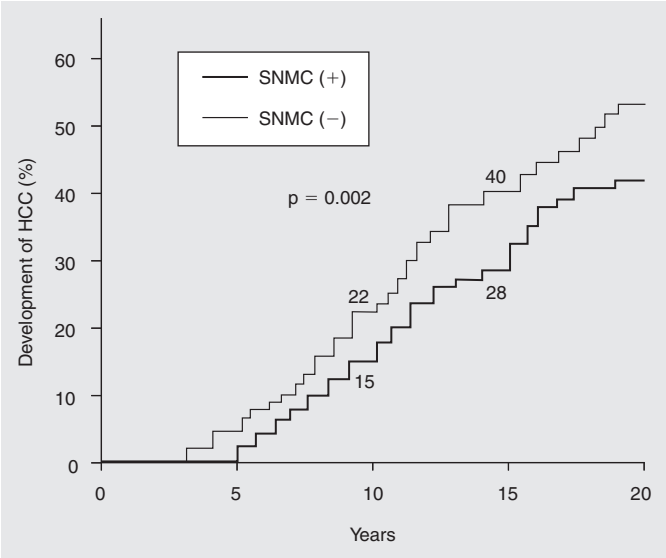

Fig. 6. Development of HCC in patients with chronic hepatitis C who did or did not receive SNMC.

not positively influence the efficacy of SNMC. Hence, it is valid to compare these two groups of patients for evaluating the efficacy of SNMC in the treatment of chronic hepatitis C.

Figure 5 compares ALT levels of patients with and without SNMC. Despite higher pretreatment ALT levels, the normalization of ALT levels was accomplished significantly more frequently in the patients with SNMC than those without (47 of 181 or 26% vs. 17 of 221 or 8%, $p < 0.001$). Furthermore, liver cirrhosis occurred less frequently in 178 patients on long-term SNMC than in 100 controls (28 vs. 40% at year 13, $p < 0.002$). The most remarkable merit of long-term SNMC was the significantly less frequent development of HCC in the patients with than those without it (fig. 6). The observed differences indicate that a long-term SNMC would be able to decrease the incidence of HCC. These observations confirm our previous results that the incidence of HCC was significantly less frequent in the patients who had received SNMC for 15 years (25 vs. 12%, $p < 0.032$) [15].

The stage of fibrosis found in pretreatment liver biopsies influences the development of HCC in the patients with or without SNMC (fig. 7). A lower incidence of HCC in the patients on SNMC than in those without it holds in all the three groups of patients with initial fibrosis at F1, F2 and F3, respectively. A significant difference in the incidence of HCC at 15 years was achieved in the patients with an initial fibrosis stage of F1 ($p = 0.040$) and F2 ($p = 0.015$). Based on these results, SNMC is effective for preventing HCC in patients with the stage of fibrosis of F2 or lower. Factors influencing the development of HCC were evaluated by multivariate Cox regression analysis (table 3). Only the administration of SNMC [relative risk 0.40 (range 0.16–0.98), $p = 0.044$] and the stage of fibrosis [relative risk 13.8 (range 5.4–34.5), $p < 0.0001$] were found to be significant influential factors.

SNMC is found to be effective in preventing HCC even in high-risk patients who are older than 50 years and with liver histology at stage F3 (fig. 8). HCC developed less frequently in the patients in whom serum ALT levels were suppressed below than in those with levels above 75 IU/l (1.5 times the upper limit of normal). The difference is remarkable, considering that they all were on SNMC; and the patients with higher serum levels of ALT also would have benefited from SNMC.

Taken together, as with most medical interventions the earlier a long-term treatment with SNMC for preventing HCC associated with HCV infection is started the better, and it is the principal goal to keep serum ALT levels low.

Discussion

There are compelling lines of evidence for the efficacy of SNMC in patients with chronic hepatitis C for suppressing necro-inflammation. Long-term treatment with

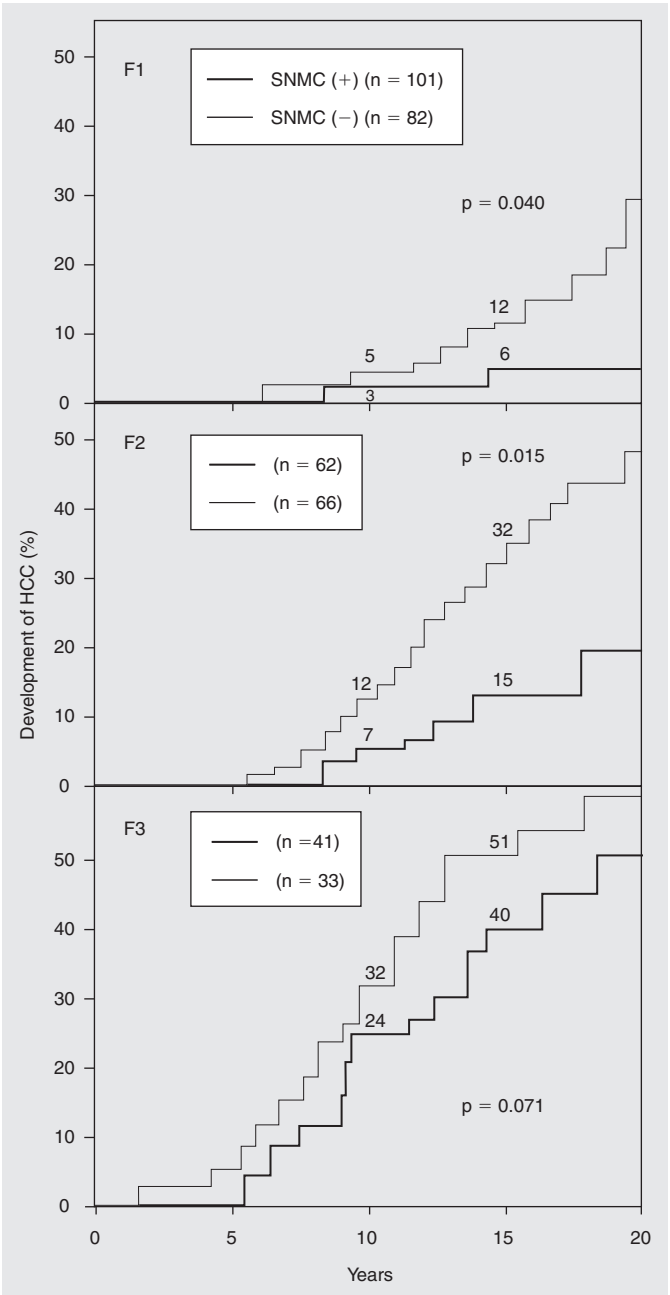

Fig. 7. Development of HCC in patients with chronic hepatitis C at various stages of fibrosis who did or did not receive SNMC.

SNMC, therefore, can prevent the development of HCC. Up until the present, SNMC has been used to this end in China, Indonesia, India, Japan, Taiwan and Korea, and in clinical trials in the Netherlands and Germany [14–21]. The popularity of SNMC in Japan is related to the high incidence rate of HCC associated with HCV infection, which is increasingly coming to the fore, and on which many Japanese researchers focus their efforts.

Table 3. Relative risk for the development of HCC

Features	Relative risk	p value
Fibrosis: F2/F3 vs. F1	13.8 (5.4–34.5)	<0.0001
Glycyrrhizin: (+) vs. (–)	0.40 (0.16–0.98)	0.044
Age: ⩾ 50 vs. <50		NS
Sex: Male vs. female		NS
Mean ALT: ⩾100 vs. <100		NS
Mean ICG_{15}%: ⩾10 vs. <10		NS
Transfusion: (+) vs. (–)		NS
HCV genotypes: 1b vs. 2a/2b	0.40 (0.16–0.98)	NS

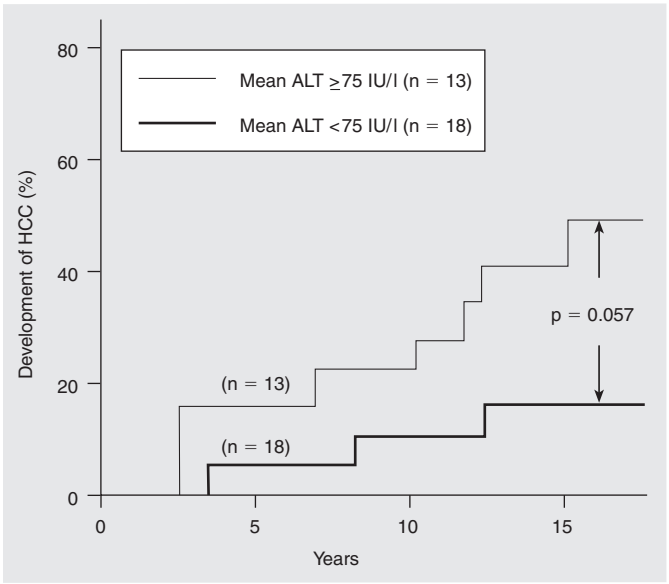

Fig. 8. Development of HCC in high-risk patients with chronic hepatitis C who received long-term SNMC. All the patients were older than 50 years and had liver histology at stage F3. The patients with mean ALT ⩾ 75 IU/l were compared with those with mean ALT < 75 IU/l.

Suzuki et al. [14] compared the efficacy of SNMC at a daily dose of 40 ml with placebo. Initially, we started treating patients with 40 ml of SNMC, and increased the dose to 100 ml per day every day. I believed that the maximal effect of SNMC would be achieved by a large dose and high frequency for maintaining effective circulating levels, provided that such a regimen does not bring about serious side effects. Actually, side effects of SNMC such as pseudo-aldosteronism are infrequent, predictable and readily treated [22]. Iino et al. [17] conducted a meticulous randomized trial, and have shown scientifically that the patients who fail to respond to 40 ml of SNMC can still benefit by receiving 100 ml of SNMC. Likewise, the superiority of SNMC 6 times in week (t.i.w.) to 3 t.i.w.

has been reproduced in the Netherlands by the group of Schalm and van Rossum [20].

The therapeutic schedule of SNMC should aim at suppressing ALT levels below 1.5 times the upper limit of normal. In our practice, we prescribe 40 ml of SNMC 5–6 t.i.w. for 4 weeks, and when the ALT level is lowered below 1.5 times the upper limit of normal, we reduce it to 3 t.i.w. If this initial attempt fails, the dose is increased to 100 ml per day 5–6 t.i.w. until patients respond.

Intravenous SNMC at a dose of 40 ml per day has been approved by the Japanese health insurance policy since 1979. Hino et al. [16] including myself conducted a controlled clinical trial with an increased dose of 100 ml to evaluate the efficacy of SNMC in improving histology. SNMC at this dose is effective in ameliorating histological changes in chronic hepatitis C. Taken along with the results of Iino et al. [17], a large dose of SNMC of 100 ml was approved by the Japanese health insurance policy in 1994.

In summary, SNMC can suppress necro-inflammation in chronic hepatitis C. Long-term treatment with SNMC, therefore, would be able to prevent liver cirrhosis and the development of HCC. Furthermore, SNMC is much cheaper than any commercial products of interferon on the market and has few untoward effects. Hence, the indication of SNMC will be extended to patients with chronic liver disease in many more countries. We are facing the reality that interferon combined with or without ribavirin can save at most half of the patients with chronic hepatitis C [1]. SNMC is useful for the patients who do not respond to interferon/ribavirin or who cannot be treated with them.

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Protective Mechanism of Glycyrrhizin on Acute Liver Injury Induced by Carbon Tetrachloride in Mice

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Glycyrrhizin is the major active component extracted from licorice (*Glycyrrhiza glabra*) roots, one of the most widely used herbal preparations for the treatment of liver disorders. This study evaluated the potential beneficial effect of glycyrrhizin in a mouse model of carbon tetrachloride (CCl_4)-induced liver injury. The mice were treated intraperitoneally with CCl_4 (0.5 ml/kg). They received glycyrrhizin (50, 100, 200, 400 mg/kg) 24 h and 0.5 h before and 4 h after administering CCl_4. The serum activities of aminotransferase and the hepatic level of malondialdehyde were significantly higher 24 h after the CCl_4 treatment, while the concentration of reduced glutathione was lower. These changes were attenuated by glycyrrhizin. CCl_4 increased the level of circulating tumor necrosis factor-α markedly, which was reduced by glycyrrhizin. The levels of hepatic inducible nitric oxide synthase, cyclooxygenase-2, and heme oxygenase-1 protein expression were markedly higher after the CCl_4 treatment. Glycyrrhizin diminished these alterations for inducible nitric oxide and cyclooxygenase-2 but the protein expression of heme oxygenase-1 was further elevated by the treatment of glycyrrhizin. CCl_4 increased the level of *tumor necrosis factor-α*, *inducible nitric oxide synthase*, *cyclooxygenase-2*, and *heme oxygenase-1* mRNA expressions. The mRNA expression of *heme oxygenase-1* was augmented by the glycyrrhizin treatment, while glycyrrhizin attenuated the increase in *tumor necrosis factor-α*, *inducible nitric oxide synthase*, and *cyclooxygenase-2* mRNA expressions. These results suggest that glycyrrhizin alleviates CCl_4-induced liver injury, and this protection is likely due to the induction of heme oxygenase-1 and the downregulation of proinflammatory mediators.

Key words carbon tetrachloride; glycyrrhizin; heme oxygenase-1; hepatoprotective activity; oxidative stress; proinflammatory mediator

Acute and chronic liver diseases constitute a global concern, and the medical treatments for these diseases are often difficult to handle and have limited efficacy. Therefore, there has been considerable interest in the role of complementary and alternative medicines for the treatment of liver diseases.[1] Developing therapeutically effective agents from natural products may reduce the risk of toxicity when the drug is used clinically.

Carbon tetrachloride (CCl_4) is a well-known hepatotoxin that is widely used to induce toxic liver injury in a range of laboratory animals. CCl_4-induced hepatotoxicity is believed to involve two phases. The initial phase involves the metabolism of CCl_4 by cytochrome P450 to the trichloromethyl radical ($CCl_3\cdot$), which leads to lipid peroxidation.[2] Heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme catabolism, is known to be induced by oxidative stress and to confer protection against oxidative stress injuries.[3] The second phase of CCl_4-induced hepatotoxicity involves the activation of Kupffer cells, which is accompanied by the production of proinflammatory mediators.[4] Several microarray studies have been reported describing gene expression changes caused by acute CCl_4 toxicity,[5] although the significance of these changes has not been fully understood.

Licorice, the root of *Glycyrrhiza glabra*, is one of the oldest and most commonly prescribed herbs in Eastern traditional medicine, and has been used to treat tuberculosis, peptic ulcers, and liver injury in a number of clinical disorders.[6] Glycyrrhizin is a major active constituent isolated from licorice that scavenges reactive oxygen species (ROS) and has an anti-inflammatory action.[7,8] A recent report suggested that glycyrrhizin also inhibits anti-Fas antibody-induced hepatitis by acting upstream of the activation of CPP32-like protease.[9] However, there is limited information available on the *in vivo* hepatoprotective effect of glycyrrhizin.

This study investigated the effect of glycyrrhizin on acute hepatic injury, the specific molecular mechanisms of protection, and the effect of glycyrrhizin on both hepatic oxidative stress and inflammation.

MATERIALS AND METHODS

Isolation and Purification of Glycyrrhizin The dried roots of *Glycyrrhiza glabra* from the Shenyang province of China were purchased from Kwanglim Co. (Daegu, Korea) and were authenticated by Dr. J. H. Lee, an Oriental medicine specialist. A voucher specimen (#06-04-0002) was deposited at the College of Pharmacy, Yeungnam University, Korea. The roots of *Glycyrrhiza glabra* (10 kg) were extracted with methanol (50 l) at room temperature. The methanol extract was evaporated under reduced pressure to obtain a residue (2.6 kg), which was then dissolved in water (3.5 l) and partitioned with methylene chloride (3.5 l×3). The methylene chloride soluble fraction (230 g) was chromatographed on silica gel (6.2 kg), with gradient elution using *n*-hexane/ethyl acetate mixtures (100 : 0, 98 : 2, 95 : 5, 90 : 10, 85 : 15, 80 : 20, 5 l for each gradient) to give sixteen fractions (G01—G16). Fraction G04 (3200 ml, *n*-hexane–ethyl acetate, 98 : 2) was purified by crystallization from cold methanol to yield glycyrrhizin (450 mg), which was subjected to analytical HPLC (25—75 μm, 5 mm i.d.×20 cm, Shim-pack ANAL-ODS column; Shimadzu, Japan) with elution by methanol–water–acetic acid (65 : 34 : 1 (v/v), 1 ml/min) to afford its purity

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(>95%) and retention time (26 min). The structure of glycyrrhizin was identified with an authentic sample by comparing their NMR and MS spectral data.[10,11]

Animals and Treatment Regimens Male ICR mice weighing 25—30 g were fasted overnight but given tap water *ad libitum*. All the animals were treated humanely under the Sungkyunkwan University Animal Care Committee Guidelines. The animals were randomly assigned to 7 groups containing 8 animals per group. The mice in group I (control) received only olive oil (10 ml/kg, i.p.). In groups II to VII, CCl_4 was dissolved in olive oil (1 : 19, v/v) and administered intraperitoneally (final concentration; 0.5 ml/kg). The animal groups I and II (vehicle) were treated intraperitoneally with saline (10 ml/kg). The animals in groups III to VI were treated intraperitoneally with glycyrrhizin (50, 100, 200, 400 mg/kg), and the animals in group VII were treated with silymarin (positive control, 200 mg/kg, i.p.), 24 h and 0.5 h before and 4 h after administering CCl_4. The dose and timing of the glycyrrhizin treatment were selected based on previous reports,[12] as well as its efficacy in a retrorsine-induced hepatotoxicity model[13] and an anti-Fas antibody-induced mice hepatitis model.[9] Blood was collected from the abdominal aorta 24 h after CCl_4 administration. The liver was isolated and used immediately to prepare the mRNA, and was stored at −75 °C for later analysis, except for the part in the left lobe, which was used for histological analysis.

Assessment of Serum Aminotransferase Activities The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using a Hitachi 747 automatic analyzer (Hitachi, Tokyo, Japan).

Determination of Hepatic Lipid Peroxidation and Glutathione Contents The steady-state level of malondialdehyde (MDA), a lipid peroxidation end product, was analyzed by measuring the level of thiobarbituric acid reactive substances (TBARS) spectrophotometrically at a wavelength of 535 nm, according to the method reported by Buege and Aust[14] using 1,1,3,3-tetraethoxypropane (Sigma, St. Louis, MO, U.S.A.) as the standard. The total glutathione level was measured spectrophotometrically at a wavelength of 412 nm, with yeast glutathione reductase, 5,5′-dithio-bis(2-nitrobenzoic acid), and NADPH, according to the methodology reported by Tietze.[15] The oxidized glutathione (GSSG) level was measured using the same method in the presence of 2-vinylpyridine,[16] and the reduced glutathione (GSH) level was determined by the difference between the total glutathione and the GSSG levels.

Histological Analysis Twenty-four hours after administering CCl_4, a small piece of liver tissue from the anterior portion of the left lateral lobe was removed for histological analysis. The sample was fixed by immersing it in 10% neutral-buffered formalin. The sample was then embedded in paraffin, sliced into 5-μm sections, and stained with hematoxylin–eosin for a blinded histological assessment. The degree of portal inflammation, hepatocellular necrosis, and inflammatory cell infiltration was evaluated semiquantitatively according to the method reported by Frei *et al.*[17] The stained 5-μm sections were graded as follows: 0, absent; I; minimal; II, mild; III, modest; and IV, severe. The histological changes were evaluated in nonconsecutive, randomly chosen ×200 histological fields.

Measurement of Serum Tumor Necrosis Factor-α (TNF-α) Levels The serum TNF-α level was quantified using enzyme-linked immunosorbent assay (ELISA) with a commercial mouse TNF-α ELISA kit (eBioscience, San Diego, CA, U.S.A.) according to the manufacturer's instructions.

Western Blot Immunoassay Freshly isolated liver tissue was homogenized in a lysis buffer. In order to determine the level of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and heme oxygenease-1 (HO-1) protein expression, 10 μg of protein samples from the liver homogenates were loaded per lane on 10% polyacrylamide gels. The protein samples were then separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to nitrocellulose membranes using a semi-dry transfer process. After transfer, the membranes were washed with Tris buffered saline (TBS) and blocked for 1 h at room temperature with 5% (w/v) skim milk powder in TBS. The blots were then incubated overnight at 4 °C with the polyclonal antibodies against mouse iNOS (Transduction Laboratories, San Jose, CA, U.S.A.; 1 : 1000 dilution), COX-2 (Cayman, Ann Arbor, MI, U.S.A.; 1 : 1000 dilution), and HO-1 (Transduction Laboratories, San Jose, CA, U.S.A.; 1 : 1000 dilution), and with the monoclonal antibodies against mouse β-actin (Sigma, St. Louis, MO, U.S.A.; 1 : 10000 dilution). On the next day, the primary antibody was removed and the blots were washed thoroughly with T-TBS (0.05% Tween 20 in TBS). The binding of all the antibodies was detected using an ECL detection system (iNtRON Biotechnology Co., Ltd., Korea), according to the manufacturer's instructions. The visualized immunoreactive bands were evaluated densitometrically with ImageQuant™ TL software version 2005 (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Total RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR) The total RNA was extracted using the method reported by Chomczynski and Sacchi.[18] Reverse transcription of the total RNA extracted from the tissue samples was carried out in order to synthesize the first strand cDNA using the oligo$(dT)_{12-18}$ primer and SuperScript™ II RNase H^- Reverse Transcriptase (Invitrogen Tech-Line™, Carlsbad, CA, U.S.A.). The PCR reaction was carried out with a diluted cDNA sample and was amplified in a 20 μl reaction volume. The final reaction concentrations are as follows: primers, 10 pmol; dNTP mix, 250 μM; ×10 PCR buffer; and Ex *Taq* DNA polymerase, 0.5 U per reaction. RT-PCR was carried out with an initial denaturation step at 94 °C for 5 min and a final extension step at 72 °C for 7 min using GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA, U.S.A.). The amplification cycling conditions are as follows: for *TNF-α*, 28 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s; for *iNOS*, 35 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s; for *COX-2*, 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; for *HO-1*, 30 cycles, and for *β-actin*, 25 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. After RT-PCR, 10 μl samples of the PCR products were visualized by ultraviolet illumination after electrophoresis through 1.5% agarose gel and ethidium bromide staining. The intensity of each PCR product was analyzed semiquantitatively using a digital camera (DC120, Eastman Kodak, New Haven, CT, U.S.A.) and analyzing software.

Statistical Analysis The overall significance of the results was examined using one-way analysis of variance (ANOVA). The differences between the groups were considered statistically significant at a p value <0.05 with the appropriate Bonferroni correction made for multiple comparisons. The results are presented as a mean±S.E.M.

RESULTS

Serum Aminotransferase Activities The serum levels of ALT and AST in the control animals were 66.6±5.5 and 128.0±14.3 U/l, respectively. 24 h after the CCl_4 treatment, the serum ALT and AST levels increased to approximately 174.7 and 94.1 times that in the control animals, respectively. Glycyrrhizin, at the doses of 200 mg/kg and 400 mg/kg, significantly reduced these increases. The ALT and AST activities were also decreased in the silymarin-treated group compared with the vehicle-treated CCl_4 group (Fig. 1).

Lipid Peroxidation and Hepatic Glutathione Contents The administration of CCl_4 increased the hepatic level of MDA to approximately 1.3 times that of the control animals. This elevation was attenuated by 200 mg/kg of either glycyrrhizin or silymarin. The GSH level in the control animals was 6.3±0.4 μmol/g liver. The GSH content decreased significantly 24 h after CCl_4 administration but was markedly attenuated by 200 mg/kg glycyrrhizin. Hepatic GSSG concentration was unchanged among any of the experimental groups. The ratio of GSH to GSSG, an indicator of the hepatocellular redox state, markedly declined after the CCl_4 treatment. The decrease in the ratio of GSH to GSSG was attenuated by glycyrrhizin (Table 1).

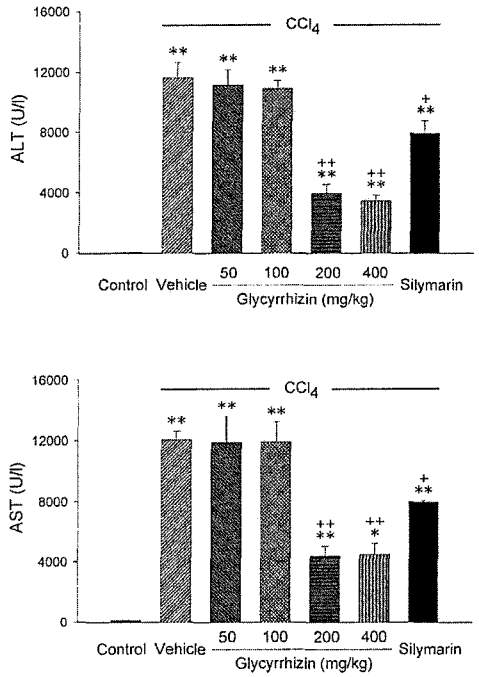

Fig. 1. Effect of Glycyrrhizin on the Serum Aminotransferase Activity after CCl_4 (0.5 ml/kg) Administration

The results are presented as the mean±S.E.M. of 8 animals per group. *, ** Denotes significant differences from the control group ($p<0.05$ and $p<0.01$); +, ++ denotes significant differences from the vehicle-treated CCl_4 group ($p<0.05$ and $p<0.01$).

Histological Analysis The histological features shown in Fig. 2 and Table 2 show a normal liver lobular architecture and cell structure of the livers in the control animals. However, the livers exposed to CCl_4 showed multiple and extensive areas of portal inflammation and hepatocellular necrosis, randomly distributed throughout the parenchyma, as well as a moderate increase in inflammatory cell infiltration. These pathological changes were inhibited by glycyrrhizin and silymarin at the doses of 200 mg/kg.

Serum TNF-α Levels The serum levels of TNF-α were low in the control animals. However, in the CCl_4-treated animals, the serum level increased 2.3-fold 24 h after the CCl_4 treatment. This increase was reduced by glycyrrhizin (200 mg/kg). Glycyrrhizin treatment alone did not affect the serum levels of TNF-α (Fig. 3).

iNOS, COX-2, and HO-1 Protein Expression The amount of iNOS, COX-2, and HO-1 protein in the livers increased markedly 24 h after CCl_4 administration. The increases in iNOS and COX-2 protein levels were significantly attenuated by glycyrrhizin, while the level of HO-1 protein expression was further elevated by the treatment of glycyrrhizin. Glycyrrhizin treatment alone did not alter the protein level of iNOS, COX-2, and HO-1 (Fig. 4).

***TNF-α, iNOS, COX-2,* and *HO-1* mRNA Expression** As shown in Fig. 5, the levels of *TNF-α*, *iNOS*, *COX-2*, and *HO-1* mRNA in the CCl_4 group were 4.5-, 3.2-, 5.0-, and 1.7-fold higher than the control level, respectively. The increase in *TNF-α*, *iNOS*, and *COX-2* mRNA levels were significantly suppressed by glycyrrhizin, while the level of *HO-1* mRNA expression was augmented by the glycyrrhizin treatment. The mRNA expression of *TNF-α*, *iNOS*, *COX-2*, and *HO-1* was unaffected by the glycyrrhizin treatment itself.

DISCUSSION

In this study, the protective effect of glycyrrhizin was ex-

Table 1. Effect of Glycyrrhizin on Lipid Peroxidation and Glutathione Contents in the Liver after CCl_4 (0.5 ml/kg) Administration

Groups	Dose (mg/kg)	MDA (nmol/g liver)	GSH (μmol/g liver)	GSSG (μmol/g liver)	GSH/GSSG ratio
Control		37.8±1.9	6.3±0.4	0.3±0.1	15.6±0.9
CCl_4					
Vehicle		49.1±1.1**	3.4±0.3**	0.4±0.0	9.8±0.7*
Glycyrrhizin	50	49.3±0.5**	3.8±1.1	0.3±0.1	10.5±1.1
	100	50.0±1.4**	3.6±0.6*	0.2±0.0	11.3±2.5
	200	43.9±1.1*,†	5.6±0.4††	0.3±0.0	14.5±3.6
	400	47.2±2.0**	4.3±1.0	0.2±0.0	15.3±1.2†
Silymarin	200	41.3±1.9††	3.8±1.2	0.4±0.1	9.1±0.9

The results are presented as a mean±S.E.M. for 8 animals per group. *,** Denotes significant differences from the control group, $p<0.05$ and $p<0.01$, respectively; †,†† denotes significant differences from the vehicle-treated CCl_4 group, $p<0.05$ and $p<0.01$, respectively.

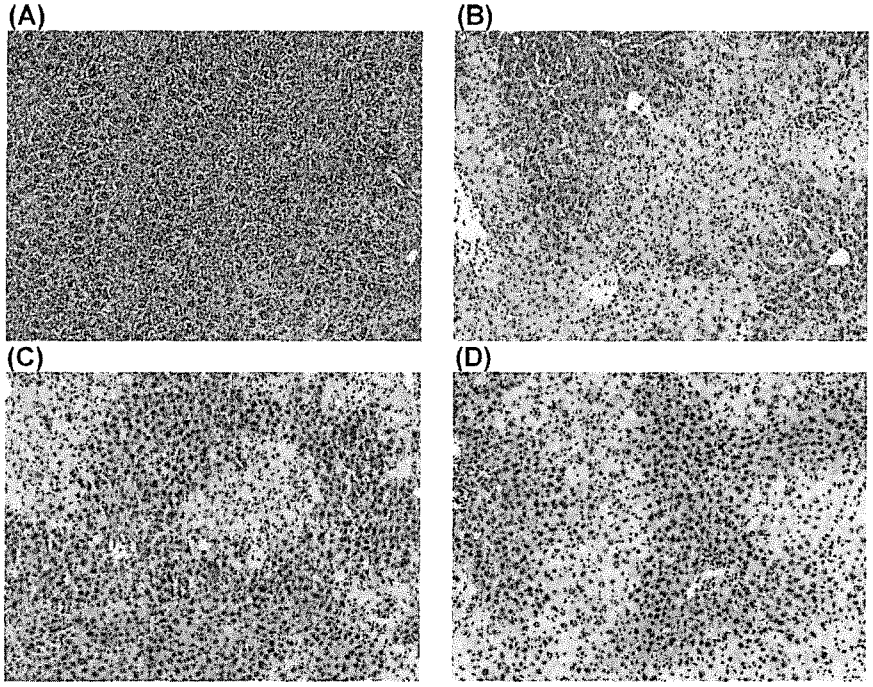

Fig. 2. Histological Analysis of the Livers after CCl_4 (0.5 ml/kg) Administration

Typical images were chosen from the different experimental groups (original magnification ×100). (A) Control group: normal lobular architecture and cell structure; (B) vehicle-treated CCl_4 group: multiple and extensive areas of portal inflammation and hepatocellular necrosis, and a moderate increase in inflammatory cell infiltration; (C) CCl_4 and glycyrrhizin (200 mg/kg)-treated group: minimal hepatocellular necrosis and inflammatory cell infiltration, and mild portal inflammation; and (D) CCl_4 and silymarin (200 mg/kg)-treated group: minimal portal inflammation and inflammatory cell infiltration, and mild hepatocellular necrosis.

Table 2. Quantitative Summary of the Histological Observations on Glycyrrhizin-Dependent Protection of CCl_4-Induced Hepatic Damage

Histopathologic grading	Control	CCl_4		
		Vehicle	Glycyrrhizin (200 mg/kg)	Silymarin (200 mg/kg)
Portal inflammation				
Grade 0	7	0	0	0
Grade I	1	0	1	5
Grade II	0	1	5	3
Grade III	0	5	2	0
Grade IV	0	2	0	0
Hepatocellular necrosis				
Grade 0	6	0	1	0
Grade I	2	0	6	3
Grade II	0	0	1	3
Grade III	0	6	0	2
Grade IV	0	2	0	0
Inflammatory cell infiltration				
Grade 0	8	0	5	3
Grade I	0	4	3	4
Grade II	0	4	0	1
Grade III	0	0	0	0
Grade IV	0	0	0	0

Liver samples were isolated 24 h after administering the CCl_4 (0.5 ml/kg, i.p.). The samples were fixed in 10% neutral-buffered formalin prior to paraffin-embedding, and stained with hematoxylin and eosin. The histological changes were graded according to the following criteria: 0, absent; I, minimal; II, mild; III, modest; and IV, severe. 8 samples per group.

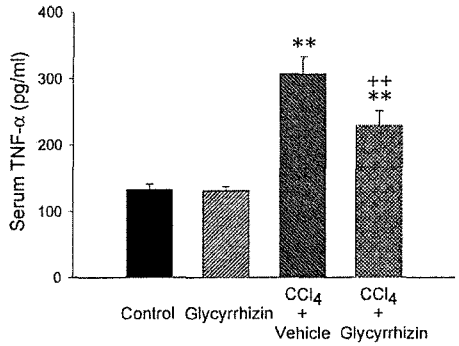

Fig. 3. Effect of Glycyrrhizin (200 mg/kg) on Serum TNF-α Secretion after CCl_4 (0.5 ml/kg) Administration

The results are presented as the mean±S.E.M. for 8 animals per group. ** Denotes significant differences from the control group ($p<0.01$); ++ denotes significant differences from the vehicle-treated CCl_4 group ($p<0.01$).

amined using a model of CCl_4-induced hepatotoxicity. The susceptibility of the liver to chemical injury is as much a function of its anatomical proximity to the bloodstream and gastrointestinal tract as to its ability to biotransform and concentrate xenobiotics. CCl_4-induced liver injury in a range of laboratory animals is considered to be an analogue of the liver damage caused by various hepatotoxins in humans.[19]

In the vehicle-treated CCl_4 group, the ALT and AST levels increased dramatically compared with the control group, indicating severe hepatocellular damage. In contrast, a treatment with 200 and 400 mg/kg of glycyrrhizin markedly attenuated the release of ALT and AST. Furthermore, the hepatoprotective effect of glycyrrhizin appeared to be higher than that of silymarin, which is used as a potent hepatoprotective agent. The histological observations of the liver samples strongly support the release of aminotransferases by the

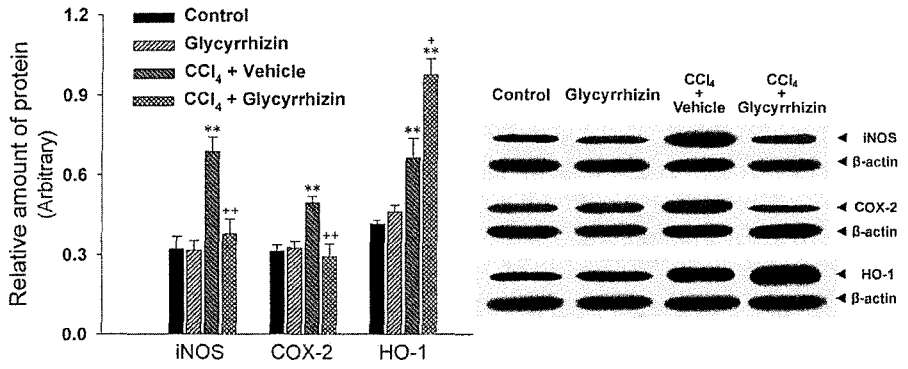

Fig. 4. Effect of Glycyrrhizin (200 mg/kg) on iNOS, COX-2, and HO-1 Protein Expression after CCl_4 (0.5 ml/kg) Administration

The results are presented as a mean±S.E.M. for 8 animals per group. ** Denotes significant differences from the control group ($p<0.01$); +, ++ denotes significant differences from the vehicle-treated CCl_4 group ($p<0.05$ and $p<0.01$).

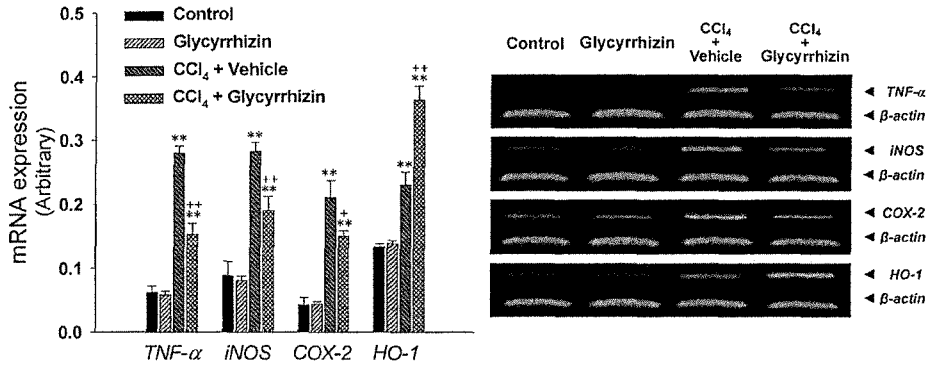

Fig. 5. Effect of Glycyrrhizin (200 mg/kg) on *TNF-α*, *iNOS*, *COX-2*, and *HO-1* mRNA Expression after CCl_4 (0.5 ml/kg) Administration

The results are presented as a mean±S.E.M. for 8 animals per group. ** Denotes significant differences from the control group ($p<0.01$); +, ++ denotes significant differences from the vehicle-treated CCl_4 group ($p<0.05$ and $p<0.01$).

damaged hepatocytes as well as the protective effect of glycyrrhizin (Fig. 2, Table 2). CCl_4 caused various histological changes to the liver, including cell necrosis, fatty metamorphosis in the adjacent hepatocyte, ballooning degeneration, cell inflammation, and the infiltration of lymphocytes and Kupffer cells. These alterations were significantly attenuated by glycyrrhizin with the livers showing only minor hepatocellular necrosis and inflammatory cell infiltration, and mild portal inflammation. These results suggest that glycyrrhizin may have potential clinical applications for treating liver disorders.

In contrast to other known hepatotoxins, CCl_4 is not toxic *per se* but is responsible for oxidative stress and lipid peroxidation through the cytochrome P450-mediated generation of the highly reactive $CCl_3\cdot$, leading to eventual cellular damage characterized by hepatocellular necrosis.[20] The subsequent chloromethylation, saturation, peroxidation, and the progressive destruction of the unsaturated fatty acids of the membrane phospholipids are collectively known as lipid peroxidation, which leads to a functional and structural disruption.[21] The hepatoprotective effect of glycyrrhizin can also be ascribed to the suppression of lipid peroxidation as well as its propagation in the liver because glycyrrhizin at a dose of 200 mg/kg could attenuate the CCl_4-induced increase in the hepatic MDA content. Another study suggested that glycyrrhizin may provide the maximum conjugation with detrimental free radicals and deprive them of their toxic properties.[22] A more definitive characterization of CCl_4-induced oxidative stress was evidenced by the decrease in the level of hepatic GSH. The GSH system acts as a major antioxidant defense mechanism against the toxic effects of free radicals.[23] These results suggest that CCl_4 causes direct cellular damage through thiol oxidation and subsequent lipid peroxidation. Moreover, the glycyrrhizin treatment attenuated the lipid peroxidation and decrease in the hepatic GSH content, which suggests that glycyrrhizin increases the hepatic pool of GSH and reduces oxidative stress.

Excessive oxidative stress has been suggested as a reason for the upregulation of HO-1, as this enzyme is known to be readily inducible upon such stressors.[24] HO-1 is a rate-limiting enzyme in the catabolism of heme and a heat shock protein (HSP32). By the equimolar production of the antioxidant bilirubin, free iron, and vasodilative carbon monoxide, HO-1 represents a cytoprotective enzyme and, when expressed, produces therapeutic benefits in a number of different conditions and diseases, such as sepsis, inflammation, and ischemia/reperfusion injury.[25] In line with this, HO-1 induction has been shown to confer protection in CCl_4-induced hepatotoxicity, as assessed by the measurements of liver transaminase levels and cytological examination of liver histology.[3] In our study, the expression of HO-1 protein was significantly increased after the CCl_4 treatment. This is in concordance to the results reported by Nakahira *et al.*[3]; following CCl_4 treatment, hepatic HO-1 expression was

markedly increased both at transcriptional and protein levels in hepatocytes, especially around the central vein. Additionally, treatment with glycyrrhizin markedly augmented HO-1 protein expression after CCl_4 treatment, which suggests that a strong induction response of HO-1 by glycyrrhizin is to protect liver cells from CCl_4-induced oxidative cellular injuries.

The liver is a major inflammatory organ, and inflammatory processes contribute to a number of pathological events after exposure to various hepatotoxins. Kupffer cells release proinflammatory mediators either in response to necrosis or as a direct action by the hepatotoxin, activated, which are believed to aggravate CCl_4-induced hepatic injury.[26] TNF-α, a pleiotropic proinflammatory cytokine, is rapidly produced by macrophages in response to tissue damage.[27] While low levels of TNF-α may play a role in cell protection, excessive amounts cause cell impairment. An increase in the TNF-α level has been directly correlated with the histological evidence of hepatic necrosis and the increase in the serum aminotransferase levels.[28] DeCicco *et al.*[29] have reported the stimulation of TNF-α production in both serum and liver following CCl_4 administration, and it is suggested that $CCl_3\cdot$ activates Kupffer cells to release TNF-α. TNF-α also stimulates the release of cytokines from macrophages and induces the phagocyte oxidative metabolism and nitric oxide production.[30] Nitric oxide is a highly reactive oxidant that is produced through the action of iNOS, and plays a role in a number of physiological processes, such as, vasodilation, neurotransmission, and nonspecific host defense.[31] Nitric oxide can also exacerbate oxidative stress by reacting with reactive oxygen species, particularly with the superoxide anion, and forming peroxynitrite.[32] As nitric oxide has a range of effects on a variety of biological processes, it is unclear if it is beneficial or detrimental in the liver injury induced by hepatotoxins. This study confirmed a significant increase in the serum TNF-α level and iNOS protein expression in the liver after CCl_4 administration. These alterations were attenuated by the glycyrrhizin treatment, which suggests that glycyrrhizin suppresses the TNF-α and iNOS protein secretion and/or enhances the degradation of their protein.

Previous studies reported that the induction of cyclooxygenase in inflammatory response is the secondary effect of CCl_4-induced hepatotoxicity.[33] COX-2 is the mitogen-inducible isoform of cyclooxygenase and is induced in macrophages by several proinflammatory stimuli, such as cytokines and growth factors, leading to COX-2 expression and the subsequent release of prostaglandins.[4] Arachidonic acid is a well-known substrate of cyclooxygenases or lipoxygenases that is metabolized to produce a variety of proinflammatory substrates called eicosanoids, and COX-2 is the key enzyme in the cascade. Free radical mediated oxidative stress or lipid peroxidation can further activate cyclooxygenases and the subsequent prostaglandin formation from arachidonic acid. The results of this study showed an increase in the expression of COX-2 protein after CCl_4 administration. Glycyrrhizin markedly attenuated this increase, suggesting a suppression of inflammatory responses.

Recent developments in genomic technology have led to new investigations into the changes in gene expression caused by an acute treatment with CCl_4. The acute administration of CCl_4 to rats caused significant changes in the gene expression profiles.[34] The most notable changes in the CCl_4-treated animals were the expression of the genes involved in stress, DNA damage, cell proliferation, and metabolic enzymes.[35] These gene expression profiles have catalogued the molecular responses to acute CCl_4 toxicity and revealed the genetic basis of hepatic toxicity. In this study, it was observed that the levels of *TNF-α*, *iNOS*, *COX-2*, and *HO-1* mRNA expression were increased significantly by the acute CCl_4 treatment. Glycyrrhizin attenuated the increase in *COX-2* mRNA, and the level of *HO-1* mRNA expression was augmented by glycyrrhizin treatment. This indicates that although posttranscriptional modifications may occur, COX-2 and HO-1 are controlled primarily at the level of transcription in response to an acute dose of CCl_4. However, glycyrrhizin slightly attenuated the increase in *TNF-α* and *iNOS* mRNA expression, which was not well correlated with the levels of their proteins. Therefore, glycyrrhizin may largely regulate the TNF-α and iNOS production by the posttranscriptional level. Additional studies are required to examine this effect in further detail.

These results provide evidence for the pharmacological effect of glycyrrhizin in CCl_4-induced hepatotoxicity. Overall, glycyrrhizin not only provides maximum conjugation with injurious free radicals and diminishes their toxic properties but also suppresses the inflammatory responses of a CCl_4-induced liver injury. Further studies will be needed to fully understand the association between oxidative stress and the inflammatory responses in the hepatoprotective effect of glycyrrhizin against CCl_4-induced hepatotoxicity.

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Review Article

Glycyrrhizic Acid in the Treatment of Liver Diseases: Literature Review

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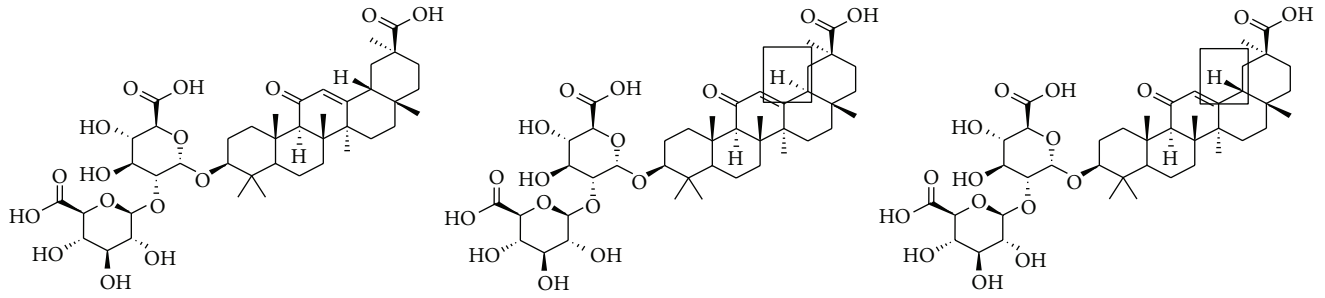
Glycyrrhizic acid (GA) is a triterpene glycoside found in the roots of licorice plants (*Glycyrrhiza glabra*). GA is the most important active ingredient in the licorice root, and possesses a wide range of pharmacological and biological activities. GA coupled with glycyrrhetinic acid and 18-beta-glycyrrhetic acid was developed in China or Japan as an anti-inflammatory, antiviral, and antiallergic drug for liver disease. This review summarizes the current biological activities of GA and its medical applications in liver diseases. The pharmacological actions of GA include inhibition of hepatic apoptosis and necrosis; anti-inflammatory and immune regulatory actions; antiviral effects; and antitumor effects. This paper will be a useful reference for physicians and biologists researching GA and will open the door to novel agents in drug discovery and development from Chinese herbs. With additional research, GA may be more widely used in the treatment of liver diseases or other conditions.

1. Introduction

The application of natural compounds in the treatment of refractory diseases is a new trend in modern clinical medicine. Because of their satisfactory efficacy in clinic and low toxicity, more natural products are being used as alternative treatments for many diseases. Many hepatoprotective monomers are derived from natural herbs, especially those from China. Glycyrrhizic acid (GA) is an example of one of these hepatoprotective compounds.

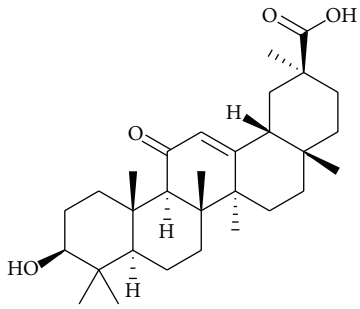
The traditional Chinese medicine Gancao (licorice root) is the dried roots of *Glycyrrhiza uralensis* Fisch (licorice), *G. inflata* Bat., or *G. glabra* L. Gancao which was first described in the Chinese book "Shen Nong Ben Cao Jing" in 200 A.D. as an antidote to toxic substances, ache, and other diseases. Gancao can complement other drugs to reduce toxicity and increase efficacy. The traditional use of Gancao involves a decoction of dried plant roots and stems. Some of the possible therapeutic properties of Gancao include antiarthritic [1], antiallergic [2], antiviral [3], antihepatotoxic [4], anticholinergic [5], antiestrogenic [6], anti-inflammatory [6], antileukemogenic [7], and anticarcinogenic effects [8]. It is commonly used for the treatment of acute and chronic liver injury, viral hepatitis, hepatic steatosis, liver fibrosis, hepatoma, viral myocarditis [9], and other diseases like psoriasis [10] or prostate cancer [11].

The known chemical components of Gancao include saponins (mainly glycyrrhizin (GA), 3.63–13.06%), flavonoids (1.5%), coumarin, alkaloids, polysaccharides, sitosterol, and amino acids [12]. GA (Figure 1) and glycyrrhetinic acid (Figure 2) are well-characterized components of Gancao. GA has been developed as a hepatoprotective drug in China and Japan. GA can generate glycyrrhetinic acid through metabolic processes in the human body. Therefore, the pharmacological effects of GA are essentially the same as glycyrrhetinic acid [13]. GA, also called glycyrrhizin, is a triterpene glycoside from licorice root (*Glycyrrhiza glabra*) and consists of one molecule of 18β-glycyrrhetinic acid and two



Glycyrrhizin $C_{42}H_{61}O_{16}NH_4$ Alpha-glycyrrhizin $C_{42}H_{61}O_{16}NH_4$ Beta-glycyrrhizin $C_{42}H_{61}O_{16}NH_4$

Figure 1: Chemical structure of glycyrrhizin (GA) and its derivatives.



Glycyrrhetinic acid $C_{30}H_{46}O_4$

Figure 2: Chemical structure of glycyrrhetinic acid.

molecules of glucuronic acid (18β-glycyrrhetinic acid-3-O-β-D-glucuronopyranosyl-(1 → 2)-beta-D-glucuronide) [14, 15]. Glycyrrhizin is considered to be the major active component of Gancao as demonstrated by studies with experimental animal models [16] and clinical studies [17]. GA has been used clinically for more than 20 years in patients with chronic hepatitis in China and Japan [18] and shows a satisfactory therapeutic effect in many other diseases. GA is also widely used as a sweetening and flavoring agent in food.

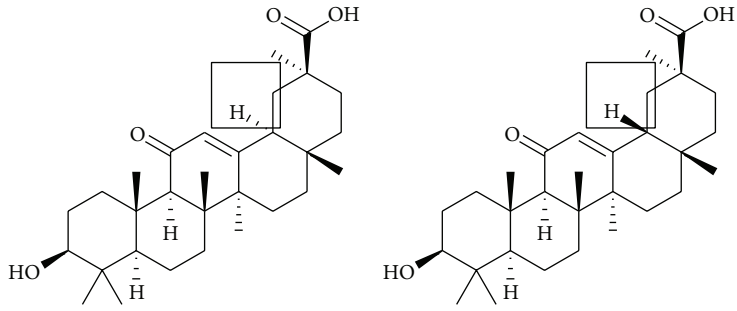
GA is a main substance of licorice, which is one of the most important substances utilized as traditional medicine for almost 2000 years. Moreover, GA was reported to have antiallergic, antiviral, and anti-inflammatory activities. GA was also found to suppress the rise in fasting blood glucose and insulin levels and improve glucose tolerance. Additionally, GA may act as an antidiabetic substance without inducing side effects, although the mechanism is unclear [19].

GA can form two epimers: α-GA and β-GA (Figure 3). α-GA is derived from β-GA by isomerization, and the α- and β-forms differ only in their C_{18}–H–, *trans*-, and *cis*-configuration, respectively. Some scholars examined their distribution characteristics in rat tissue and found that the concentrations of α-GA in the liver and duodenum were significantly higher than those of β-GA after *i.v.* administration. However, the concentrations of α-GA in the other tissues were lower than or similar to those of β-GA and declined rapidly. This indicates that the protective and anti-inflammatory effects of α-GA on the liver may be better than those of β-GA [20].

Several clinical studies reported that GA was efficacious in the treatment of various types of inflammation (mainly in liver [21–30] (Table 1), but also in lung, kidney, intestine, and spinal cord [31]). The most common use of GA is in the treatment of liver disease [32]. GA can reduce steatosis and necrosis of liver cells significantly [33] to inhibit the inter-interstitial inflammation and liver fibrosis and promote cell regeneration. GA has few side effects and is therefore considered to be a drug worth attention and promotion for liver disease.

2. Mechanisms of GA Effects

2.1. Inhibition of Hepatic Apoptosis and Necrosis. Tumor necrosis factor-alpha (TNF-α) is an important cytokine, which is a key mediator of hepatic apoptosis and necrosis in LPS/D-GaAlN-induced liver failure [34]. Plasma TNF-α level is also elevated in patients with chronic hepatitis caused by hepatitis B viral [35] and acute alcoholic hepatitis [36]. Therefore, TNF-α plays a key role in the pathogenesis of not only endotoxin-induced experimental liver injury but also many human liver diseases. Caspase-3 activation is an indicator of almost all apoptosis systems [37]. GA has anti-inflammatory and antiapoptotic effects via suppression of TNF-α and caspase-3 and these are used to explain the hepatoprotective effect of GA (Table 2) [38]. GA also significantly inhibits the release of cytochrome C from mitochondria into the cytoplasm. The anti-inflammatory activity of GA may rely on the inhibition of release of TNF-α, myeloperoxidase activity, and translocation of nuclear factor-κB (NF-κB) into the nuclei. GA also upregulated the expression of proliferating cell nuclear antigen, implying that it might be able to promote regeneration of liver injury [39]. Activated Kupffer cells are involved in ischemia-reperfusion- (I/R-) induced liver injury and high-mobility group box 1 (HMGB1) production. GA was shown to inhibit HMGB1 production by Kupffer cells and prevented I/R-induced liver injury [40]. GA could also alleviate I/R-induced [41] and spinal cord [42] injury via this



18-Alpha-glycyrrhetinic acid $C_{30}H_{46}O_4$ 18-Beta-glycyrrhetinic acid $C_{30}H_{46}O_4$

Figure 3: Chemical structure of 18α-glycyrrhetinic acid and 18β-glycyrrhetinic acid.

mechanism. In addition, GA conjugates free radicals, which might explain the protective action of GA [43]. For example, GA can be an effective chemopreventive agent against lead acetate mediated hepatic oxidative stress in rats because it binds lead [44]. In concanavalin A- (ConA-) induced mouse model, GA alleviated ConA-induced inflammation and fibrosis progression in livers via inhibition of CD4+ T cell proliferation in response to ConA via the Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and phosphoinositide 3-kinase (PI3K)/AKT pathways [45].

2.2. Anti-Inflammation and Immunity Regulation. GA suppressed interleukin-6 (IL-6) and TNF-α production induced by the lipid A moiety of lipopolysaccharides (LPS) in RAW264.7 cells. It inhibited lipid A-induced NF-κB activation in Ba/F3 cells expressing toll-like receptor 4 (TLR4)/myeloid differentiation protein-2 (MD-2), cluster of differentiation 14 (CD14), and bone marrow-derived macrophages (BMMs). GA also inhibited activation of mitogen-activated protein kinase (MAPKs), including JNK, p38 protein, and ERK in BMMs. In addition, GA inhibited NF-κB activation and IL-6 production induced by paclitaxel, a nonbacterial TLR4 ligand. It attenuated the formation of the LPS-TLR4/MD-2 complexes, resulting in inhibition of homodimerization of TLR4. Therefore, GA modulated the TLR4/MD-2 complex at the receptor level, leading to suppression of LPS-induced activation of signaling cascades and cytokine production. This indicates that GA can attenuate inflammatory responses and modulate innate immune responses [46]. Moreover, GA can prevent the activation of signal transducers and activators of transcription-3 (STAT-3), reduce the upregulation of intercellular cell adhesion molecule (ICAM-1) and P-selectin expression, reduce formation of poly(adenosine diphosphate-ribose) (PAR) and nitrotyrosine, and reduce polymorphonuclear neutrophil (PMN) infiltration. Some observations suggest that broad anti-inflammatory activity of GA is mediated by interaction with the lipid bilayer, thereby attenuating receptor mediated signaling [47]. GA inhibited the lytic pathway of the complement system and may prevent tissue injury caused by the membrane attack complex. Therefore, GA could be a potent agent for suppressing complement-dependent tissue injury in autoimmune and inflammatory diseases [48]. GA can suppress systemic inflammatory response syndrome (SIRS) associated anti-inflammatory response manifestation via inhibition of CC chemokine ligand 2 (CCL2) production by PMN. It may also have the potential to inhibit anti-inflammatory response-associated opportunistic infections in critically ill patients with severe SIRS [49]. There are also other studies that indicated the same anti-inflammatory mechanisms of GA [50].

2.3. Antiviral Effects. The antiviral mechanisms of GA mainly include the inhibition of viral replication and immunity regulation. GA affects cellular signaling pathways such as protein kinase C and casein kinase II and transcription factors such as activator protein 1 and NF-κB. Furthermore, nitrous oxide (NO) inhibits replication of several viruses like Japanese encephalitis virus 4 (a member of the Flaviviridae family), which can also be inhibited by GA. The powerful anti-inflammatory capabilities of GA make it effective in the treatment of various types of hepatitis like viral hepatitis and nonalcoholic hepatitis. GA was found to inhibit the replication of the SARS-associated virus [51]. In the treatment of HCV (hepatitis C virus) infection, GA can inhibit HCV full-length viral particles and HCV core gene expression or function in a dose-dependent manner and have a synergistic effect with interferon [52]. GA is also involved in biliary secretion and excretion. GA can increase hepatic glutathione levels by the inhibition of biliary excretion of glutathione partly through the inhibition of MRP2 [53], an efflux transporter located at the canalicular membrane of a hepatocyte. MRP2 translocates glutathione, LTC4, bilirubin, methotrexate (MTX), glucuronide (e.g., estradiol-17-β-glucuronide [$E_2$17G]), or sulfate conjugates and other organic anions from a hepatocyte into the bile canaliculus [54–58].

GA can activate certain immune functions, such as IFN production, augmentation of NK cell activity, and modulation of the growth response of lymphocytes via augmentation of IL-2 production [70]. GA can enhance immune function

Table 1: Clinical trials using compound glycyrrhizic acid injection.

Experimental drugs	Dose and course of treatment	Combined medication	Case/control	Disease type	Indications and symptoms	Efficacy	Positive control	Side effect	Reference
Glycyrrhizin	200 mg + NaCl 100 mL, i.v., q.d., 4 weeks	Prednisolone (20–60 mg)	31/14	Acute onset autoimmune hepatitis (AIH)	Fever, general malaise, fatigue, nausea, vomiting, and right upper quadrant discomfort	Recovery rate was higher in the SNMC group than in the SNMC + CS group ($P = 0.035$)	Glycyrrhizin and corticosteroids (CS)	None	[21]
Glycyrrhizin	200 mg, i.v., q.d., 52 weeks	None	374/129	Chronic hepatitis C	Inflammatory effect	The proportion of patients with ALT reduction ≥50% after 12 weeks was significantly higher with 5×/week glycyrrhizin (28.7%, $P < 0.0001$) and 3×/week glycyrrhizin (29.0%, $P < 0.0001$) compared with placebo (7.0%).	Placebo-controlled	None	[22]
Glycyrrhizin	200 mg + NaCl 100 mL, i.v., 6c/week, 4 weeks	None	69/13	Chronic hepatitis C	HCV and HCV-RNA positive; serum ALT at least 1.5 times; liver fibrosis or cirrhosis	The mean percentage ALT decrease from baseline at the end of treatment was 26% and 47% for the three times per week and six times per week treatment group, respectively (both $P < 0.001$ versus placebo)	Placebo-controlled	None	[23]
Glycyrrhizin	200 mg + NaCl 100 mL, i.v., q.d. for 8 weeks, then 2–7c/week for 2–16 years	None	453/109	Hepatocellular carcinoma (HCC) occurs in patients with hepatitis C virus-RNA positive chronic liver disease	Inflammatory effect after HCC	Patients treated with SNMC; the 10-year HCC appearance rates in histologic Stages I, II, and III were 3%, and 13%, respectively	Other herbal medicines	None	[24]
Glycyrrhizin	200 mg + NaCl 100 mL, i.v., q.d., 0.1–14.5 years	None	1249/796	Interferon-resistant hepatitis C	Chronic hepatitis effect	Crude carcinogenesis rates in the treated and untreated group were 13.3%, 26.0% at the fifth year and 21.5% and 35.5% at the 10th year, respectively ($P = 0.021$)	Others without glycyrrhizin therapy	Hypertension skin rash without itching	[25]

Table 1: Continued.

Experimental drugs	Dose and course of treatment	Combined medication	Case/control	Disease type	Indications and symptoms	Efficacy	Positive control	Side effect	Reference
Diammonium glycyrrhizinate	150 mg + 5–10% glucose injection liquid 250 mL, i.v., q.d., 1 month, 1-2 cycles	None	629/127	Chronic hepatitis, liver cirrhosis	Fatigue, gastrointestinal symptoms, and discomfort in liver area	After 17-day therapy, there are 93.3% patients with ALT normal level in treating group, but 73.3% in contrast group ($P < 0.05$). At day 10, the patient'srecovered normal SB were 86.7% in treatment group, but that was 40% in contrast group ($P < 0.01$)	Compound ammonium glycyrrhetate injection	Headache, facial edema, and blood pressure increased	[26]
β-glycyrrhetinic acid	80 mg + 10% glucose injection liquid 250 mL, i.v., q.d., 4 weeks, 100 mg, p.o. tid, 12 weeks	None	80/40	Chronic Hepatitis B	Chronic hepatitis effect	Compared with control group, the TBil, ALT, AST, HA, and IVC are significantly ameliorated in treatment group ($P < 0.01$)	α-glycyrrhizic acid	Edema, blood pressure increased, and serum potassium mildly low	[27]
Magnesium isoglycyrrhizinate	80 mg + 10% glucose injection liquid 250 mL, i.v., q.d., 4 weeks	Hepatoprotective drugs	80/40	Chronic Hepatitis B	Fatigue, gastrointestinal symptoms, and discomfort in liver area	Compared with control group, the TBil, ALT, AST are significantly ameliorated in treatment group ($P < 0.05$)	Diammonium glycyrrhizinate injection	Headache,and blood pressure increased	[28]
Magnesium isoglycyrrhizinate	150 mg + 5–10% glucose injection liquid 250 mL, i.v., q.d., 4 weeks	None	60/30	Chronic severe hepatitis	Fatigue, gastrointestinal symptoms, discomfort in liver area, and yellow urine	Compared with control group, the TBil, PTA, ALT, and AST are significantly ameliorated in treatment group ($P < 0.01$)	Hepatocyte generation drugs	None	[29]
Magnesium isoglycyrrhizinate	150 mg + 5–10% glucose injection liquid 250 mL, i.v., q.d., 2 weeks	None	56/28	Liver lesion induced by chemotherapy in cancer	Liver injury effect	Compared with control group, the TBil, PTA, ALT, and AST are significantly ameliorated in treatment group ($P < 0.01$)	Diammonium glycyrrhizinate injection	None	[30]

GA: glycyrrhizic acid; TBil: total bilirubin; IVC: type IV collagen; ALT: alanine aminotransferase; AST: aspartate transaminase; PTA: prothrombin time activity.

Table 2: Mechanism of action of glycyrrhizin compound chemotherapy.

Compound	Pharmacological activities	Mechanisms of action	Reference
Glycyrrhizic acid	Anti-inflammatory antiviral inhibition of hepatic fibrosis	Regulating the expression of inflammation-related factors; inhibition replication of viral mRNA	[46–49] [51, 59] [45]
Compound glycyrrhizin tablet	Improving the liver dysfunction augmented the entire cytotoxic function mediated by hepatic lymphocytes inhibiting the cascade leading to apoptosis	Regulating the expression of inflammation-related factors; promoting the growth of hepatocyte; inhibition replication of viral mRNA	[60] [61] [62]
Glycyrrhetinic acid	Anti-inflammatory antiviral antiallergic antitumor proliferation	Regulating the expression of inflammation-related factors; inhibition replication of viral; inhibition of the expression of sensitizing factors and tumor-associated factor;	[63] [64] [65] [66]
18β-glycyrrhetic acid	Antiviral anti-inflammatory	Regulating the expression of inflammation-related factors; inhibition replication of viral mRNA	[67]
Diammonium glycyrrhizinate	Anti-inflammatory, resistance to biologic oxidation and membranous protection neuroprotective effect	Regulating the expression of inflammation-related factors; regulating the enzymatic reactions' related oxidation	[68]
Dipotassium glycyrrhizinate	Anti-inflammatory	Regulating the expression of inflammation-related factors	[69]

Table 3: Patents of glycyrrhizin extracts.

Patent	Patent number
Acetylated 18-alpha glycyrrhizic acid and preparation method thereof	CN102351937 A
Ammoniated glycyrrhizin modified sweetened beverage products	US2008226787(A1)
Application of beta-glycyrrhizic acid and derivatives thereof for radiation protection	CN102206242 A
Application of glycyrrhetinic acid and glycyrrhizic acid in preparing medicaments for preventing or treating pulmonary fibrosis	CN101919870 B
Application of glycyrrhizic acid and glycyrrhetic acid in preparing medicine for inflammatory enteropathy	CN1846705 A
Application of glycyrrhizic acid in preparation of sunitinib malate cardiotoxicity reduction drug	CN103285020 A
Application of glycyrrhizic acid on treating dilated cardiomyopathy cardiac remodeling and cardiac dysfunction	CN102247392 A
Application of glycyrrhizic acid, glycyrrhetinic acid, or salt thereof as well as gel composition and preparation method for gel composition	CN102614213 A
Application of glycyrrhizic acid and its breakdown product glycyrrhetinic acid for the manufacture of a medicament for the treatment of inflammatory bowel disease	US2010087385(A1)
Aqueous pharmaceutical solutions with trisubstituted glycyrrhizic acid salts	EP1226831 A1
Aspergillus niger bacterial strain and glycyrrhizic acid used for production thereof	CN101255401 B
Berberine glycyrrhizic acid enantiomer salt and preparation method and usage thereof	CN101747405 A
Biological extraction process of glycyrrhizic acid	CN101067146 B
Carboxymethyl chitosan nanoparticles modified with glycyrrhizic acid, preparation method, and application thereof	CN102357079 A
Chitosan glycyrrhizic acid nanoparticle and its preparing method	CN1586488 A
Composite glycyrrhizic acid amino acid injection and preparation method as well as applications thereof	CN101669962 A
Compositions containing glycyrrhizin	US4678772(A)
Compound for the control of herpes simplex virus using glycyrrhizic acid, lipoic acid, allantoin, and slippery elm	US2011229584(A1)
Compound glycyrrhizin capsule composition	CN103230407 A
Compound glycyrrhizin soluble powder for livestock and preparation method thereof	CN102526082 B
Dispersed compound tablet of glycyrrhizic acid and glycyrrhizinate and its preparing process	CN100386086 C
Enteric-coated formulation of glycyrrhizic acid and its salt and its preparing method	CN1274309 C
Film-coated tablet of glycyrrhizinic acid monopotassium salt and method for preparing the same	CN100341515 C
Glycyrrhizic acid compounds as foamer in chemically derived surfactant-free dentifrice	US2008274062(A1)
Glycyrrhizic acid and its derivative used as RANTES inducer	CN1498623 A
Glycyrrhizic acid antibody and its preparing method and use	CN1293097 C
Glycyrrhizic acid aureola dimer mediated targeted medication body as well as preparation method and purpose of glycyrrhizic acid aureola dimer mediated targeted medication body	CN102716488 A
Glycyrrhizic acid composition	CN101081227 B
Glycyrrhizic acid derivatives having amino acid, its preparation method, and medicinal composition containing them	CN1911954 A
Glycyrrhizic acid double salt and preparation thereof	CN100537593 C
Glycyrrhizic acid matrine salt and glycyrrhizic acid marine salt, its preparing method and use	CN100564391 C

Table 3: Continued.

Patent	Patent number
Glycyrrhizic acid organic salt phospholipid ligand and preparation thereof	CN102716463 A
Glycyrrhizic acid removal glycyrrhiza flavonoid and medicament composition thereof	CN101747307 A
Glycyrrhizic acid sustained-release dropping pills and preparation method thereof	CN101269020 A
Glycyrrhizic acid transdermal formulation and preparation technique thereof	CN101433529 A
Glycyrrhizic acid, biogastrone acid or its salt, derivative temperature sensing gel rubber, preparation method, and application thereof	CN101292952 B
Glycyrrhizin high-concentration preparation	US2006160754(A1)
Glycyrrhizin or derivatives thereof for treating or preventing severe acute respiratory syndrome (SARS)	US2007099855(A1)
Glycyrrhizin preparations for transmucosal absorption	US6890547(B1)
Glycyrrhizin-free fractions from licorice root and process for obtaining such fractions	US4163067(A)
Inclusion compound of glycyrrhizic acid or its derivative and alkaloid and its preparation method	CN1301717 C
Magnetic resonance imaging contrast medium with glycyrrhizic acid as carrier	CN101002950 B
Medicine composition of glycyrrhizic acid or its salt and reduced glutathione	CN1985987 B
Medicine composition of glycyrrhizic acid or its salt, ginseng and astragalus root	CN1985873 B
Medicine composition prepared mainly from glycyrrhizic acid or its salt, ginseng and glossy ganoderma	CN1985864 B
Method for determining glycyrrhizic acid content in extract after polysaccharide extraction of glycyrrhiza by virtue of vanillin-sulfuric acid	CN102621089 A
Method for extracting and purifying glycyrrhizic acid by ion-exchange fibers	CN102304165 B
Method for measuring paeoniflorin, hesperidin, and glycyrrhizic acid in stomach-nourishing granules	CN103175915 A
Method for producing glycyrrhizin sodium aliphatate or glycyrrhizin potassium aliphatate	CN101830962 B
Method for producing glycyrrhizic acid through enzymolysis	CN102219824 B
Method for separating and purifying glycyrrhizic acid extracting solution through macroporous resin separation	CN103242393 A
Method of preparing 18 alpha type glycyrrhizic acid and its salt using nonhomogeneous phase reaction	CN100522985 C
Nanocapsule containing glycyrrhizic acid medicine and its preparing method	CN1319537 C
Novel glycyrrhizic acid double salt and preparation and application thereof	CN103242392 A
Pharmaceutical antiviral composition comprising glycyrrhizic acid and at least one protein endowed with antiviral activity	US6329339(B1)
Pharmaceutical antiviral composition, comprising glycyrrhizic acid and at least one protein endowed with antiviral activity	CN1114447 C
Pharmaceutical applications of glycyrrhizic acid or salt and derivative thereof	CN102552280 A
Potassium-magnesium-calcium glycyrrhizin	US4176228(A)
Potentiation of chocolate flavor with ammoniated glycyrrhizin	US3356505(A)
Powder injection of compound glycyrrhizic acid glycosides and preparation method thereof	CN101317852 B
Preparation method for trans-glycyrrhizic acid	CN102584928 A
Preparation method of high-purity glycyrrhizic acid	CN103159809 A
Preparation method of glycyrrhizic acid	CN101759757 A
Process for extracting purified glycyrrhizic acid from licorice residue	CN1450081 A
Process for producing glycyrrhizic acid	CN102617694 A

Table 3: Continued.

Patent	Patent number
Products sweetened with alpha-glycosyl glycyrrhizin	US4537763(A)
Separation and purification process of glycyrrhizic acid	CN102453075 A
Separation of glycyrrhizic acid from licorice extract by ultrafiltration	US2011196138(A1)
Separation, purification, and concentration device for glycyrrhizic acid extract	CN202909639 U
Silver glycyrrhizic acid and its producing process and use thereof	CN1063184 C
Slow-released compound preparation of glycyrrhizic acid and glycyrrhizinate and its preparing process	CN1857288 A
Sucrose-ammoniated glycyrrhizin sweetening agent	US3282706(A)
Supercritical CO2 extraction method for extracting glycyrrhizinic acid from licorice	CN1136225 C
Technique for extracting glycyrrhizin using hot reflux method	CN103130863 A
The application of glycyrrhizic acid and its breakdown product glycyrrhetinic acid for the manufacture of a medicament for the treatment of inflammatory bowel disease	WO2007093090 A1
The application of glycyrrhizic acid and its breakdown product glycyrrhetinic acid for the manufacture of a medicament for the treatment of inflammatory bowel disease	EP2067476 A1
Ultrasonic extracting method for changing glycyrrhizic acid leaching phase balance	CN101486750 A
Use of glycyrrhetic acid and/or glycyrrhizin for producing cosmetic preparations for tanning the skin	US2009280074(A1)
Use of glycyrrhetinic acid, glycyrrhizic acid, and related compounds for prevention and/or treatment of pulmonary fibrosis	US2012053141(A1)
Use of glycyrrhizin and its derivatives as MCP-1 production inhibitors	US2004138171(A1)
Use of glycyrrhizin and its derivatives as RANTES inducers	US2004142882(A1)
Use of glycyrrhizin for the treatment of standard therapy-resistant hepatitis C patients	WO2004056374 A1
Use of iso-glycyrrhizic acid and salt thereof in treating allergic rhinitis	CN101396368 B
Use of one or more of glycyrrhizic acids for reducing the irritating action of surfactants in cosmetic compositions	US2011015143(A1)

in mice [71]. GA treatment could significantly reduce blood immunoglobulin E (IgE), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), NO, TNF-α levels, and nitrous oxide synthase (NOS) activity dose-dependently. GA could also enhance blood immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), interleukin-2 (IL-2), and interleukin-12 (IL-12) levels in AR mice. Gr-1^+CD11^+b cells are responsible for numerous pathological processes such as T cell dysfunction after severe trauma or major surgery, leading to increased susceptibility to infection [72]. These cells exercise an inhibitory effect on MBD-1 production of EKs mediated via the suppressor molecules CCL-2 and IL-10. GA acts as a potent inhibitor of these cells and therefore restores MBD-1 levels. This restoration affects T cell dysfunction [73]. In thermally injured mice, GA regulates the burn-associated type 2 T cell responses to recover IL-12 and make it unresponsive, thus restoring the impaired cells [74]. GA acts as a promoter of the late signal transduction of T lymphocytes for IL-2 production. The balance between augmenting and suppressing effects might be dependent on the level of stimulation and stage of the cell. Therefore, this determines quality and quantity of the immunomodulatory action of GA [75]. In blood and nasal mucosa, GA consumption decreases antioxidant enzyme activity, lipid peroxidation, Glutathione levels, and IL-4 levels and enhances IFN-γ, thus protecting the nasal mucosa from oxidative injury and improving immunity activity [76].

GA interferes with some viruses, such as H5N1 [77]. The replication and virus-induced proinflammatory gene expression include inhibition of the virus-induced formation of reactive oxygen species and reduced activation of NF-κB, JNK, and p38, which are redox-sensitive signaling events known to be relevant for replication.

2.4. Antitumor Effects. CYP enzymes are mainly found in the liver and bowel wall. They are responsible for the bulk of phase I or oxidative metabolism of xenobiotics including dietary toxins, carcinogens, mutagens, and drugs. Administration of GA was able to significantly induce CYP content, which reduces the incidence of cancer [78]. GA can also protect against aflatoxin-induced oxidative stress. The protective effect is likely from its capacity to inhibit the metabolic activation of hepatotoxin, a critical factor in the pathogenesis of chemical-induced carcinogenicity [79]. O-carboxymethyl chitosan nanoparticles (CMCNP) modified by GA with

various substitution degrees can efficiently deliver paclitaxel (PTX) to hepatocellular carcinomas (HCC). CMCNP-GA significantly facilitated the increased accumulation of PTX in hepatic tumor tissues and the targeted delivery of PTX to hepatoma carcinoma cells, which resulted in remarkably enhanced *in vitro* cytotoxicity and *in vivo* antitumor efficacy [80]. In a diethylnitrosamine-treated experimental animal study, as a chemopreventive agent of HCC, modulation of cell proliferation and apoptosis by GA may be associated with inhibition of HCC. Therefore, GA treatment may inhibit the occurrence of HCC [81].

2.5. Inductive Effect of Liver Enzyme Activity. Some studies showed that GA has an inductive effect on CYP3A activity. Therefore, clinicians should pay attention to other drugs catalyzed by CYP3A, especially those substrates with a narrow therapeutic range such as cyclosporine A, to avoid possible clinically significant interactions with GA [82]. Some studies revealed that the area under concentration-time curve and the mean retention time of methotrexate (MTX) were significantly increased by GA, which increases the adverse reactions of MTX [83]. MTX is an antifolate agent, anticancer agent, and immunosuppressant and is commonly used for anticancer chemotherapy [84], rheumatoid arthritis [85], and severe psoriasis [86]. The adverse reactions of MTX include nausea, vomiting, diarrhea, and hepatotoxicity [87, 88]. A case report showed that combined administration of GA and cilostazol caused pseudoaldosteronism [89]. Therefore, the concurrent use of GA with MTX or cilostazol is not recommended. One report shows a case of hypokalemic rhabdomyolysis secondary to chronic GA intoxication [90]. GA ingestion could therefore potentially aggravate hypokalemia in patients with chronic laxative abuse [91], indicating that the use of GA in hypokalemia should be treated with caution.

3. Other Pharmacological Activities

GA is effective in combating hyperglycemia and associated pathological complications such as hyperlipidemia, abnormal histoarchitectures of different organs, and oxidative stress including hemoglobin-induced iron-mediated free radical reactions. The effects of GA on diabetes-associated changes are almost comparable with those of glibenclamide, a standard antihyperglycemic drug, suggesting a possible use of the herbal agent as a drug to prevent complications of diabetes mellitus [92]. Furthermore, GA regulates renal function through the regulation of water channels [93], and GA administration ameliorates the renal concentrating ability and structural lesions in renal tissues in rats with early-phase of ischemia-acute renal failure [94]. As a reduction inhibitor, GA reduces the therapeutic loss of methylprednisolone produced from methylprednisolone 21-sulfate sodium in the large intestine, thus improving the therapeutic property of the prodrug against inflammatory bowel disease [95]. GA also offers protection from the damage induced by UVB radiation in humans. Therefore, it could be considered as a promising agent for addition to topical formulations for the prevention of skin cancer [96]. GA significantly alleviates asthma symptoms [97], inhibits lung inflammation [98], and relieves acute lung injury [35, 99]. It can directly affect cardiac performance and play a role in myocardial and coronary protection in the presence of cardiovascular diseases [100]. GA may prevent brain tissue damage [101], can be a putative therapeutic drug for neurodegenerative diseases associated with cognitive deficits and neuroinflammation such as Alzheimer's disease [102], and could suppress ocular hypertension with potential therapeutic effects in eye disease [103]. GA improves resistance to *C. albicans* infection by inducing CD4+ T cells, which suppress type 2 cytokine production by Th2 cells [104]. GA inhibits activated macrophage (M2M) generation stimulated with neutrophils. The regulation of neutrophil-associated M2M generation by GA may provide a new therapeutic strategy, which could influence the outcome of certain severe infections in hosts with M2M generation [105].

4. Drugs That Include GA

Drugs made with GA have been on the market for many years, and most have important therapeutic uses. Magnesium isoglycyrrhizinate injection (TianQing GanMei, Chia Tai Tainqing, JiangSu, China) is one example of a drug with GA. Magnesium isoglycyrrhizinate is an effective and safe treatment for chronic liver diseases [106] and is capable of slowing down the progress of pulmonary fibrosis [107]. Moreover, diammonium glycyrrhizinate enteric-coated capsules (TianQing GanPing, Chia Tai Tainqing, JiangSu, China) and diammonium glycyrrhizinate injection (GanLiXin, Chia Tai Tainqing, JiangSu, China) are used for acute and chronic hepatitis associated with elevated alanine aminotransferase. Stronger neo-minophagen C (SNMC, Minophagen Pharmaceutical, Tokyo, Japan) is often used in the treatment of chronic liver disease and can improve liver dysfunction [60]. SNMC is a compound GA tablet that includes GA (2 mg) with glycine acid (20 mg) and L-cysteine hydrochloride (1 mg). SNMC has anti-inflammatory, antiallergic, steroid-like, anticomplementary, and immunoregulatory effects.

5. GA Combined with Matrine

GA combined with matrine (Mat) can improve CCL4-induced liver fibrosis effectively. This is evidenced by lower levels of collagen, hyaluronic acid (HA), and laminin (LN), less hepatic stellate cells (HSC) proliferation, collagen I, and HA levels secreted by HSC *in vitro* with combined therapy compared with GA or Mat alone. GA combination with Mat could protect liver cells and inhibit hepatic fibrosis and may therefore be a safe and effective strategy for improving hepatic fibrosis [108]. In an animal model, GA combined with Mat reduced the mortality of acetaminophen overdosed mice, attenuated acetaminophen-induced hepatotoxicity, and reduced the number and area of γ-GT positive foci, thus protecting liver function and preventing HCC from occurring [109]. Additionally, the combination of GA and cyclosporine was an effective treatment for nonsevere aplastic anemia [110].

6. Common Derivatives of Glycyrrhizin

Glycyrrhetinic acid (3β-hydroxy-11-oxo-oleana-12-en-28-oic acid), the aglycone of GA, stimulates glucose-induced insulin secretion in isolated pancreatic islets. Glycyrrhetinic acid treatment enhances plasma insulin levels and reduces the levels of gluconeogenic enzymes in liver. It is a pentacyclic triterpene acid with numerous biological activities, including anti-inflammatory [63], antiviral [64], antiallergic [65], and antitumor proliferative effects [66].

Glycyrrhetinic acid restrains the proliferation of skin tumors in mice and human breast cancer cells (MCF7) and induces apoptosis of cancer cells. The mechanism of apoptosis might be via increased free Ca^{2+} level in the cells [111]. Mizushina et al. [112] demonstrated that glycyrrhetinic acid potently inhibited the activity of mammalian polymerases, including pol λ. Glycyrrhetinic acid also reduced TNF-α production and NF-κB activation and suppressed mouse ear inflammation stimulated by tissue plasminogen activator. Therefore, glycyrrhetinic acid could be an anti-inflammatory agent based on pol λ inhibition.

Another licorice acid derivative is 18β-glycyrrhetic acid. The triterpene structure of the HMGB1-binding compound is capable of binding to HMGB1 and altering its proinflammatory properties, inhibiting HMGB1-dependent cyclooxygenase (COX) 2 induction [113]. 18β-glycyrrhetic acid has significant antiviral activity against rotavirus replication *in vitro*, and studies to determine whether 18β-glycyrrhetic acid attenuates rotavirus replication *in vivo* are underway, although the exact mechanism is unclear. However, some reports show that 18β-glycyrrhetic acid inhibits NF-κB activation, which has been interpreted as 18β-glycyrrhetic acid-mediated regulation of the inflammatory response [114]. 18β-glycyrrhetinic acid can also inhibit the activity of tyrosine and prevent melanin growth and whitening. Some reports show that 18β-glycyrrhetinic acid is likely responsible for amelioration of dysfunction of glutamate transport in astrocytes, and the inhibition of protein kinase C activity might be related to its pharmacological efficacy [67].

7. Conclusions and Future Perspectives

This review summarized the efficacy of GA in liver disease from clinical trials and its mechanisms of action *in vitro* and *in vivo*. Studies indicate that GA could modulate various molecular pathways in liver disease. There are numerous patents for drugs including GA (Table 3). Studies described here highlight the use of GA as a novel chemopreventive agent for liver injury. It is expected that future studies with GA will help to define various molecular mechanisms and targets for inflammation and steatosis. At present, the number of multicenter, large sample, randomized, double-blind, controlled chemoprevention clinical trials with GA is very limited. Extensive clinical research is warranted to evaluate the safety and chemopreventive efficacy of GA alone or in combination with chemotherapy agents.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review

The Role of Oxidative Stress and Antioxidants in Liver Diseases

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Abstract: A complex antioxidant system has been developed in mammals to relieve oxidative stress. However, excessive reactive species derived from oxygen and nitrogen may still lead to oxidative damage to tissue and organs. Oxidative stress has been considered as a conjoint pathological mechanism, and it contributes to initiation and progression of liver injury. A lot of risk factors, including alcohol, drugs, environmental pollutants and irradiation, may induce oxidative stress in liver, which in turn results in severe liver diseases, such as alcoholic liver disease and non-alcoholic steatohepatitis. Application of antioxidants signifies a rational curative strategy to prevent and cure liver diseases involving oxidative stress. Although conclusions drawn from clinical studies remain uncertain, animal studies have revealed the promising *in vivo* therapeutic effect of antioxidants on liver diseases. Natural antioxidants contained in edible or medicinal plants often possess strong antioxidant and free radical scavenging abilities as well as anti-inflammatory action, which are also supposed to be the basis of other bioactivities and health benefits. In this review, PubMed was extensively searched for literature research. The keywords for searching oxidative stress were free radicals, reactive oxygen, nitrogen species, anti-oxidative therapy, Chinese medicines, natural products, antioxidants and liver diseases. The literature, including ours, with studies on oxidative stress and anti-oxidative therapy in liver diseases were the focus. Various factors that cause oxidative stress in liver and effects of antioxidants in the prevention and treatment of liver diseases were summarized, questioned, and discussed.

Keywords: oxidative stress; antioxidant; liver diseases; foods; medicinal plants

1. Introduction

Free radicals are atoms or molecules that have unpaired electrons, usually unstable and highly reactive [1]. In biology system, oxygen based radicals and nitrogen based radicals are two types of free radicals. Oxygen free radicals, such as superoxide, hydroxyl radicals, and peroxyl radicals, with the addition of non-radicals, such as hydrogen peroxide, hypochlorous acid and ozone, are known as reactive oxygen species (ROS), which are generated during the metabolism process of oxygen. Reactive nitrogen species (RNS), including nitrogen based radicals and non-radicals, such as nitrogen dioxide, nitric oxide radicals and peroxynitrite, are derived from nitric oxide and superoxide via inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, respectively [2,3]. Due to their special chemical characteristics, ROS/RNS can initiate lipid peroxidation, cause DNA strand breaks, and indiscriminately oxidize virtually all molecules in biological membranes and tissues, resulting in injury. However, since the body is able to remove ROS/RNS to a certain degree, these reactive species are not necessarily a threat to the body under physiological conditions [3,4]. As a matter of fact, ROS are required at certain level in the body to

perform its important physiological functions. The generation of ROS is a natural part of aerobic life, which is responsible for the manifestation of cellular functions including signal transduction pathways, defense against invading microorganisms and gene expression to the promotion of growth or death [1]. Oxidative/nitrosative stress represents the bodies' imbalance in the production and the elimination of reactive oxygen and nitrogen species as well as decreased production of antioxidants. In terms of oxidative stress, in specific physiological conditions, it is actually useful. For example, it could strengthen biological defense mechanisms during appropriate physical exercise and ischemia, and induce apoptosis to prepare the birth canal for delivery [2,3]. However, this is confined to particular situations, and in most other cases, large levels of ROS and oxidative stress will induce cell death through necrotic and/or apoptotic mechanisms, leading to cellular and tissue injury.

Liver is a major organ attacked by ROS [5]. Parenchymal cells are primary cells subjected to oxidative stress induced injury in the liver. The mitochondrion, microsomes and peroxisomes in parenchymal cells can produce ROS, regulating on PPARα, which is mainly related to the liver fatty acid oxidation gene expression. Moreover, *Kupffer* cells, hepatic stellate cells and endothelial cells are potentially more exposed or sensitive to oxidative stress-related molecules. A variety of cytokines like TNF-α can be produced in *Kupffer* cells induced by oxidative stress, which might increase inflammation and apoptosis. With regard to hepatic stellate cells, the proliferation and collagen synthesis of hepatic stellate cells is triggered by lipid peroxidation caused by oxidative stress [6–8]. In mammals, a sophisticated antioxidant system has been developed to maintain the redox homeostasis in the liver (Figure 1). When the ROS is excessive, the homeostasis will be disturbed, resulting in oxidative stress, which plays a critical role in liver diseases and other chronic and degenerative disorders [9]. The oxidative stress not only triggers hepatic damage by inducing irretrievable alteration of lipids, proteins and DNA contents and more importantly, modulating pathways that control normal biological functions. Since these pathways regulate genes transcription, protein expression, cell apoptosis, and hepatic stellate cell activation; oxidative stress is regarded as one of the pathological mechanisms that results in initiation and progression of various liver diseases, such as chronic viral hepatitis, alcoholic liver diseases and non-alcoholic steatohepatitis [10,11]. It has also been suggested that there are complicated cross-talks among pathological factors, inflammation, free radicals and immune responses [11,12]. The general mechanism scheme of oxidative stress induced by various factors on liver disease is concluded in Figure 2. Moreover, systemic oxidative stress arising during liver disease can also cause damage to extra-hepatic organs, such as brain impairment and kidney failure [13]. It was suggested systemic oxidative stress might be a significant "first hit", acting synergistically with ammonia to induce brain edema in chronic liver failure [14]. With regard to kidney failure, systemic oxidative stress is considered to play a critical role in the pathophysiology of several kidney diseases [15,16].

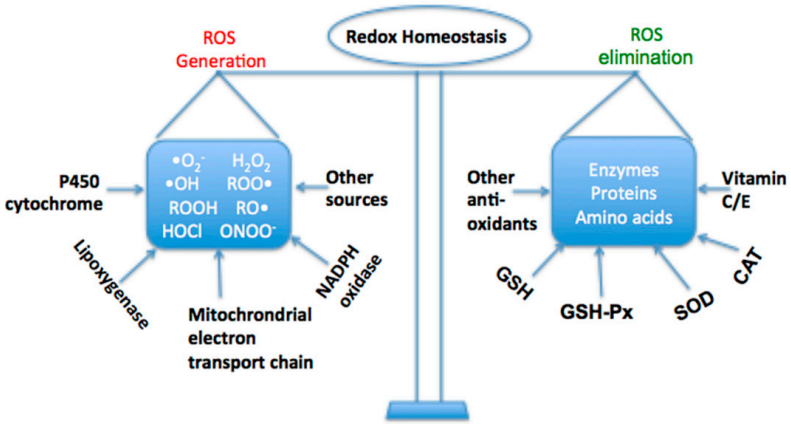

Figure 1. The redox homeostasis in the liver.

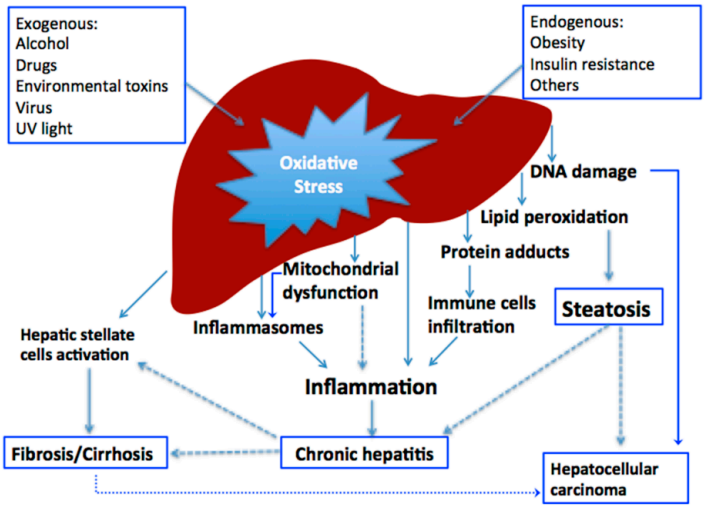

Figure 2. The general mechanism scheme of oxidative stress induced by various factors on liver disease.

Both enzymatic and non-enzymatic antioxidant system are essential for cellular response in order to deal with oxidative stress under physiological condition. Therefore, antioxidant enzyme such as CAT, SOD, and GSH-Px and non-enzymatic electron receptors such as GSH are affected and used as indexes to evaluate the level of oxidative stress [12,17–19]. Notably, erythroid 2-related factor 2 (Nrf2) is a major regulator of cellular redox balance [20]. Under physiological condition, Nrf2 binds to kelch-like ECH-associated protein-1 (Keap1) in the cytoplasm, and the ones remaining are inactivated and easily to be degraded. Under oxidative stress, however, Nrf2 dissociates form Keap1 by Keap1 modification or Nrf2 phosphorylation and are thus activated. The activated Nrf2 translocates into the nucleus and interacts with antioxidant response element (ARE), promoting the expression of cytoprotective target genes including antioxidant enzymes and phase II detoxifying enzymes [21]. The enhanced activation of Nrf2 by pharmacologic molecules or genetic engineering has been shown to protect the liver in different oxidative stress models [22]. For example, in terms of pharmacologic activation of Nrf2, the use of small molecules, such as BHA, oleanolic acid, ursolic acid and CDDO-Im have been reported to show hepatoprotection against liver damage induced by acetaminophen, a famous drug possessing hepatotoxicity. During the process where mitochondria convert acetate into ATP, a significant amount of free radicals are generated, which results in cellular injuries, especially to mitochondria themselves. Activation of Nrf2 protects mitochondria from oxidative stress via a variety of mechanisms depending on different circumstances, such as increasing antioxidant levels, protecting against mitochondrial permeability transition pore opening, maintaining the mitochondrial redox state, enhancing mitochondrial biogenesis by promoting transcription of nuclear respiratory factor 1 (Nrf1). For fatty liver disease, activation of Nrf2 could facilitate fatty acid metabolism in liver by directly regulating fatty acid metabolism related genes, such as CD36 [20,22]. Furthermore, the enhanced antioxidant signaling regulated by activated Nrf2 protects mitochondria from oxidative damages, which further ensures competent hepatic fatty acid catabolism.

Regarding the vital role of oxidative stress in chain of liver diseases, various anti-oxidative therapy and antioxidants are proposed to prevent and treat liver diseases [9,12]. A series of studies have tested the effectiveness of some antioxidants in the treatment of patients with various liver diseases, such as chronic hepatitis C virus infection, alcoholic hepatitis or cirrhosis, and non-alcoholic fatty liver disease (NAFLD). The clinical effects of antioxidants as adjuvants including vitamin E/C, mitoquinone, *N*-acetylcysteine, polaprezinc silymarin, silibinin and some antioxidant cocktail on chronic hepatitis C patients have been examined has shown clear benefit of antioxidants to interferon based therapy of HCV [23,24]. However, despite some positive results were obtained, it cannot reach to the conclusion that antioxidants are useful therapeutic agents for chronic

hepatitis C partly due to the sample scale and treatment duration. Vitamins E/C, *N*-acetylcysteine, polyenylphosphatidylcholine, silymarin, and antioxidants cocktail have been attempted for the treatment of alcoholic hepatitis or cirrhosis patients [24–26]. Although some promise has been shown, results indicated that many antioxidants failed to improve the outcome of patients [27]. Additionally, a great deal of studies has investigated the therapeutic effects of vitamins E/C and *N*-acetylcysteine on NAFLD. It is worth noting that vitamin E has been demonstrated clinically to be a rather promising drug for the treatment of non-alcoholic steatohepatitis [28,29]. Although data from clinical studies is yet to prove the efficacy of antioxidant, application of antioxidants is a rational curative strategy for prevention and treatment of liver diseases involving oxidative stress [17,30]. Natural antioxidants have been found in many edible (such as fruits, vegetables, cereals and tea) and medicinal plants, which often possess strong antioxidant and free radical scavenging abilities as well as anti-inflammatory action [9]. Several well-elaborated reviews concerning antioxidants as therapeutic agents for diverse liver diseases in clinic have been published [11,31,32], therefore, in this review, particular attention will be drawn on the factors causing oxidative stress in liver and *in vivo* effects of antioxidants for the prevention and treatment of liver diseases. Moreover, although oxidative stress has been suggested to exist in almost all liver diseases, since the fact that there are no animal models with virus-induced liver disease, including hepatitis A, hepatitis B, and hepatitis C, the role of oxidative stress in viral hepatitis are not included in this review.

2. Oxidative Stress in Liver Diseases

2.1. Oxidative Stress Caused by Alcohol

Alcohol beverages are widely consumed all over the world; however, excessive alcohol consumption may cause a series of health problems. It was reported that alcohol consumption accounting for an estimated 3.8% of global mortality. Alcoholic liver disease (ALD) is one of the most important causes of liver-related death, which is associated with increased dose and time of alcohol intake. In 2003, it has been reported that age- and sex-adjusted mortality rate of ALD was 4.4/100,000. Although reductions in overall ALD mortality were observed in several reports on a country scale, it is more likely due to advances in disease management rather than a decrease in the prevalence of ALD, which could be supported by increases in hospital admissions for alcoholic hepatic failure and alcoholic hepatitis [33–35]. ALD may progress from steatosis to more severe liver diseases form, such as hepatitis, fibrosis, and cirrhosis [36,37]. As a matter of fact, more than 90% heavy drinkers develops fatty liver, and about 30% of heavy drinkers further develops advance forms of ALD. Although pathogenesis of ALD has not been fully elaborated, the direct consequence of ethanol metabolism seems to be related to ROS production, mitochondrial injury and steatosis, which are the common features of acute and chronic alcohol exposure [32,38,39]. It is well illustrated that at least three distinct enzymatic pathways are involved in the process of ethanol oxidation [15]. The primary pathway for the ethanol metabolism is dehydrogenase system. It is initiated by alcohol dehydrogenase (ADH), a NAD^+-requiring enzyme expressed at high levels in hepatocytes, which oxidizes ethanol to acetaldehyde. Then, acetaldehyde enters the mitochondria where it is oxidized to acetate by aldehyde dehydrogenases (ALDH). The second major pathway to oxidize ethanol is the microsomal ethanol oxidizing system (MEOS), which involves an NADPH-requiring enzyme, the cytochrome P450 enzyme CYP2E1. The MEOS pathway is prompted in individuals who consume alcohol chronically. In addition, infrequently, ethanol can also be oxidized by catalase in peroxisomes. Since this oxidation pathway requires the presence hydrogen peroxide (H_2O_2), under normal conditions, this pathway plays no major role in alcohol metabolism [15–17]. During the metabolism processes via dehydrogenase system and MEOS system, NADH or $NADP^+$ will be produced in bulk, leading to the increase of ROS, which cause oxidative stress resulting in hepatocyte injury, and finally trigger various liver diseases (Figure 3).

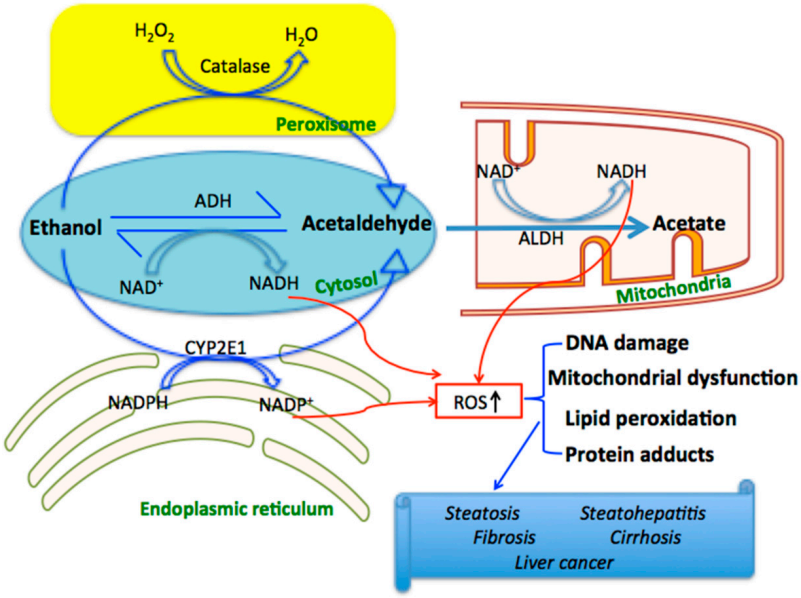

Figure 3. The metabolic process of ethanol in hepatocyte and the generation of ROS contributing to the liver diseases.

Studies have demonstrated that enzymatic as well as non-enzymatic systems which maintaining cellular homeostasis are remarkably affected by alcohol in diverse models. In particular, the activities of SOD, CAT, GSH-Px, GRD, and GST, as well as the level of lipid peroxidation were changed in animals treated with alcohol [19,40–42]. For example, SOD and CAT activities were decreased and the lipid peroxidation level was significantly increased in the liver of 30 days alcohol-treated diabetic rats [40]. An increase of lipid peroxidation and hepatic cytochrome P450, and decrease of hepatic SOD, GSH-Px, GRD, GST, and GSH were also observed in mice treated with dimethoate in combination with ethanol [41]. Furthermore, oxidative stress and antioxidant enzyme were measured in patients with ALD [32]. It was found that as the severity of the disease increased, followed by elevation of serum level of lipid peroxidation indicator malondialdehyde (MDA) and the concentrations of serum vitamins E and C, which act as indexes of antioxidant status, were decreased in ALD patients. The pro-oxidant and antioxidant status in chronic alcoholics have been detected in several studies. The significant decreases of GSH levels in liver and blood of patients with alcoholic liver disease were observed when compared to controls. However, the activity/content of SOD and CAT after alcohol exposure are rather controversial, with reports of increases, no changes, or decreases, depending on the amount and time of alcohol consumption [43,44]. Nevertheless, the increased oxidative stress in patients with ALD has been demonstrated. It was argued that the increases of antioxidants enzymes such as SOD, CAT and GSH-Px might be a compensatory regulatory response to increased oxidative stress [45]. The level of ALT was increased significantly while the level of AST was decreased significantly in patients with ALD [32,46,47].

2.2. Oxidative Stress Caused by Drugs

The liver is the most frequently targeted organ in terms of drug toxicity. The production of radical species, specifically ROS and RNS, has been proposed as an early event of drugs hepatotoxicity and as an indicator of hepatotoxic potential [48]. It has been discovered that a lot of drugs could induce oxidative stress including increase of cellular oxidants and lipid peroxidation, depletion of antioxidants in the liver, such as anti-inflammation drugs, anti-analgesic drugs, anti-cancer drugs and antidepressants. For example, sulfasalazine, a drug to treat inflammatory bowel diseases, has been found to induce hepatic oxidative damage [49]. Oral sulfasalazine administration could reduce SOD but increase CAT activity significantly. It is also suggested that

oxidative damage is involved in hepatotoxicity of sulfasalazine treatment. As for zoledronic acid, it is a nitrogen-bearing bisphosphonate, and used to treat the cancer-associated hypercalcemia. It has been shown that zoledronic acid significantly elevated MDA and nitric oxide levels, whereas reduced GSH levels, which indicated that zoledronic acid could induce oxidative stress and decrease antioxidant level in liver [18]. Furthermore, liver antioxidant capacity in hepatic injury induced by paracetamol, an extensively used analgesic compound in mice was evaluated [50]. It was shown that paracetamol induced a remarkable increase of MDA and nitrite as well as nitrate in the liver, with potent decrease of total SOD and Cu/Zn-SOD activity. Samarghandian *et al*. [51] studied effect of long-term treatment of morphine on enzymes, oxidative stress indices and antioxidant status in male rat liver. The results showed that the levels of ALT, AST and lactate dehydrogenase (LDH) in serum as well as MDA in liver were significantly elicited, while the activities of SOD, glutathione-s-transfrase and CAT were remarkably reduced by morphine. Oxidative stress generated by anticancer drugs including doxorubicin, paclitaxel and docetaxel in the liver of rats have been indicated. It was found that all three drugs increased thiobarbituric acid-reactive substances (TBARS), and the administration of docetaxel significantly decreased the activity of SOD. Furthermore, combined administration of two drugs generated greater changes in oxidative stress related molecules than single agents [52]. Nimesulide, nonsteroidal anti-inflammatory drug, could increase the activities of ALT, AST, ALP and the content of bilirubin in the serum. The activities of SOD and CAT and GSH-Px in the liver were decreased by nimesulide in mice [53]. Chronic administration of fluoxetine (15 mg/kg/day) or clozapine (20 mg/kg/day) was measured in rats exposed to chronic social isolation and controls. The increased serum ALT activity, MDA, decreased GSH levels and compromised SOD expression suggests a link between drugs and hepatic oxidative stress [54]. Anti-tuberculosis agent isoniazid (INH) resulted in both oxidative and nitrosative stress, but the correlation of hepatotoxicity severity with RNS rather than ROS suggested that $ONOO^-$ generation and mitochondrial dysfunction are responsible mechanisms for hepatotoxicity of INH *in vivo* [55,56].

Although hepatotoxicity induced by various drugs in humans has been demonstrated in a great number of clinical trials, report concerning the role of oxidative stress in patients with drug induced liver disease is limited by far. For example, mitochondrial dysfunction and DNA damage are found to be critical events in the underling mechanism of paracetamol induced hepatotoxicity in patients, which is supposed to partly attribute to oxidative stress, but, accurate and direct evidence to show the status and role oxidative stress in patients is lacking [57]. As a matter of fact, currently, in addition to animal model study, the investigation of hepatotoxicity induced by drugs is mainly based on the results of retrospective study, whereas there are few clinical studies with large numbers of patients. Moreover, models using human cells have been attempted to mimic pathogenesis of drug induced hepatotoxicity in humans [55]. Overall, clinical data and appropriate experimental model, which could closely resemble the human pathophysiology, is critical for future study of antioxidant treatment for hepato-toxicity caused by drugs.

2.3. Oxidative Stress Caused by Environmental Pollutants

Environmental pollutants such as heavy metals and microcystin have been shown to cause oxidative damage in liver of animal models. Antioxidant defense system in rat liver was damaged after mercury chloride treatment [58]. Mercury chloride at the dose of 0.1 mg/kg could induce a significant decrease in both Mn-dependent SOD and Cu- and Zn-dependent SOD activities, and progressive changes of CAT, GSH-Px, GRD and glucose-6-phosphate dehydrogenase activities. This is also accompanied by a minor increase in serum ALT and γ glutamyltransferase. The results showed that low dose of mercury could incur oxidative stress and hepatic damage. Besides mercury, lead was also found to exacerbate liver lipid peroxidation in protein-undernutrited rats, in which the study also suggested that free radicals is a pathological mechanism for hepatotoxicity of lead [59]. Microcystins are algae toxins produced by cyanobacteria, kind of cyclic nonribosomal peptides, possessing hepatotoxicity that may cause severe injury to the liver. The effect of microcystin LR,

the most studied toxic variants, on antioxidant enzymes and lipid peroxidation was investigated in liver rats after acute exposure [60]. The reduction of enzymes activities of GSH-Px, GRD, SOD and CAT as well as significant increase of lipid peroxidation levels were observed in the liver of microcystin LR-treated rat. These results showed that acute exposure of microcystin LR could result in perturbation of the antioxidant enzymes, suggesting the involvement of oxidative stress in the pathogenesis of microcystin LR-induced toxicity.

2.4. Oxidative Stress Caused by Other Factors

Other factors such as radiation and temperature may also induce hepatic oxidative stress. The oxidative stress induced through exposure of mobile phone-like radiation has been investigated in the liver of guinea pigs [61]. The results showed that after radiation exposure, the levels of MDA and total nitric oxide were significantly increased and the activities of SOD, myeloperoxidase and GSH-Px were reduced in the liver of guinea pigs. Additionally, the severity of oxidative damage was increased along with the duration of radiation exposure. The results suggested that mobile phone-like radiofrequency radiation could induce oxidative damage in liver, implying the adverse effect of mobile phone use. Moreover, study observed that cold stress could lead to decrease in CAT, SOD and GSH-Px activities in rat liver when the rats were kept at 10 °C for a week, which indicated that cold stress may cause hepatic damage which is associated with oxidative stress [62].

Benzoyl peroxide is a substance with strong oxidizing capacity, and broadly used as flour bleaching agent. The hepatic antioxidant status and ATPases were affected by benzoyl peroxide in mice [63]. Following benzoyl peroxide exposure, SOD activity was reduced significantly, whereas the content of MDA was increased in liver tissue. The activities of Ca^{2+}-ATPase and Mg^{2+}-ATPase in liver were also significantly decreased by benzoyl peroxide. In another study, the effect of ZnO_2 nanoparticles, a common cosmetic component, on cellular oxidative stress in mouse liver was investigated [64]. After exposure to ZnO_2 nanoparticles, viability of hepatic cells was decreased in concentration-dependent manner, and decrease in antioxidant enzyme levels as well as increase in DNA adduct.

Studies have suggested that maternal high-fat diet feeding could raise the incidence of metabolism-related diseases in offspring, including chronic liver disease. Zhang *et al*. [65] found that maternal high-fat diet increased the level of plasma triglyceride and hepatic TBARS significantly. The size of lipid droplets in the liver of rat offspring was also increased. Expression of antioxidant defense genes, such as GSH-Px-1, Cu/Zn-SOD, and paraoxonase enzymes, were significantly lowered in the liver. Up-regulation of the inhibitor of cyclooxygenase-2 and cyclin dependent kinase 4a, and down-regulation of cyclin D1 and phosphorylation of retinoblastoma protein were found in the offspring. These results suggested that maternal high-fat diet might reduce the capacity of antioxidant defense and speed up cellular senescence in hepatic tissue of older offspring. In another study, the effect of high dietary salt on hepatic antioxidant defensing enzyme of fructose-fed rats was investigated [66]. Feeding fructose-fed rats with high-salt diet could trigger hyperinsulinemia and insulin resistance resulting in membrane perturbation. This potentially enhanced hepatic lipid peroxidation in the presence of steatosis, and led to decrease in antioxidant defenses, as observed by reduction of GSH, SOD and CAT activities. These results indicated that consumption of salt-rich diet by insulin-resistant subjects could lead to sodium reabsorption, which may aggravate hepatic lipid peroxidation related to damage antioxidant defenses.

In addition to those liver injury induced by exogenous substances, hepatic oxidative stress has been revealed in other liver diseases and functional disorders. For instance, Messarah *et al.* [67] has found that thyroid dysfunction would increase lipid peroxidation and oxidative stress status in rat liver. In another study, oxidative stress and antioxidant status in patients with autoimmune cholestatic liver diseases (AC) or autoimmune hepatitis (AIH) were investigated [68]. Several markers of oxidative injury and antioxidant components in whole blood, serum, and urine of 49 patients with AC and 36 patients with AIH as well as healthy subjects were assessed. The results showed that both

AC and AIH patients had increased levels in oxidation products of lipid and protein while significant decreased of whole blood GSH level. Protein carbonyl and isoprostane levels were increased and GSH level was gradually decreased with disease severity level (mild to severe fibrosis and cirrhosis) in both AC and AIH patients. In addition, AIH patients had higher levels of aldehydes and GSH-Px activity and lower protein carbonyl levels compared to AC patients. In patients with nonalcoholic fatty liver disease (NAFLD), the oxidative stress and antioxidant status were changed as well [69]. It was shown that level of TBARS in NAFLD patients was significantly higher than subjects with viral hepatitis or healthy controls. Moreover, the ferric reducing ability of plasma in patients with NAFLD was significantly higher than healthy controls, and diseased control group of patients. These results implied that lipid peroxidation and oxidative stress were significantly increased in patients with NAFLD. Although existence of hepatic oxidative stress in various liver diseases was commonly observed, the relationship between oxidative damage and diseases are causal and not strictly defined.

3. Antioxidants for Prevention and Treatment of Liver Diseases

3.1. Antioxidants for Prevention and Treatment of Alcoholic Liver Diseases

An obvious avenue of alcoholic liver diseases (ALD) prevention would be abstinence; however, abstinence is not easy to maintain due to the high rate of recidivism in alcoholics [14]. As mentioned above, ALD develops from simple steatosis to more severe disease forms including hepatitis, fibrosis, cirrhosis, and even hepatocellular carcinoma, which implies that preventing disease development at the early stage would be more effective than receiving treatment at end-stage of liver disease. Notably, TNF, a group of cytotoxic pro-inflammatory cytokines, is thought to play a vital role in initiation of liver damage [70]. Increasing evidence has indicated that oxidative stress might act together with endotoxins to increase TNF production. Increased circulating TNF-α stimulates TNF-α receptors of cell surface, which leads to activation of the stress-related protein kinases JNK and IKKβ, resulting in increased production of additional inflammatory cytokines, and reduced insulin sensitivity. Consequently, the inhibition of TNF is regarded as a therapy to block fatty liver and relieve liver injury [70,71]. Pharmacological and genetic manipulation of TNF have been attempted to treat liver disease. For example, anti-TNF antibodies or knocking out TNF-R1 have been treated to mice to protect against the development of ALD. However, since liver regeneration requires low "basal" contents of TNF, down regulating but not blocking totally TNF activity is a preferred therapeutic intervention for liver disease [71,72]. With better understanding of the mechanism that regulates the initiation and advancement of ALD, antioxidant therapy could be developed as directed therapy to prevent or treat ALD [32,37,73,74]. It has been demonstrated that many food and plants, such as vegetables, fruits, tea, cereals, medicinal plants, microalgae, edible macro-fungi, and wild flowers, have abundant natural antioxidants, and possess the ability of eliminating free radicals and protecting the liver from oxidative stress [75–83], and thus might be beneficial for liver diseases.

In recent years, a great number of natural plants has been attempted to eliminate hepatic damage induced by ethanol in animal models, and the bioactive compounds that are responsible for relieving oxidative stress are usually indistinctly ascribed to polyphenols and flavonoids compounds [42,84–87]. For example, it has been found that green tea, containing abundant water-soluble antioxidants, showed positive effect on the antioxidant abilities in rat liver with chronic ethanol treatment [84]. It was shown that significant reduction of enzymatic and non-enzymatic antioxidants levels, as well as increased levels of lipid and protein modifications was induced by ethanol diet. After administration of green tea, interestingly, the enzymes activity and level of non-enzymatic antioxidants as well as lipid and protein oxidation products were partly normalized. The effects of some natural products on hepatic alcoholic damage associated with oxidative stress were summarized in Table 1, which indicate that anti-oxidative treatment is an encouraging method to reduce alcoholic liver injury. Besides phenolic compounds, more specific bioactive compounds should be further identified and isolated in the future.

Table 1. The effects of antioxidants/plants on alcoholic liver damage. Up-arrow means increase and up-regulation, and down-arrows means decrease and down-regulation.

Models (Prevent/Treatemnt)	Materials	Effect	Dose (Dose-Effect)	Bioactive Compounds	References
Rats treated with ethanol diet (Prevent)	Green tea	↑ Enzymes, non-enzymatic antioxidants; ↓ lipid and protein oxidation	7 g/L in ethanol Lieber-DeCarli diet	Epicatechin, epicatechin gallate	[84]
Rats treated with ethanol (Prevent)	*Ziziphus mauritiana* leaf	↓ ALT, AST, ALP, total bilirubin, CAT; ↑ GSH-Px, glutathione reductase and SOD	200 and 400 mg/kg b.w. (Dose-effect)	Tannins, saponins and phenolic compounds	[42]
Rats sub-chronically exposed to ethanol (Prevent)	*Amaranthus hypochondriacus* seed	↓ MDA, NADPH; ↑ Cu, Zn-SOD	140 g/kg in diet	Total phenols	[87]
Mice with acute alcohol-induced liver injury (Prevent)	Peduncles of Hoveniadulcis	↓ ALT, AST, MDA; ↑ SOD, GSH-Px	100, 350 and 600 mg/kg b.w. (Dose-effect)	Non-starch polysaccharide	[86]
Rats treated with ehanol (Prevent)	Methanolic extract from *Hammada scoparia* leaves	↓ Aminotransferase, glycogen synthase kinase-3 β, lipid peroxidation; ↑ GSH-Px	200 mg/kg b.w.	Phenolic compounds	[85]
Mice with chronic alcoholic liver damage (Prevent)	Jujube honey	↓ Lipoprotein oxidation, AST, ALT, MAD, 8-hydroxy-2-deoxyguanosine; ↑ GSH-Px	27 and 54 g /kg b.w. (Dose-effect)	Phenolic acids	[88]
Mice with alcohol-induced hepatotoxicity (Treatment)	Freeze-dried, germinated and fermented mung bean	↑ Antioxidant levels, NO	200 and 1000 mg/kg b.w.		[89]
Chronic ethanol exposure in rats (Prevent)	Virgin olive oil	↓ Transaminases levels, hepatic lipid peroxidation; ↑ GSH-Px, SOD and CAT	5% (wt/wt) in diet	Tocopherols, chlorophyll, total polyphenols	[90]

In addition to these natural products, many single compounds have been investigated for their role in eliminating oxidative stress, such as L-theanine, vitamin E, *N*-acetyl cysteine, raxofelast and betaine [91]. L-theanine, a unique amino acid in green tea, has been proven to possess the ability to prevent alcoholic hepatic damage via augmenting antioxidant capacities [91]. The ethanol-stimulated increase of ALT, AST, and MDA and reduction of antioxidant enzymes activities including the activities of SOD, and CAT, as well as level of GSH were significantly inhibited by L-theanine. The regulation of L-theanine on alcohol-induced fat droplets was further confirmed by histopathological examination. Besides, vitamin E is considered to be beneficial for prevention of diseases associated with oxidative stress because of its remarkable anti-oxidative properties. Kaur *et al*. [92] has proven that vitamin E could restore the redox status, prevent oxidative stress and reduce apoptosis, and could be used as a prospective curative agent for ethanol-induced hepatic oxidative injury. Moreover, raxofelast, an analog of vitamin E, possesses the ability to inhibit lipid peroxidation in mice exposed to ethanol [93]. Raxofelast diminished the increased hepatic NF-κB activity, reduced serum ALT and liver triglycerides, lowered hepatic MAD levels, prevented liver GSH depletion, decreased Toll-like receptor-4, TNF-α, IL-6 and intercellular adhesion molecule-1 hepatic gene expression. It has been suggested that raxofelast blunted the inflammatory cascade and liver damage during chronic ethanol exposure. *N*-acetyl cysteine, a scavenger of ROS, may reverse alcoholic liver damage, and alter activities of matrix metalloproteinases [94]. Furthermore, it was shown that the ethanol-induced oxidative stress could be inhibited effectively by betaine, which is also responsible to its hepatoprotection [95].

Betulinic acid is a pentacycliclupane-type triterpene, and has a wide range of bioactivities. Yi *et al*. [96] has reported that pre-treatment of betulinic acid could significantly reduce the serum levels of ALT, AST, total cholesterol, and triacylglycerides in the mice treated with alcohol. Hepatic levels of GSH, SOD, GSH-Px, and CAT were remarkably increased, while MDA contents and microvesicular steatosis in the liver were decreased by betulinic acid. It was suggested that the hepatoprotective effect of betulinic acid is associated with the improvement of antioxidant enzymes capacity, primarily via enhancement of the tissue redox system and protection of the antioxidant system in the liver. Demethyleneberberine, a natural mitochondria-targeted antioxidant found in Chinese herb Cortex *Phellodendri chinensis*, has been demonstrated the ability of inhibiting oxidative stress and steatosis in acutely/chronically ethanol-fed mice [97].

3.2. Antioxidants for Prevention and Treatment of Non-Alcoholic Fatty Liver Diseases

NAFLD is characterized by abnormal fatty acids deposition in the liver cells of patients without excessive alcohol intake, viral infection or other hepatoxins, including a broad spectrum of histological irregularities [98]. Notably, obesity is considered to be the main risk factor for the development of NAFLD and the main driver of rapid rise of NAFLD prevalence [99]. The oxidative stress of endoplasmic reticulum induced by free fatty acid in the liver might contribute to the hepatic injury, progressive fibrosis and even cirrhosis [100]. In Table 2, certain antioxidants or plants were attempted to reduce liver injury induced by high fat diet in experimental animals, which indicated that most of them showed both antioxidant and hepato-protective effects. Furthermore, in a clinical trial that aims to systematically evaluate the effect of antioxidant supplements, it was found that AST levels, but not of ALT levels were reduced significantly in patients with NAFLD by antioxidant intervention. It should be pointed out that, however, data obtained is so far insufficient to figure out whether dietary supplements is beneficial or useless for patients with NAFLD [98]. To address this issue, large-scaled of prospective randomized clinical studies on this topic is quite necessary.

It has also been indicated that insulin resistance, oxidative stress, and the inflammatory cascade play a vital role in the pathogenesis of NAFLD by animal study. Data from clinic trial indicated that insulin resistance is a high risk factor of NAFLD. Recent studies have shown that insulin resistance is present in surrounding tissue and live of almost all NAFLD patients [44]. The severity of insulin resistance is correlated with the progression of disease. However, the role of oxidative

stress and inflammation in the pathogenesis of NAFLD cascade need to be further studied in human. In the setting of obesity, increased fatty acids and other related metabolites enhance oxidative phosphorylation and ATP generation, leads to increase ROS/RNS production and oxidative stress. Multiple stress-sensitive kinase signaling cascades, such as JNK and IKKβ, are activated by the increased oxidative stress. Once activated, these kinases are able to phosphorylate multiple targets, including the insulin receptor and the family of IRS proteins [101]. Insulin action is impaired by the abnormal serine/threonine phosphorylation in insulin receptor and IRS proteins such as IRS-1 and IRS-2, resulting in insulin resistance. In hyperglycemia caused by insulin resistance, intensive redox reactions occur during the process of protein glycation, generating a great deal of ROS [102]. Additionally, hyperglycemia and high insulin levels stimulate fatty acids synthesis and result in increasing lipid droplets storage within hepatocytes. The excessive intracellular levels of lipid can induce hepatocytes dysfunction or death. The increased ROS also act on large molecules such as poly-unsaturated fatty acids to initiate lipid per-oxidation, which further change the fluidity and permeability of the cell membrane. The inflammatory infiltration induced by lipid per-oxidation may also result in liver inflammation and necrosis, and even fibrosis. In mitochondrion, lipid peroxidation reduces the activity of mitochondrial respiratory chain, and thereby produces more ROS and increase oxidative stress. The prolonged oxidative stress may favor insulin resistance circularly, acting like a vicious circle. Then, the persistent exposure of oxidative stress and hyperglycemia contribute to NAFLD [103,104]. In addition to obesity, other risk factors such as drugs, re-feeding syndrome and other disorders are considered. For example, streptozotocin-induced diabetic rats constitutes as the model of oxidative stress. It was indicated that supplementation of alpha-tocopherol increased alpha-tocopherol in liver, but not in plasma [105]. Diet supplementation of acai, a promising source of natural antioxidants, could increase mRNA levels of gamma-glutamylcysteinesynthetase and GSH-Px in liver tissue, and decrease ROS produced by neutrophils. In addition, supplementation with acai could decrease thiobarbituric acid-reactive substances levels, and increase reduced GSH content in the liver. Moreover, the effect of dietary supplementation of vitamins C and E on oxidative stress and antioxidant redox systems was studied in streptozotocin-induced aged diabetic rats [106]. GSH-Px activity and the concentration of vitamin E in liver were lower, whereas lipid peroxidation levels in liver, and contents of ALT and AST in plasma were higher in the diabetic group than in the control group and were mostly restored by vitamins C and E treatment. Furthermore, the combined treatment with vitamin C, vitamin E, and Se showed a curative effect against the liver injury in streptozotocin-induced diabetic rats [107]. The effects of some antioxidants/plants on liver of streptozotocin-induced diabetic rats are summarized in Table 3.

Table 2. The effects of some antioxidants/plants on NAFLD.

Models (Prevent/Treatment)	Antioxidant/Plants	Effects	Dose (Dose-Effect)	Bioactive Compounds	References
Diabetic rats fed on a high fat thermolyzed diet (Prevent)	Omega 3-polyunsaturated fatty acids	↑ SOD, CAT; ↓ triglycerides, non-esterified fatty acid, lipoperoxidation	3.0% in diet	Omega 3-polyunsaturated fatty acids	[108]
Mice fed with high-fat diet (Prevent and treatment)	*Moringa oleifera* leaves; haw pectic oligosaccharide; *Thymbra spicata*	↑ GSH; ↓ ALT, AST, ALP, lipid peroxidation	50, 150 and 300 mg/kg b.w. (No dose–effect)	Haw pectic oligosaccharide	[109–111]
Liver damage in diet-induced atherosclerotic rats (Prevent)	*Tulbaghia violacea* rhizomes	↓ LDH, AST, ALT, ALP, bilirubin antioxidation	100 mg/kg b.w.		[112]
Rabbits with high-fat diet (Prevent)	Apolipoprotein A–I	↑ SOD, GSH-Px; ↓ iNOS, MDA	15 mg/kg b.w.		[113]
WeRats fed a high-fat diet (Prevent)	Black cabbage sprout	↑ SOD, CAT, NADPH, GSH-Px, GRD GST	250 and 500 mg/kg b.w. (Dose–effect)		[114]

Table 3. The effects of some antioxidants/plants on liver of streptozotocin-induced diabetic rat.

Models (Prevent/Treatment)	Materials	Effects	Dose (Dose-Effect)	References
Streptozotocin-induced diabetic aged rats (Prevent)	Vitamins C and E	Antioxidation, hepatoprotection		[106]
Streptozotocin-induced diabetic rats (Prevent)	Acai	Antioxidation, hepatoprotection	2% (*w*/*w*) in standard diet	[115]
Streptozotocin-induced diabetic rats (Prevent)	*Herba bidentis*	Antioxidation, hepatoprotection	5 mL/kg	[116]
Streptozotocin-induced diabetic rats (Prevent)	(−)-Epicatechin	Antioxidation	15 and 30 mg/kg (Dose–effect)	[117]
Streptozotocin-induced diabetic rats (Treatment)	Stobadine		24.7 mg/kg	[118]
Streptozotocin-induced diabetic mice (Prevent)	*Terminalia glaucescens* leaves	Antioxidation	100 and 300 mg/kg (Dose–effect)	[119]
Streptozotocin-induced diabetic rats (Treatment)	Berberine	Antioxidation	75, 150 and 300 mg/kg (Dose–effect)	[120]
Streptozotocin-induced diabetic rats (Prevent)	*Aloe vera* leaves		300 mg/kg	[121]
Streptozotocin-induced diabetic rats (Treatment)	*N*-Acetylcysteine	Antioxidation	1.5 g/kg	[122]
Streptozotocin-induced diabetic rats (Treatment)	*Oroxylum indicum* stem bark	Antioxidation	250 mg/kg	[123]
Streptozotocin-induced diabetic rats (Treatment)	Maslinic acid	Antioxidation	40, 80 and 160 mg/kg (Dose–effect)	[124]
Streptozotocin-induced diabetic rats (Treatment)	Resveratrol	Antioxidation	20 mg/kg	[125]
Streptozotocin-nicotinamide induced diabetic rats (Prevent)	*Stevia rebaudiana*	Antioxidation		[126]

3.3. Antioxidants for Prevention and Treatment of Liver Diseases Induced by Other Factors

Since liver is an essential organ for detoxification and metabolism, and all pharmaceuticals make their way to the liver, for storage and therefore it is more prone to damage [127,128]. Paracetamol is widely used to relieve pain and reduce fever. Although use of paracetamol at its recommended dose is generally safe, overdose could still cause severe hepatic damage in many cases. As mentioned above, paracetamol may induce a remarkable increase of MDA and nitrite as well as nitrate in the liver, apart from a significant reduction in total SOD and Cu/Zn-SOD activity. Models of paracetamol-induced liver damage in mice/rats are widely used to study antioxidant and hepatoprotective effects of antioxidants/plants. For example, Rasool *et al*. [129] studied hepatoprotective and antioxidant effects of Gallic acid in paracetamol-induced liver damage in mice. It was shown that Gallic acid possessed antioxidant and hepatoprotective effects. In addition to paracetamol, some other drugs such as doxorubicin, tert-butyl hydroperoxide and D-galactosamine may also induce liver injury, which is possibly associated with the rise of oxidative stress. The effects of certain antioxidants/plants on paracetamol and other drugs-induced liver damage are summarized in Table 4. As seen from Table 4, a conclusion could be drawn that materials possessing antioxidant activity also hold capacity of hepatoprotection in animal model, which implies the correlation between antioxidative property of these compounds and their hepatoprotective effect.

Table 4. The effects of some antioxidants/plants on drugs-induced liver damage.

Models (Prevent/Treatment)	Materials	Effects	Dose (Dose-Effect)	References
Paracetamol-induced liver toxicity in mice (Prevent)	Gallic acid	Antioxidation, hepatoprotection	100 mg/kg	[129]
Paracetamol-induced liver toxicity in mice (Prevent)	Sauchinone	Antioxidation, hepatoprotection	30 mg/kg	[130]
Paracetamol-induced liver toxicity in mice (Prevent)	Genistein	Antioxidation, hepatoprotection	50, 100 and 200 mg/kg (Dose-effect)	[131]
Paracetamol-induced liver toxicity in mice (Prevent)	*Phyllanthus niruri*	Antioxidation, hepatoprotection	100 mg/kg	[132]
Paracetamol-induced liver toxicity in mice (Prevent)	*Polyalthia longifolia* leaves	Antioxidation, hepatoprotection	200 mg/kg	[133]
Paracetamol-induced liver damage in rats (Prevent)	*Boerhaavia diffusa* leaves	Antioxidation, hepatoprotection	100, 200, 300 and 400 mg/kg/day (No dose-effect)	[134]
Paracetamol-induced liver damage in rats (Prevent)	Saponarin from *Gypsophila trichotoma*	Antioxidation, hepatoprotection	80 mg/kg/week	[135]
Lipopolysaccharide-induced liver injury in rats (Prevent)	Carnosic acid	Antioxidation, hepatoprotection	15, 30 and 60 mg/kg (Dose-effect)	[136]
D-Galactosamine-induced liver injury in rats (Prevent)	Combination of selenium, ascorbic acid, β-carotene, and α-tocopherol	Antioxidation, hepatoprotection		[137]
D-Galactosamine-induced liver injury in rats (Prevent)	*Leucasaspera*	Antioxidation, hepatoprotection	200 and 400 mg/kg (No dose-effect)	[138]

Table 4. *Cont.*

Models (Prevent/Treatment)	Materials	Effects	Dose (Dose-Effect)	References
D-Galactosamine-induced liver injury in rats (Prevent)	Swertiamarin from *Enicostemma axillare*	Antioxidation, hepatoprotection	100 and 200 mg/kg (No dose-effect)	[139]
Lipopolysaccharide/ D-galactosamineinduced liver injury in rats (Prevent)	Curcumin	Antioxidation, hepatoprotection	100 mg/kg	[140]
Lipopolysaccharide/ D-galactosamineinduced liver injury in rats (Prevent)	betulinic acid	Antioxidation, hepatoprotection	20 and 50 mg/kg (No dose-effect)	[141]
Lipopolysaccharide/ D-galactosamine induced hepatitis in rats (Prevent)	*Tridaxprocumbens*	Antioxidation	300 mg/kg	[142]
Doxorubicin-induced liver injury in rats	*N*-acetylcysteine	Antioxidation, hepatoprotection	10 mg/kg	[143]
Cisplatin-induced liver injury in rats (Prevent)	Tomato juice	Antioxidation, hepatoprotection		[144]
Tert-butyl hydroperoxide-induced liver injury in rats (Prevent)	Propolis	Antioxidation, hepatoprotection	50 and 100 mg/kg (No dose-effect)	[145]
Tamoxifen-induced liver injury in mice (Prevent)	Catechin	Antioxidation	40 mg/kg	[146]
Hepatic steatosis stimulated with tunicamycin (Treatment)	Melatonin	↓ ER stress, expression of miR-23a		[147]
Ethionine-induced liver injury in mice (Prevent)	Melatonin	Antioxidation, hepatoprotection	3 mg/kg	[148]

Many pollutants and toxic substances could cause oxidative stress/damage of liver as mentioned above. Among pollutants and toxins that have been used to model hepatic injury in animals for studying effects of antioxidants/plants on pollutant-induced liver damage, carbon tetrachloride (CCl_4) is most widely used. In CCl_4-induced liver injury model, oxidative stress could be provoked, which prompts lipid peroxidation that injure hepatocellular membrane, followed by substantial release of pro-inflammatory chemokines and cytokines, which in consequence of liver damage [10]. A large amount of plants, especially medicinal plants, has been investigated to eliminate the hepatic damage stimulated by CCl_4. For example, *Coptidis rhizome*, a traditional Chinese medicinal plant used to clear heat and scavenge toxins, belongs to liver meridian in Chinese medicinal practice [149,150]. The effect of *Coptidis rhizome* and its bioactive compound berberine on CCl_4-induced chronic and acute hepatotoxicity in rats has been thoroughly studied by our research group [10,30,127]. We have found that *Coptidis rhizome* might act as an antioxidant to relieve CCl_4-induced oxidative stress and hepatic damage. The mechanism may partly be ascribed to the reduced phosphorylation of Erk1/2 expression when exposed to oxidative stress [10]. The effects of some antioxidants/plants on toxic substances-induced liver damage are summarized in Table 5. It is particularly worth noting that Nrf2 could be activated by several antioxidants/plants in dimethylnitrosamine or cadmium induced hepatic injury models [151–153]. Antioxidant could induce both modification of inhibitor of Nrf2 (INrf2) cysteine 151 and PKC-mediated phosphorylation of Nrf2 serine 40 to release Nrf2 from INrf2. The dissociated and activated Nrf2 then translocates to the nucleus, binds to ARE and up-regulates antioxidants gene expression, which protects cells and relieves injury induced by oxidative stress [154]. Although most of the studies shown in Table 5 suggested the simultaneous role of these natural products as antioxidative and hepatoprotective agents, the related mechanisms and signal pathways have not yet fully studied.

Accumulating evidence demonstrated that ROS could lead to protein modification, lipid peroxidation, DNA damage and therefore acts as the initiator or promoter of carcinogenesis [155–157]. As the first line defense in suppressing tumor initiation, antioxidants are treated as one of the promising strategies to prevent liver cancer. Furthermore, it has been reported that the combination of certain chemotherapeutic drugs and antioxidants could reduce drug resistance, sensitizing the liver cancer cells to chemotherapeutics and therefore improving the efficacy of anti-cancer therapy [158]. Our previous studies demonstrated that *Coptidis rhizome* and berberine are promising agents to fight against liver cancer due to their hepatoprotective and antioxidant properties [155,157,159,160]. In all, cumulative evidence from epidemiological and clinical studies showed that consumption of suitable antioxidants from natural sources may beneficial in fighting against cancer without obvious adverse effects. Besides liver cancer, oxidative injury-associated liver damage induced by other disorders has also been mentioned for confirming the use of antioxidants in the related diseases. For example, it was found that taking catechin from green tea could reduce injury of liver in cholestatic rats induced by bile duct ligation [161]. Allopurinol, a competitive xanthine oxidase inhibitor, has also been used to reduce systemic oxidative stress. The xanthine oxidase over-activity is suggested to play a role in the altered intestinal permeability in cirrhosis, it was found in an open-label pilot study that changes in intestinal permeability correlated to changes in MDA serum values after allopurinol treatment [162]. Additionally, treatment with allopurinol in bile-duct ligation rats and TAA induced liver injury was shown to reduce ROS and thus attenuate brain edema [163]. Effects of certain antioxidants/plants on other substances-induced liver damage are summarized in Table 6, which suggested that some antioxidants possess anti-tumor and hepatoprotective effects collectively *in vivo*, but the relationship and mechanisms need further exploration.

Notably, melatonin, *N*-acethyl-5-metoxytryptamine, a famous hormone synthesized mainly by the pineal gland, has been demonstrated as having striking antioxidant properties in numerous studies. It has the remarkable capability to scavenge both ROS and RNS, and block transcriptional factors of pro-inflammatory cytokines. Recently, it has been applied to the treatment of liver disease in terms of reducing oxidative stress [164]. A variety of liver disease models, such as streptozocin-induced diabetic rats and TAA-induced or bile-duct ligated fibrosis rats, melatonin administration showed hepato-protection partially via improving oxidative damage. As a matter of fact, it has been demonstrated that melatonin is even better antioxidant than vitamin E and C in the contexts of certain disease. A comparative study of the protective effects of melatonin and vitamin E on extra-hepatic bile duct ligation in rats indicated that melatonin is much more efficient than vitamin E in reducing the cholestasis parameters, decreasing lipid peroxidation and restoring anti-oxidative enzymes [165,166]. Further investigations are required to evaluate antioxidant and hepato-protective effect of melatonin in clinic.

Table 5. The effects of some antioxidants/plants on toxins-induced liver damage.

Model (Prevent/Treatment)	Antioxidant/Plant	Effects	Dose/(Dose–Effect)	Bioactive Compounds	References
CCl_4-induced liver damage in rats (Prevent)	*Coptidis rhizome* and berberine	↑ SOD; ↓ ALT, AST, Erk1/2	Berberine: 120 mg/kg b.w. Extract: 800 mg/kg b.w.	Berberine	[10]
CCl_4-induced liver damage in rats (Prevent)	Friedelin isolated from *Azima tetracantha* leaves	↑ SOD, CAT, GSH, GSH-Px; ↓ ALT, AST, LDH			[59]
CCl_4-induced liver damage in rats (Treatment)	*N*-butanol fraction of *Actinidias deliciosa* roots	↑ GSH; ↓ ALT, AST, MDA	(Dose–effect)	Oleanolic acid	[167]
CCl_4-induced liver damage in rats (Prevent)	*Silybum marianum* seeds	↑ GSH; HDL/LDL; hepatoprotection	100 mg/kg b.w.		[168]
CCl_4-induced liver damage in rats (Prevent)	*Dioclea reflexa* seeds	↑ SOD, CAT; ↓ Transaminases, MDA	5 mg/kg (acute) 2.5 mg/kg b.w. (chronic)		[169]
CCl_4-induced liver damage in rats (Prevent)	*Morus bombycis*, 2,5-dihydroxy-4,3′-di (β-D-glucopyranosyloxy)-*trans*-stilbene	↓ Lipid peroxidation; hepatoprotection	100, 300 and 500 mg/kg b.w. (No dose–effect)		[170,171]
CCl_4-induced liver damage in rats (Prevent)	*Nigella sativa*, *Urticadioica*	↑ Antioxidant enzyme; ↓ lipid peroxidation; hepatoprotection	*Nigella sativa*: 0.2 mg/mL *Urtica dioica*: 0.2 mg/mL		[172]
CCl_4-induced liver damage in rats (Prevent)	*Pleurotusostreatus* (oyster mushroom)	↑ GSH, CAT, SOD, GSH-Px; ↓ ALT, AST, ALP, MDA	200 mg/kg b.w.		[173]
CCl_4-induced liver damage in rats (Prevent)	*Cytisusscoparius*	↑ GSH, CAT, SOD, GSH-Px, GST, GRD; ↓ ALT, AST, LDH	250 and 500 mg/kg (No dose–effect)		[174]
CCl_4-induced liver damage in rats (Prevent)	Ethanol extract of *Phellinusmerrillii*	↑ CAT, SOD, GSH-Px; ↓ ALT, AST	0.5, 1 and 2 g/kg b.w. (No dose–effect)		[175]
CCl_4-induced liver damage in rats (Prevent)	*Ginkgo biloba*	↑ GSH, SOD, CAT, GSH-Px, GRD, albumin; hepatoprotection	25 and 50 mg/kg b.w. (No dose–effect)		[176]

Table 5. *Cont.*

Model (Prevent/Treatment)	Antioxidant/Plant	Effects	Dose/(Dose–Effect)	Bioactive Compounds	References
CCl_4-induced liver damage in mice (Prevent)	Protein isolate from *Phyllanthus niruri*	↑ SOD, CAT; ↓ ALT, ALP; lipid peroxidation	5 mg/kg b.w.		[177]
CCl_4-induced liver damage in mice (Prevent)	Kahweol and cafestol (Coffee)	↓ ALT, AST, cytochrome P450 2E1, lipid peroxidation	Kahweol or cafestol: 10–100 mg/kg b.w. (Dose–effect)	Kahweol and cafestol	[178]
CCl_4-induced liver damage in rats (Prevent)	*Cirsium setidens*	↑ GSH-Px; SOD; hepatoprotection	500 mg/kg b.w.		[179]
CCl_4-induced liver damage in rats (Prevent)	Curcumin and saikosaponin A	↑ SOD, GSH; ↓ MDA; hepatoprotection			[180]
CCl_4-induced liver damage in rats (Prevent)	Ethanolic extract of *Momordica tuberosa* tubers	Antioxidation, hepatoprotection			[181]
CCl_4-induced liver damage in rats (Prevent)	Oregano and rosemary	↓ AST, ALT, ALP; antioxidation	20 g/kg b.w.		[182]
CCl_4-induced liver damage in rats (Prevent)	*Enicostemma axillare*	Antioxidation, hepatoprotection	100 and 200 mg/kg b.w. (No dose–effect)		[139]
CCl_4-induced liver damage in rats (Prevent)	*Ficuscarica* leaves and fruits, *Morus alba* root barks	↑ CAT, SOD, GSH; ↓ MDA, AST, ALT, ALP	50 and 150 mg/kg b.w. (No dose–effect)		[183]
CCl_4-induced liver damage in rats (Prevent)	*Podophyllum hexandrum*	↑ GSH, GSH-Px, GRD, SOD, GST; ↓ AST, ALT, LDH	20, 30 and 50 mg/kg b.w. (No dose–effect)		[184]
CCl_4-induced liver damage in rats (Prevent)	*Ficusreligiosa* roots	↑ CAT, GSH-Px, GRD, SOD, GST; ↓ lipid peroxidation; hepatoprotection			[185]
CCl_4-induced liver damage in rats (Prevent)	Dehydroabietylamine, *Carthamus tinctorious*	↓ AST, ALT, ALP; antioxidation			[186]

Table 5. *Cont.*

Model (Prevent/Treatment)	Antioxidant/Plant	Effects	Dose/(Dose–Effect)	Bioactive Compounds	References
CCl_4-induced liver damage in rats (Prevent)	Artemetin, *Vitexglabrata*	↑ SOD, CAT, GSH-Px; ↓ AST, ALT, ALP, lipid peroxidation, TB			[187]
CCl_4-induced liver damage in mice (Prevent)	Blueberry anthocyanins	↑ SOD, CAT, GRD, glycogen; ↓ AST, ALT, MDA			[188]
CCl_4-induced liver damage in rats (Prevent)	*Matricaria chamomilla*	↑ SOD, CAT, GSH-Px, GSH; ↓ AST, ALT, MDA	50, 100 and 200 mL/kg b.w. (No dose–effect)		[189]
CCl_4-induced liver damage in mice (Prevent)	*Lysimachia clethroides*	↑ SOD; ↓ AST, ALT, MDA	150, 300 and 600 mg/kg b.w. (No dose–effect)		[190]
CCl_4-induced liver damage in rats (Prevent)	*Garcinia indica* fruit rind	↑ SOD, CAT, GRD, GSH-Px, GSH; ↓ AST, ALT, MDA	400 and 800 mg/kg b.w. (No dose–effect)		[191]
CCl_4-induced liver damage in rats (Prevent)	*Agaricus blazei*	↑ GSH, GRD; ↓ AST, ALT, MDA	500 mg/kg b.w.		[192]
CCl_4-induced liver damage in rats (Prevent)	*Nerium oleander* flowers	↑ SOD; ↓ AST, ALT, ALP, MDA	100, 200 and 400 mg/kg b.w. (No dose–effect)		[193]
CCl_4-induced liver damage in rats (Prevent)	*Hybanthus enneaspermus*	↓ AST, ALT, ALP, TB; antioxidation	200 and 400 mg/kg b.w. (No dose–effect)		[194]
CCl_4-induced liver damage in mice (Treatment)	Anthocyanins in black rice bran	↑ SOD, GSH-Px; hepatoprotection	200, 400 and 800 mg/kg b.w. (No dose–effect)		[195]
CCl_4-induced liver damage in rats (Prevent)	*Roureainduta*	↑ SOD, CAT, GSH, GSH-Px; ↓ AST, ALT, total bilirubin;	500 mg/kg b.w.		[196]
CCl_4-induced liver damage in rats (Prevent)	Proanthocyanidins extracted from grape seeds	↑ SOD, GSH, GSH-Px, CAT; ↓ lipid accumulation, liver injury, DNA damage	400 mg/kg b.w.	Proanthocyanidins	[197]

Table 5. *Cont.*

Model (Prevent/Treatment)	Antioxidant/Plant	Effects	Dose/(Dose–Effect)	Bioactive Compounds	References
CCl_4-induced liver damage in mice (Prevent)	*Veronica ciliata*	↑ SOD, GSH; ↓ ALT, AST, ALP	150, 300 and 600 mg/kg b.w. (No dose–effect)		[198]
CCl_4-induced liver damage in rats (Prevent)	*Subereamollis*	↑ SOD, GSH, GSH-Px, CAT; ↓ ALT, AST, ALP, MDA	100, 200 and 400 mg/kg b.w. (No dose–effect)		[199]
CCl_4-induced liver damage in rats (Prevent)	*Solanum xanthocarpum* leaves	↑ SOD, CAT, GSH, GST; ↓ ALT, AST, ALP, LDH	100 and 200 mg/kg b.w. (No dose–effect)		[200]
CCl_4-induced liver damage in rats (Prevent)	*Allopurinol*	Modulation of NF-κB, cytokine production and oxidative stress	50 mg/kg b.w.		
CCl_4 and H_2O_2 induced liver damage in goat (Prevent)	*Ocimumbasilicum*, *Trigonellafoenum-graecum*	Antioxidation			[201]
TAA-induced liver injury (Prevent)	Genistein	↑ GSH; ↓ MDA, ALT, AST, TB	0.5, 1.0 and 2.0 mg/kg b.w. (No dose–effect)		[202]
TAA-Induced liver Cirrhosis in rats (Prevent)	*Andrographis paniculata* Leaf	Hepato-protection, ↓ ROS, LDH	250 and 500 mg/kg b.w. (No dose–effect)		[203]
TAA-induced hepatotoxicity in rats (Prevent)	coriander	Antioxidant; ↓ ALT, AST, ALP, TBARS, MPO, NO		Phenolic compounds	[204]
TAA-induced fibrosis in mice (Treatment)	*Ger-Gen-Chyn-Lian-Tang*	Antioxidant; anti-fibrosis; modulation on TGF-β/TGF-β receptor signaling	100 and 300 mg/kg b.w. (Dose–effect)		[205]
TAA-induced hepatotoxicity in rats (Treatment)	*Trigonella foenum-graecum*	Antioxidant; hepato-protection; ↓ ALP, MDA			[206]
TAA-induced hepatotoxicity in rats (Treatment)	Allopurinol	Regulating cellular redox-sensitive transcription factors			[163]

Table 5. *Cont.*

Model (Prevent/Treatment)	Antioxidant/Plant	Effects	Dose/(Dose–Effect)	Bioactive Compounds	References
Cigarette smoke-induced oxidative damage in liver of rats (Treatment)	*Sesbania grandiflora* leaves	↑ SOD, GSH, GSH-Px, CAT, GST, GRD, glucose-6-phosphate dehydrogenase; ↓ AST, ALT, ALP	1000 mg/kg b.w.		[207]
Cigarette smoking induced oxidative damage in liver of mice (Prevent)	Vitamin E and selenium	↑ GSH-Px, Se-GSH-Px			[208]
Atrazine exposure rats (Prevent)	Vitamin E	↑ SOD, GSH-Px, CAT, GST; ↓ lipid peroxidation			[209]
Methidathion-induced liver injury in rats (Prevent)	Vitamins C and E	↓ AST, ALT, ALP, MDA;	Vitamin E: 50 mg/kg b.w.;Vitamin C: 20 mg/kg b.w.		[210]
Pesticide (chlorpyriphos and cypermethrin) induced hepatic damage in mice (Prevent)	Black tea	↑ SOD, GSH, GSH-Px, CAT, GRD, GST; ↓ AST, ALT, ALP	200 mg/mL b.w.		[211]
Polychlorinated biphenyls induced hepatic damage in rats (Prevent)	α-Tocopherol	Antioxidation	50 mg/kg. b.w.		[212]
Aflatoxin-induced hepatic injury in rats (Prevent)	*Urticadioica* seed	↑ SOD, GSH-Px, CAT, GRD, GST; ↓ lipid peroxides, hydroxyl radical and hydrogen peroxides	2 mL/rat/day		[213]
Thioacetamide-induced hepatic damage in rats (Prevent)	eugenol	↑ COX-2; ↓ AST, ALT, ALP, bilirubin, CYP2E1, lipid peroxidation; antioxidation	10.7 mg/kg b.w.		[214]

Table 5. *Cont.*

Model (Prevent/Treatment)	Antioxidant/Plant	Effects	Dose/(Dose–Effect)	Bioactive Compounds	References
Lead-induced liver damage in rats (Prevent)	Ginger	↑ SOD, CAT; ↓ MDA	100 mg/kg b.w.		[215]
Dimethylnitrosamine-induced hepatic damage in rats (Prevent)	Anthocyanins from purple sweet potato	↑ Nrf2, NADPH, GSH, GST; ↓ yclooxygenase-2, MDA	50, 100 and 200 mg/kg b.w. (No dose–effect)	Anthocyanins	[151]
Cadmium-induced hepatic injury in rats (Prevent)	Heated garlic juice, ascorbic acid	↑ Nrf2, SOD, CAT; ↓ MDA	Heated garlic juice: 100 mg/kg b.w.; Ascorbic acid: 100 mg/kg b.w.	Ascorbic acid	[152]
Potassium bromate-induced hepatotoxicity of rat (Prevent)	*Launaea procumbens*	↑ SOD, CAT, GSH, GSH-Px, GRD, GST	200 mg/kg b.w.		[216]
Dimethylnitrosamine induced liver fibrosis in rats (Prevent)	*Platycodi radix* root	↑ Nrf2, heme oxygenase-1, NADPH, NQO1, GST; ↓ ALT, AST; anti-fibrotic action	200 mg/kg b.w.	Changkil	[153]
As_2O_3-induced hepatotoxicity in cat (Prevent)	Resveratrol	↑ GSH; ↓ ROS, MDA	3 mL/kg b.w.		[217]
Sodiumarsenite induced liver damage in rats (Prevent)	*Emblica officinalis*	Antioxidation	500 mg in 0.1 mL water, 100 g b.w.		[218]
Trichloroacetic acid induced liver injury in rats (Prevent)	Date palm fruit	↑ SOD, CAT, GSH-Px; ↓ MDA	0.5 and 2 g/L b.w. (No dose–effect)		[219]

Table 6. Effects of some antioxidants/plants on other related liver disease.

Stress (Prevent/Treatment)	Antioxidant/Plants	Effects	Dose (Dose–Effect)	Bioactive Compounds	References
Human liver cancer cell line	*Morinda pubescens* leaves	Antioxidation, cytotoxicity	25, 50, 100 and 250 μg/mL b.w. (Dose–effect)	Hyoscyamine	[220]
Liver cancer of rats (Prevent)	*Chlorella vulgaris*	Antioxidation, antitumour	50, 150 and 300 mg/mL b.w. (Dose–effect)		[221]
Hepatocellular carcinoma	*Caesalpinia bonducella* leaves	↑ SOD, GSH, CAT; ↓ MDA, AST, ALT, ALP; anticancer		Flavonoids, triterpenoids	[222]
Liver cancer of mice (Prevent)	*Pleurotus pulmonarius* (edible mushroom)	Antioxidation, anti-tumor			[158]
Rat with secondary biliary cirrhosis (Prevent)	Silybin	Antioxidation	0.4 g/kg b.w.		[223]
Cholestatic rats with bile duct ligation (Treatment)	Green tea catechin	Antioxidation, reducing hepatic fibrosis	50 mg/kg b.w.		[161]
Bile duct-ligated cholestatic rats (Treatment)	Epigallocatechin-3-gallate	Anti-fibrotic effects, ↓ phosphorylation of Smad2/3 and Akt	5 mg/kg b.w.		[224]
Bile duct-ligated cholestatic rats (Treatment)	*Holothuria arenicola*	↑ SOD, GSH, GST, CAT; ↓ MDA, AST, ALT, ALP	200 mg/kg b.w.	Phenolic compounds, chlorogenic acid, pyrogallol, rutin, coumaric acid	[225]

Table 6. *Cont.*

Stress (Prevent/Treatment)	Antioxidant/Plants	Effects	Dose (Dose–Effect)	Bioactive Compounds	References
Bile-duct ligated Rats (Treatment)	Garlic	↑ GSH; ↓ LDH, TB, MDA, MPO; ↓ TNF-α, TGF-β, MMP-13			[226]
Bile-duct ligated Rats (Treatment)	thymoquinone	↑ SOD, GSH-Px; ↓ MDA	50 mg/kg b.w.		[227]
Bile-duct ligated Rats (Treatment)	*N*-acetylcysteine	↑ GSH, CAT; ↓ MDA, ALT	300 mg/kg b.w.		[228]
Bile-duct ligated Rats (Prevent)	*Phaseolus trilobus*	↑ SOD; ↓ AST, ALT, ALP, LDH, TB, TBARS;	125, 250 and 500 mg/kg b.w. (Dose–effect)		[229]
Bile-duct ligated Rats (Treatment)	Melatonin	↓ TBARS, MPO	10 and 100 mg/kg b.w. (Dose–effect)		[230]
Ischemia/reperfusion in obese rats with fatty liver	Melatonin	↑ Antioxidant enzymes; ↓ AST, ALT, MAD, NOx metabolites	10 mg/kg b.w.		[231]
Bile-duct ligated Rats (Treatment)	*Allopurinol*	↓ ROS, brain edema	100 mg/kg b.w.		[232]
Restraint stress-induced liver injury in mice (Prevent)	*Astragali radix* and *Salviae radix*	Antioxidation, hepatoprotection	50, 100 and 200 mg/kg b.w. (No dose–effect)	Myelophil	[233]

4. Current Anti-Oxidative Therapy in Clinical Trials

Clinical trials are extremely vital and indispensable for the development of anti-oxidative therapy. We looked up the related information of current anti-oxidative therapy in clinic at http://www.ClinicalTrials.gov website. Vitamins, especially vitamin E, are the most frequently studied antioxidant as dietary supplement in clinical trials for liver disease, primarily in phase 2/3. Some other nutritional antioxidants such as zinc and Coenzyme Q10 were studied in phase 2. Compounds including silymarin, metadoxine, *N*-acetylcystein, propofol, and mitoquinone mesylate, which partially act as antioxidant, have been used as drugs or supplement for liver disease. Some of them, such as silymarin, metadoxine and *N*-acetylcysteine, are studied for NAFLD or NASH or ALD in phase 4. For example, the application of antioxidants supplement consisted of siliphos, selenium, methionine, and alpha lipoic acid has been approved in patients with fatty liver and non-alcoholic steatohepatitis in Mexico. Plants and foods such as ginger, green tea extract, and chocolate have been adopted as food supplement for their anti-oxidative properties for liver disease. Furthermore, quercetin and resveratrol, two well-known bioactive compounds isolated from plants, have been studied as food supplement as antioxidants for liver disease in phase 3. Despite certain promising results have obtained in clinical trials, anti-oxidative therapy still has a long way to go. As a matter of fact, many antioxidants are highly effective for prevention or treatment in animal models, but in humans it does not appear to be effective for the treatment of established disease. For example, anti-TNF, which shows desirable treatment effects in animal model, appears not to be effective in patients with acute alcoholic hepatitis. Therefore, translational research is highly important for the application of antioxidant therapy in clinic. In the future, natural plants and bio-active compounds isolated from plants as well as endogenous antioxidants such as melatonin, which have shown strong anti-oxidative ability and hepato-protection effects, should be studied by clinic trials with large patient samples and longer duration time.

5. Conclusions and Prospects

Anti-oxidative therapy, mainly using natural and synthetic antioxidants, represents a reasonable therapeutic approach for the prevention and treatment of liver diseases due to the role of oxidative stress in contributing to initiation and progression of hepatic damage. However, although concept of anti-oxidative therapy has been raised for decades and intensive efforts have been paid, there is a long way to go for the application of antioxidants in liver disease. In current clinical trials, mechanisms by which drugs or compounds treat liver disease might partly attribute to anti-oxidative ability, but plain antioxidants mainly used as dietary supplement to prevent the progress of disease or improve the outcome of patients might also be effective. The complex role of oxidative stress in physiological and pathological processes, lacking studies of underlying mechanisms in humans, and other difficulties in translational research are challenges ahead. In current studies, intervention of antioxidants is explored widely in prevention models rather than treatment model, without elaborated underlying mechanism investigation. For natural plants study, the dose used, especially content of antioxidants, is always blurry, not to mention the shift dose for humans. For those studies in which dose–effect has been investigated, only small portion of plants antioxidant showed dose–effect manner for reducing liver injury, suggesting the complex role of oxidative stress in pathogenesis. In animal study, antioxidants are given to animals via oral or intraperitoneal injection. The route of administration is also an influence for absorption and bio-availability of antioxidants. Additionally, since liver is a central organ for metabolism, oxidative stress in liver diseases interacts with many other diseases such as kidney failure and diabetes, certain models in animal study should be improved. These limitations in current study might result in antioxidants that showed desirable effects for prevention or treatment in animal models, but in humans they do not appear to be effective for the treatment of established disease, which is a barrier for the development of anti-oxidative therapy in clinic. Therefore, translational research is of great importance for anti-oxidative therapy. Considering ROS and oxidative stress act positively in certain circumstances and the difference between animals and

humans, the effective dose and safe dose, duration of treatment, absorption and bio-availability of antioxidants require thorough investigation. Furthermore, in the future, large-scale samples and appropriate duration of anti-oxidative treatment for liver diseases should be performed.

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Abbreviations

AC	Autoimmune cholestatic liver diseases
ADH	Alcohol dehydrogenase
AIH	Autoimmune hepatitis
ALD	Alcoholic liver disease
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ALDH	Aldehyde dehydrogenases
ARE	Antioxidant response element
AST	Aspartate aminotransferase
BHA	Butylated hydroxyanisole
bw	Body weight
CAT	Catalase
CCl_4	Carbon tetrachloride
ER	Endoplasmic reticulum
GSH-Px	Glutathione peroxidase
GSH	Glutathione
GRD	Glutathione reductase
GST	Glutathione S-transferase
HDL	High density lipoprotein
HCV	Hepatitis C virus
IL-6	Interleukin 6
INH	Anti-tuberculosis agent isoniazid
iNOS	Inducible nitric oxide synthase (iNOS)
INrf2	Inhibitor of Nrf2
IKKβ	IκB kinase-β
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinases
Keap1	kelch-like ECH-associated protein-1
LDH	lactate dehydrogenase
LDL	Low density lipoprotein
MDA	Malondialdehyde
MEOS	Microsomal ethanol oxidizing system
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NAFLD	Non-alcoholic fatty liver disease NAFLD
NO	Nitric Oxide
NQO1	NAD(P)H Dehydrogenase, Quinone 1
Nrf1	Nuclear respiratory factor 1
Nrf2	Erythroid 2-related factor 2
PKC	protein kinase C
PPARα	Peroxisome proliferator activated receptor α
RNS	Reactive nitrogen species
ROS	Reactive oxygen species (ROS)
SOD	Superoxide dismutases
TAA	Thioacetamide
TB	Total bilirubin
TBARS	Thiobarbituric acid-reactive substances
TNF	Tumor necrosis factor

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ORIGINAL ARTICLE

Basic Study

Glycyrrhizic acid inhibits apoptosis and fibrosis in carbon-tetrachloride-induced rat liver injury

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Data sharing: No additional data are available.

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Abstract

AIM: To investigate anti-apoptotic effects of glycyrrhizic acid (GA) against fibrosis in carbon tetrachloride (CCl_4)-induced liver injury and its contributing factors.

METHODS: Liver fibrosis was induced by administration of CCl_4 for 8 wk. Pathological changes in the liver of rats were examined by hematoxylin-eosin staining. Collagen fibers were detected by Sirius red staining. Hepatocyte apoptosis was determined by TUNEL assay and the expression levels of cleaved caspase-3, Bax, α-SMA, connective tissue growth factor (CTGF), matrix metalloproteinase (MMP) 2 and MMP9 proteins were evaluated by western blot analysis, and α-SMA mRNA, collagen type Ⅰ and Ⅲ mRNA were estimated by real-time PCR.

RESULTS: Treatment with GA significantly improved the pathological changes in the liver and markedly decreased the positive area of Sirius red compared with rats in the CCl_4-treated group. TUNEL assay showed that GA significantly reduced the number of TUNEL-positive cells compared with the CCl_4-treated group. The expression levels of cleaved caspase-3, Bax, α-SMA, CTGF, MMP2 and MMP9 proteins, and α-SMA mRNA, collagen type Ⅰ and Ⅲ mRNA were also significantly reduced by GA compared with the CCl_4-treated group ($P < 0.05$).

CONCLUSION: GA treatment can ameliorate CCl_4-induced liver fibrosis by inhibiting hepatocyte apoptosis and hepatic stellate cell activation.

Key words: Glycyrrhizic acid; Hepatocyte apoptosis; Liver fibrosis; Hepatic stellate cell; Matrix metalloproteinase

Core tip: This study showed that glycyrrhizic acid (GA) had inhibitory effects on hepatocyte apoptosis and liver fibrosis, which were mainly associated with down-regulation of hepatic stellate cell (HSC) activation, thus regulating fibrotic-related factors, such as expression levels of connective tissue growth factor, MMP2 and MMP9 proteins, and collagen type Ⅰ and Ⅲ mRNA. Collectively, these results demonstrate that GA treatment significantly ameliorated CCl_4-induced liver fibrosis by inhibiting hepatocyte apoptosis and HSC activation, which may provide potential therapeutic strategies for anti-fibrosis.

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INTRODUCTION

Liver fibrosis, induced by various pathological factors, is commonly encountered in many chronic liver diseases, such as chronic viral hepatitis, nonalcoholic steatohepatitis, and alcoholic liver disease[1]. The progression of fibrosis is likely to lead to liver failure, portal hypertension, and even an increased risk of hepatic carcinoma[2].

Apoptosis, a stereotypical morphologic form of cell death, results in liver damage in a wide range of acute and chronic liver diseases[3]. Following liver injury, two distinct paths are involved in the repair process: one is a regenerative path, in which the same type of cells replace injured cells; and the other path is known as fibroplasia or fibrosis, in which normal parenchymal tissue is replaced by connective tissue in an uncontrolled manner[4]. Studies have shown that hepatocyte apoptosis can induce liver fibrosis[5-7], which is an excessive wound healing response to chronic liver injury. Liver fibrosis, a dynamic and reversible process[8-10], is characterized by an imbalance between synthesis and degradation of the extracellular matrix (ECM), which is rich in fibrillar collagens (mainly collagen Ⅰ and Ⅲ). It was demonstrated that activated hepatic stellate cells (HSCs) are the main fibrogenic cells in injured liver[10]. Therefore, focusing on HSC activation, signaling pathways activating HSCs and molecules that modulate fibrolysis and fibrogenesis may be effective strategies for the treatment and prevention of hepatic fibrosis[11].

Glycyrrhiza glabra, a perennial herb, has been widely used to cure diseases for thousands of years in China. In recent years, the efficacy of Chinese herbal medicine has been appraised by modern biological technology[12,13]. Glycyrrhizic acid (GA), extracted from the roots of *G. glabra*, is a major active component, and has been found to have numerous pharmacological effects, such as anti-inflammatory, anti-viral and hepatoprotective activities[14]. GA also exerts an anti-apoptotic effect by inhibiting hepatocyte apoptosis[15,16]. As shown in our previous study[17], GA can inhibit CCl_4-induced hepatocyte apoptosis *via* a p53-dependent mitochondrial pathway to retard the progress of liver fibrosis in rats. However, the mechanism how GA exerts its anti-apoptotic effect against fibrosis in CCl_4-induced liver injury and its contributing factors are unknown.

MATERIALS AND METHODS

Materials

GA and α-smooth muscle actin (SMA) antibody were purchased from Sigma-Aldrich (St Louis, MO, United States). MMP2 and MMP9 antibodies were bought from Abcam (Cambridge, MA, United States), CTGF antibody was bought from Santa Cruz Biotechnology (Santa Cruz, CA, United States), caspase-3 and Bax antibodies were purchased from Cell Signaling Technology (Beverly, MA, United States). GAPDH and tubulin antibodies were bought from Beyotime Biotechnology (Haimen, Jiangsu, China), horseradish peroxidase (HRP)-conjugated anti-mouse, anti-goat and anti-rabbit IgG antibodies were purchased from Cell Signaling Technology. The chemiluminescence reaction kit (ECL Plus) was purchased from Millipore (Billerica, MA, United States).

Animal model of liver fibrosis and treatment

Male Sprague-Dawley rats weighing 150-200 g were supplied by the Experimental Animal Center of Zhongshan Hospital, Fudan University. The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for 2 wk prior to experimentation. Forty-five rats were randomly and equally divided into the control group, CCl_4 group and GA treatment group. In order to induce the liver fibrosis model, rats were given a 40% solution of CCl_4 in olive oil by hypodermic injection at a dose of 3 mL/kg biweekly for 8 wk, while the rats in the control group were given the same dose of olive oil by subcutaneous injection, with an initial double-dose injection. In the GA group, rats were also treated with a 40% solution of CCl_4 by hypodermic injection at a dose of 3 mL/kg plus 0.2% GA solution in water (3 mL) by intraperitoneal injection three times weekly, beginning at the first week, following a previously published method[17]. Rats in the control group were treated with the same isovolumetric dose of olive oil and water. Animals were sacrificed 24 h

after the last injection. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection. Liver tissues were removed and rinsed with 0.9% saline, some were fixed in 10% buffered formaldehyde and embedded in paraffin for hematoxylin-eosin (HE) and Sirius-red staining, and the remaining sections were stored at -70 ℃ for analysis.

HE and Sirius-red staining

Liver tissues were embedded in paraffin and 5-μm-thick slices were cut and placed on glass slides, stained with HE, and examined under a light microscope (Olympus, Tokyo, Japan). HE staining was performed to assess pathological changes in the liver. Sirius-red staining was performed to detect collagen deposition and observed under a light microscope.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay

The deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Roche, Germany) was performed following the manufacturer's protocol. Nuclei were respectively counterstained with 4, 6-diamidino-2-phenylindole (DAPI) in the same sections. Cells stained by TUNEL were evaluated using fluorescence microscopy (Olympus).

Western blot analysis

Total proteins in liver tissue were extracted and quantified using the bicinchoninic acid protein concentration assay kit (Beyotime Biotechnology). Sample proteins were separated by 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% bovine serum albumin for 2 h, and then incubated overnight at 4 ℃ with primary antibodies rabbit caspase-3, Bcl-2, Bax, MMP2 and MMP9 antibodies, mouse α-SMA, GAPDH and tubulin antibodies, and goat CTGF antibody. On the next day, the membranes were incubated with the secondary antibodies conjugated with horseradish-peroxidase goat anti-rabbit IgG, goat anti-mouse IgG and rabbit anti-goat IgG (1:5000 dilution) at room temperature for 2 h, and then washed three times with Tris-buffered saline with 0.1% Tween-20 (TBST) , and detected by enhanced chemiluminescence. The intensities of the bands were analyzed by Image J software.

Real-time polymerase chain reaction analysis

Total RNA in liver tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States), subsequently converted to cDNA, which was carried out using PrimeScript reagent kit with gDNA Eraser (Takara Bio,. Dalian, China), and subjected to real-time PCR using SYBR *Premix Ex Taq*II (Takara Bio). The results were calculated in line with the dissociation curves and normalized against a housekeeping gene (GAPDH). This was performed using a 7300HT Fast Real-time polymerase chain reaction (PCR) System. Primer sequences used for real-time PCR were as follows: α-SMA: sense 5′-AGGGAGTGATGGTTGGAATG-3′, antisense 5′-GATGATGCCGTGTTCTATCG′; collagen Ⅰ: sense 5′-TCAAGATGGTGGCCGTTACT-3′, antisense 5′-GCGGATGTTCTCAATCTGCT-3′; collagen Ⅲ: sense 5′-ACCTCCTGGTGCTATTGGTC-3′, antisense 5′-TCTCTCCATTGCGTCCATC-3′, GAPDH: sense 5′-GACATGCCGCCTGGAGAAAC-3′, antisense 5′-AGCCCAGGATGCCCTTTAGT-3′. The mRNA expression levels of samples relative to the control groups were analyzed by the comparative CT ($2^{-\Delta\Delta CT}$) method[18].

Statistical analysis

All results were expressed as mean ± SD of three independent experiments. Data were analyzed using one-way analysis of variance. Differences were considered statistically significant if P was < 0.05. All analyses in the study were carried out using SPSS for Windows version 18.0.

RESULTS

Effects of GA on hepatic apoptosis induced by CCl_4

Under fluorescence microscopy, no staining was observed and non-apoptotic nuclei were found in normal liver tissue slices using the TUNEL assay. High quantities of TUNEL cells were observed in the liver tissue slices from the CCl_4 group, and numerous condensed and fragmented nuclei were seen. In the GA-treated group, few TUNEL cells were observed in the liver tissue sections, and fewer condensed and fragmented nuclei, in the same slice, were observed. In the same view, co-location of green and blue staining indicated TUNEL-positive cells, and numerous TUNEL-positive cells were found in the CCl_4 group, while a significant reduction in TUNEL-positive cells was observed in the GA-treated group (Figure 1). Overall, these results demonstrated that GA treatment reduced apoptosis during the process of liver injury.

Expression of apoptosis-related proteins in liver tissue from the different treatment groups was evaluated by western blot analysis. As shown in Figure 2, the expression level of Bax, the pro-apoptotic protein, was markedly increased in the CCl_4-induced hepatic injury group, whereas GA treatment significantly decreased the expression level of Bax (Figure 2). Caspase activation plays a vital role in apoptosis, and cleaved caspase-3 is a typical feature of apoptosis[19]. In the present study, cleaved caspase-3 (17 kDa) was increased in the CCl_4 group, which suggested severe apoptosis. In addition, the expression level of cleaved caspase-3 significantly decreased in the GA treatment group (Figure 2).

These findings indicated that GA suppressed CCl_4-induced hepatocyte apoptosis by inhibiting the activation of caspase-3. Thus, GA treatment ameliorated CCl_4-induced hepatocyte apoptosis.

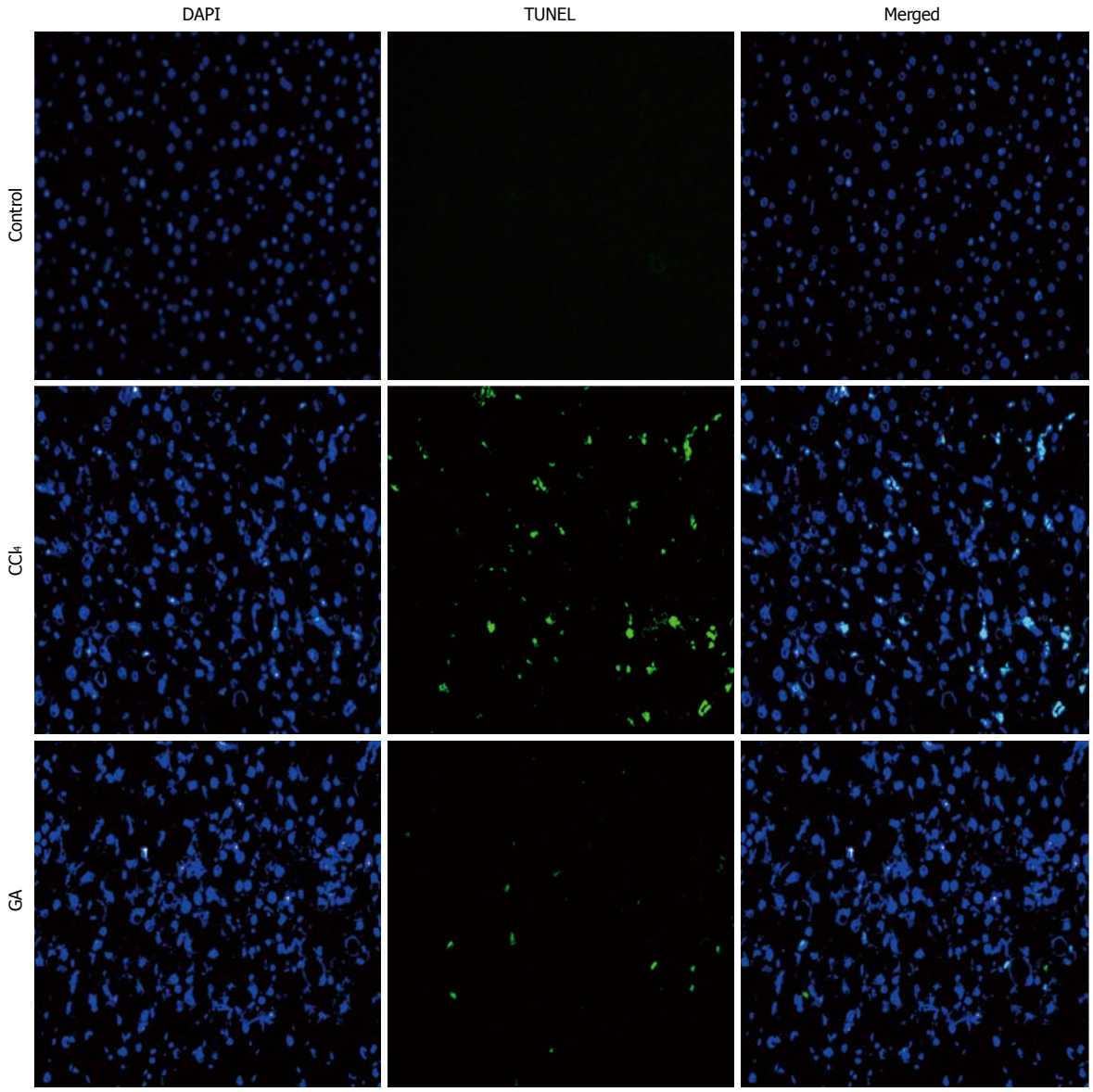

Figure 1 Effects of glycyrrhizic acid on hepatic apoptosis in carbon tetrachloride-induced rat liver fibrosis were detected by TUNEL assay. Liver tissue sections from different treatment groups were stained with TUNEL (green) and the same sections were respectively counterstained with DAPI to localize the nuclei (blue), and the co-location of green and blue indicated TUNEL-positive cells in the same view (magnification × 200).

Effects of GA on α-SMA protein and mRNA expression

We examined the effects of GA on α-SMA protein and mRNA expression in CCl_4-induced liver injury using western blotting and real-time PCR. As shown in Figure 3A, the expression level of α-SMA protein was maintained at a low level in the control group, and was up-regulated in the CCl_4-induced hepatic injury group. In contrast, GA significantly down-regulated expression of α-SMA protein. The trend in mRNA expression of α-SMA was in accordance with the results of Western blotting (Figure 3B).

Effects of GA on CTGF, MMP2 and MMP9 protein expression

Using Western blotting, we also examined the effects of GA on CTGF, MMP2 and MMP9 protein expression levels in CCl_4-induced liver injury. As indicated in Figure 4, expression of CTGF, MMP2 and MMP9 proteins was up-regulated in CCl_4-induced hepatic injury. In contrast, following GA treatment, expression of CTGF, MMP2 and MMP9 proteins was down-regulated compared to that in the CCl_4 group.

Effects of GA on collagen type Ⅰ and Ⅲ mRNA expression

We evaluated the expression levels of type Ⅰ and Ⅲ collagen mRNA using real-time PCR. In the CCl_4-treated group, expression of type Ⅰ and Ⅲ collagen mRNA was enhanced, however, the pattern and degree of change clearly differed between the two transcripts (Figure 5). Type Ⅰ collagen mRNA expression level increased in parallel with worsening liver disease. In the CCl_4-induced liver injury group, this enhancement was > 30 times higher than that in the control group,

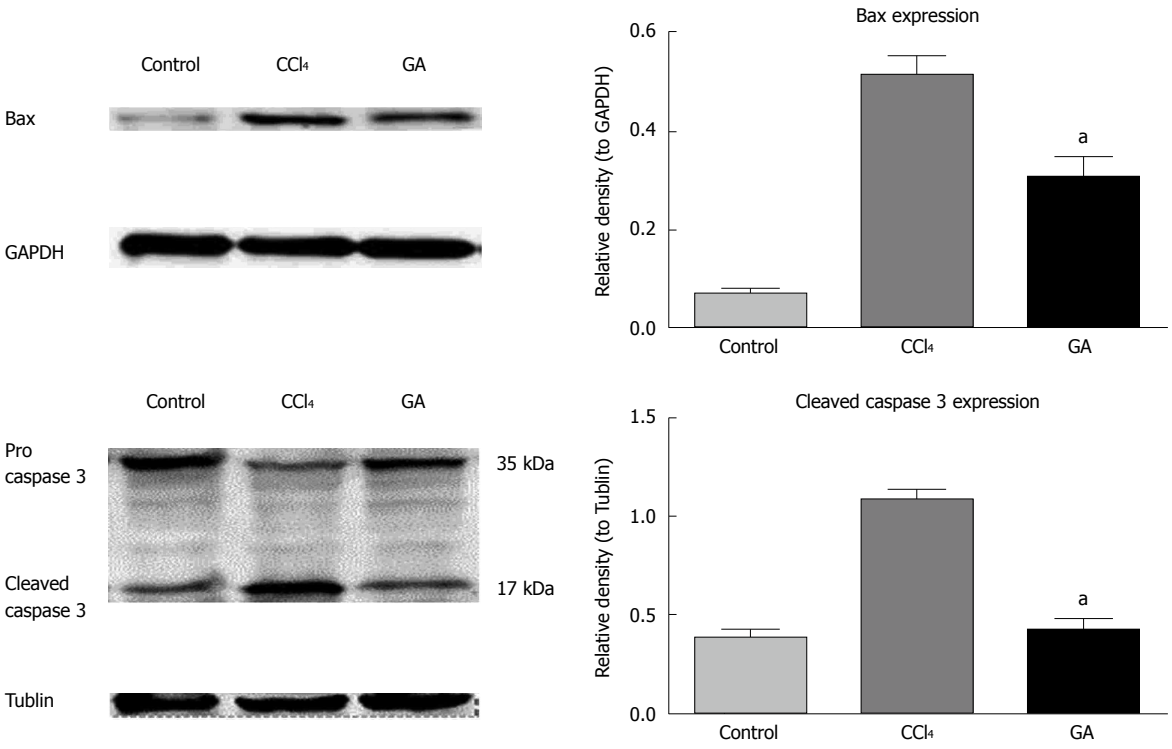

Figure 2 Effects of glycyrrhizic acid on expression of Bax and caspase-3 were evaluated by western blotting. Total proteins were extracted from the livers of rats in the different treatment groups. GAPDH and tubulin were used to confirm the same sample loading in each lane. Data are shown as mean ± SD. $^aP < 0.05$ *vs* CCl_4 group. GA: Glycyrrhizic acid; CCl_4: Carbon tetrachloride.

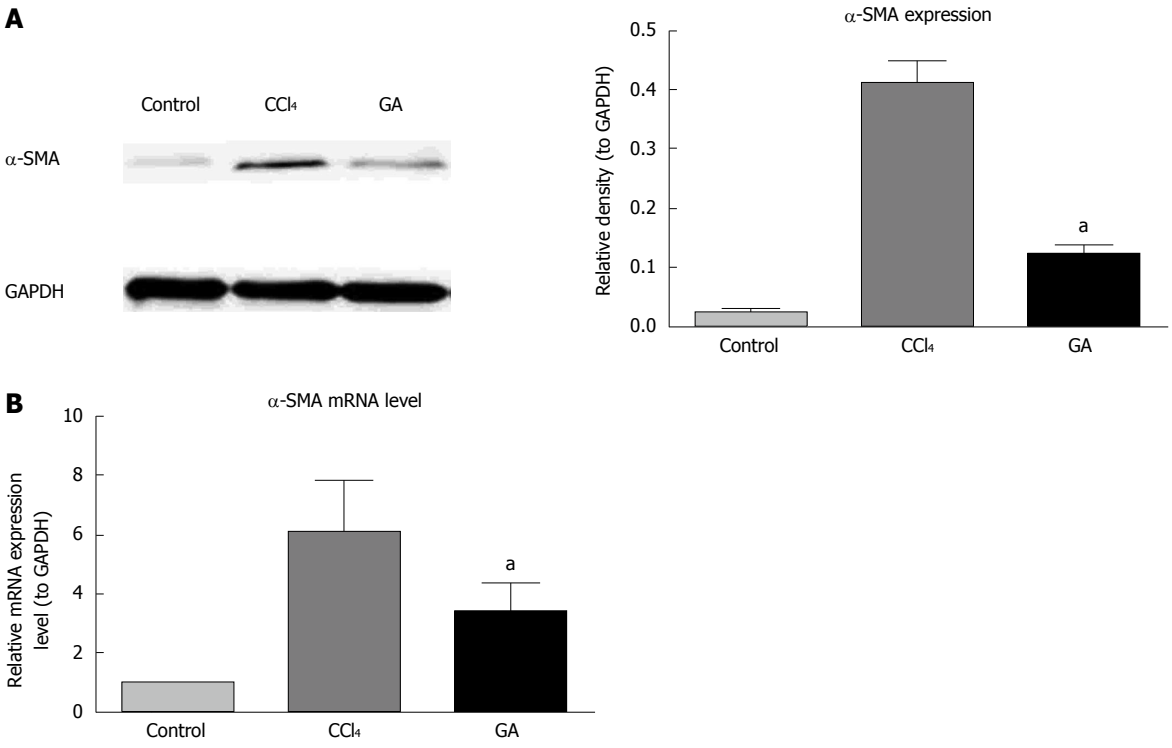

Figure 3 Effects of glycyrrhizic acid on α-SMA expression were estimated by western blotting and real-time polymerase chain reaction. A: Total proteins were extracted from the livers of rats in the different treatment groups. GAPDH was used to confirm the same sample loading in each lane; B: Total RNA was extracted from the livers of rats in the different treatment groups. GAPDH mRNA was included to normalize the expression of mRNA. Data are shown as mean ± SD. $^aP < 0.05$ *vs* the CCl_4 group.

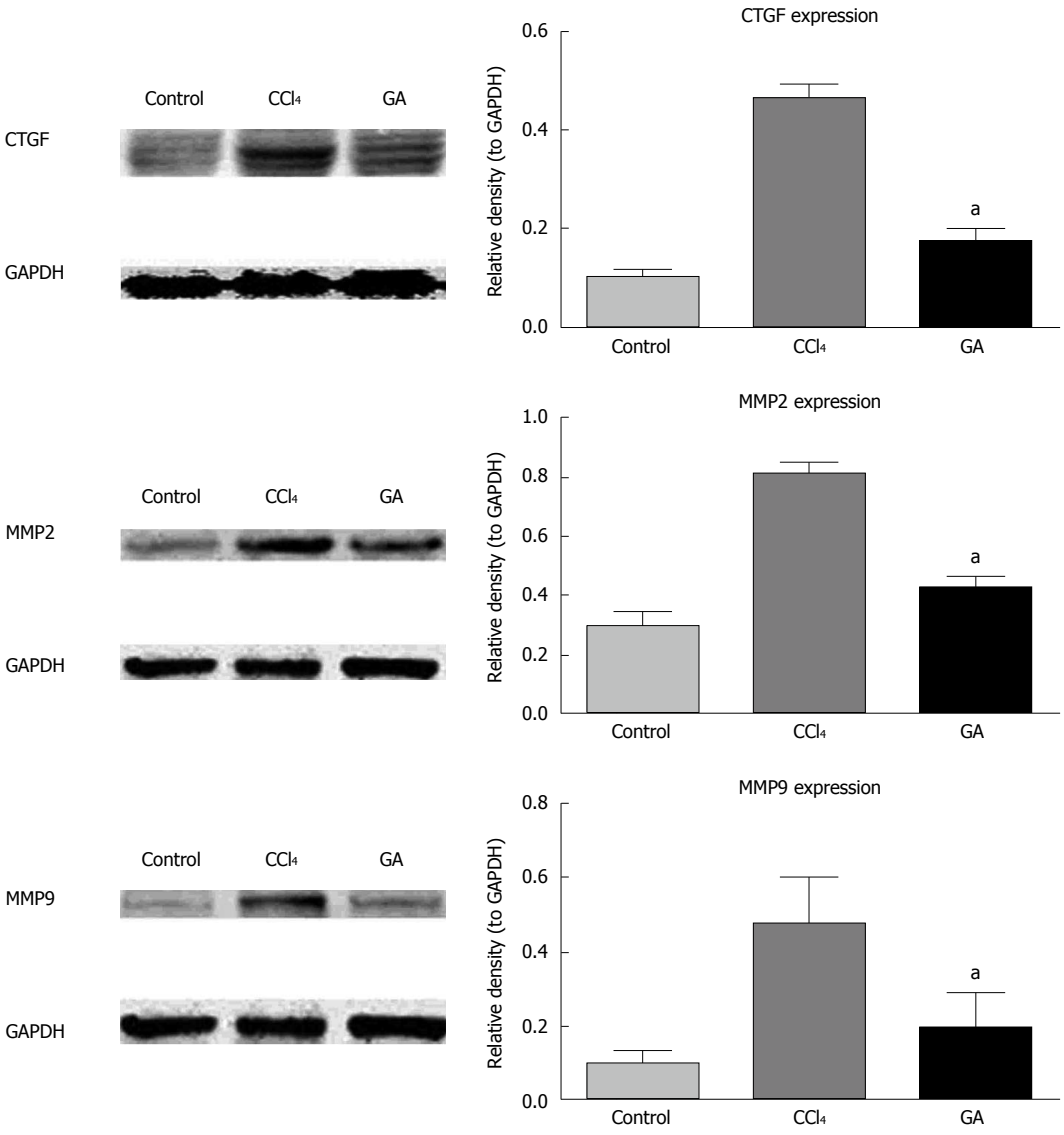

Figure 4 Effects of glycyrrhizic acid on CTGF, MMP2 and MMP9 expression were examined by Western blotting. Total proteins were extracted from the livers of rats in the different treatment groups. GAPDH was included to confirm the same sample loading in each lane. Data are shown as mean ± SD. $^aP < 0.05$ *vs* the CCl_4 group. GA: Glycyrrhizic acid; CCl_4: Carbon tetrachloride.

and type Ⅲ collagen mRNA expression was about 6 times higher than that in the control group. Moreover, GA significantly decreased expression of type Ⅰ and Ⅲ collagen mRNA, which was about 20 times and about 4 times that in the control group, respectively.

Effects of GA on collagen deposition in liver tissue induced by CCl_4

After 8 wk CCl_4 administration, HE and Sirius red staining showed an integrated lobular structure with central venous and hepatic cord radiation in liver slices from the control group (Figure 6), in which there were few positive areas of Sirius red staining around small central venous walls. These disorders of lobular structure, including wide fibrous tissue hyperplasia and fibrous septa formation, were identified in the CCl_4-treated group, and some pseudolobuli were present (Figure 6). Many positive areas of Sirius red staining were seen in the boundaries of the hepatic lobules in the CCl_4-treated group. Less fibrous tissue hyperplasia and fibrous septa formation were observed in the GA treatment group compared with the CCl_4-treated group, and positive areas of Sirius red staining were significantly reduced in the GA treatment group.

DISCUSSION

GA is a major active component of *G. glabra* roots and is commonly used in Asia to treat patients with chronic hepatitis[20,21], and has satisfactory therapeutic effects in many other diseases. GA has been reported to have numerous pharmacological effects, such as anti-inflammatory, anti-viral and hepatoprotective activity. In the present study, we demonstrated that GA significantly ameliorated liver fibrosis induced by CCl_4. GA exerted a beneficial anti-apoptotic effect and inhibited fibrosis-related factors.

In animal models, CCl_4 can induce hepatocyte

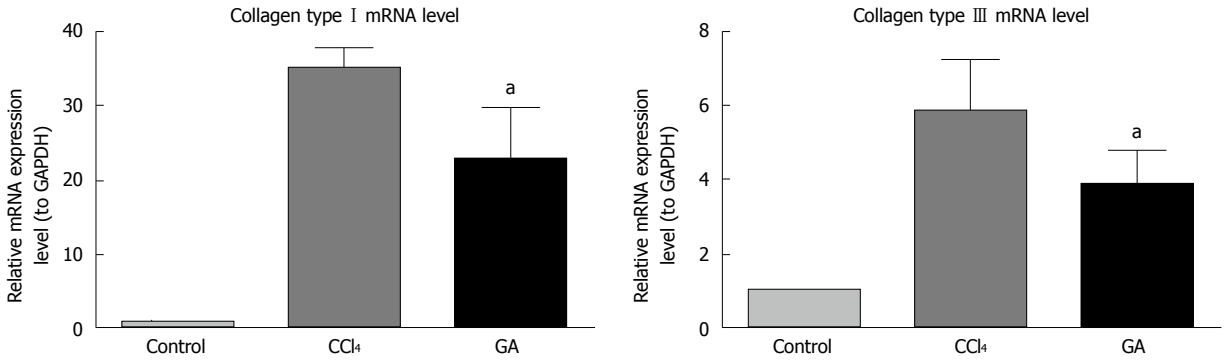

Figure 5 Effects of glycyrrhizic acid on expression of collagen type Ⅰ and Ⅲ mRNA. Total RNA was extracted from the livers of rats in the different treatment groups. GAPDH mRNA was included to normalize the expression of mRNA. Data are shown as mean ± SD. $^aP < 0.05$ *vs* the CCl_4 group. GA: Glycyrrhizic acid; CCl_4: Carbon tetrachloride.

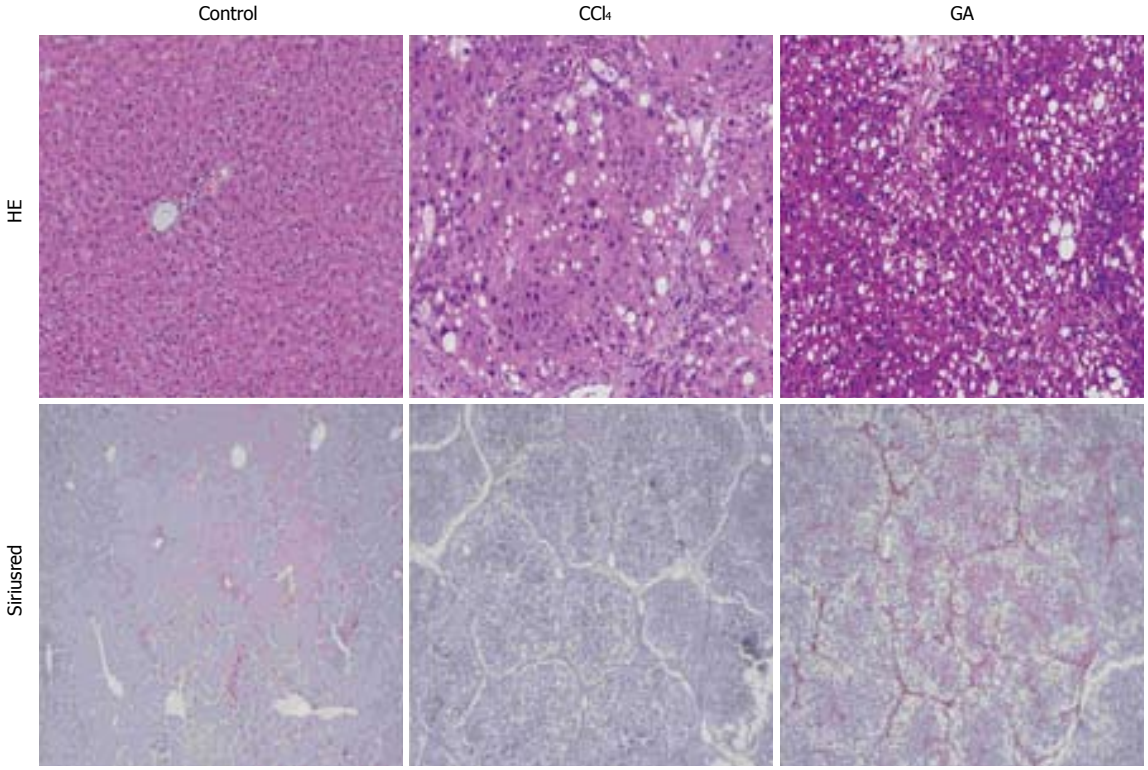

Figure 6 Effects of glycyrrhizic acid on the pathological histology and collagen of livers in carbon tetrachloride-induced rat liver fibrosis were evaluated by hematoxylin-eosin and Sirius red staining. Liver tissue sections from the different treatment groups were stained with hematoxylin-eosin (magnification × 100) and Sirius red (magnification × 40).

apoptosis and liver fibrosis[22-26]. Responses to CCl_4-induced damage in rat and mouse models are similar to responses to liver cirrhosis in humans[27], and these models can be used to screen for anti-hepatotoxic and/or hepatoprotective drugs[28].

Hepatocyte apoptosis, a cardinal feature of many liver diseases, is considered to play a part in initiating and maintaining HSC activation[29]. Apoptosis of parenchymal cells, an important inflammatory stimulus, activates HSCs, which display a surprising capacity to phagocytize apoptotic bodies, rather than being a silent consequence of liver injury[30]. Thus, factors that affect apoptosis may be used to modulate liver fibrosis[26], and may be developed as a potential anti-fibrotic strategy.

Although the incidence of hepatic fibrosis is high worldwide, few effective and accepted antifibrogenic therapies are available. However, our previous study found that GA could inhibit CCl_4-induced hepatocyte apoptosis and retard the progression of liver fibrosis in rats[17]. Similar to our previous research, DNA fragmentation of hepatocyte apoptosis was assessed using the TUNEL assay. In the CCl_4-treated group, TUNEL-positive cells were increased compared to the control group and GA treatment group, whereas GA treatment significantly decreased the number of TUNEL-positive cells, and these results agree with previous findings that GA reduced the number of TUNEL-labeled cells[16]. However, the TUNEL assay

is not a specific marker of apoptosis, thus Western blotting was conducted and found that GA treatment inhibited hepatocyte apoptosis by down-regulating expression of cleaved caspase-3 and Bax proteins. These results coincided with those from the TUNEL assay showed that GA reduced hepatocyte apoptosis.

Necrosis and apoptosis are involved in the process of liver fibrosis[26]. In our previous study, we found that hepatocyte apoptosis could induce liver fibrosis. However, the mechanism how GA exerts its anti-apoptotic effect against fibrosis in CCl_4-induced liver injury and its contributing factors are unknown. HSC activation, characterized by a high rate of proliferation, expression of fibrotic cell markers and production of ECM[29], plays a key role in liver fibrogenesis[30,31], and a positive relationship between the degree of fibrosis and HSC activation in damaged livers has been observed in animal and human fibrogenesis[32]. α-SMA, a marker of activated HSCs, was evaluated by western blotting and real-time PCR in the present study. Expression of α-SMA protein and mRNA was significantly up-regulated following CCl_4 administration and down-regulated by GA. These results indicated that HSCs were activated in CCl_4-induced liver fibrosis, and significantly inhibited by GA treatment.

An imbalance between ECM degradation and production existed during fibrosis, and both collagen type Ⅰ and Ⅲ exhibited a significant increase. Activation of HSCs, involved in the conversion of quiescent, vitamin-A-storing cells into proliferative, fibrogenic and contractile myofibroblasts, can synthesize and secrete a large number of fibril-forming collagens, especially collagen type Ⅰ and Ⅲ[33,34]. During liver fibrosis, the main components of the scar matrix are interstitial collagen type Ⅰ and Ⅲ, which replace the basal membrane of the subendothelial space of Disse and sinusoids[35,36]. In the present study, using real-time PCR, we found that expression of collagen type Ⅰ and Ⅲ mRNA was enhanced in CCl_4-induced liver fibrosis, while expression in the liver of GA-treated rats was significantly reduced, thus, GA significantly decreased accumulation of ECM.

Matrix degradation is dependent on the role of the MMP family in the extracellular space. Activation of HSCs also generates MMP2, MMP9 and MMP3, which destroy the basement membrane, leading to recruitment of inflammatory cells to the site of injury[37-39]. The activity of MMP2 and MMP9 in liver fibrosis progression increases as HSCs become activated both in humans and animals[1,10]. The present study showed that expression of MMP2 and MMP9 significantly increased in the CCl_4-treated group, while GA treatment decreased expression of MMP2 and MMP9.

CTGF, another important fibrogenic factor, is synthesized by hepatocytes and HSCs[40,41]. CTGF is a general mediator of the interactions between fiber-fiber, fiber-matrix and matrix-matrix. During liver fibrosis, CTGF is a hepatic fibrogenic master switch in the epithelial to mesenchymal transition, and plays a pivotal role in the increase of ECM-producing fibroblasts[41]. In the present study, expression of CTGF was up-regulated in the CCl_4-treated group, while GA treatment significantly reversed this increase. We also found that hepatic injury in the CCl_4 group was more serious than that in the GA treatment group on the basis of histological observation and Sirius red staining.

In the present study, we found that GA has inhibitory effects on hepatocyte apoptosis and liver fibrosis, which are mainly associated with down-regulation of HSC activation, thus regulating fibrosis-related factors, such as expression of CTGF, MMP2 and MMP9 proteins, and collagen type Ⅰ and Ⅲ mRNA. Collectively, these results demonstrated that GA significantly ameliorates CCl_4-induced liver fibrosis by inhibiting hepatocyte apoptosis and HSC activation, which may provide potential therapeutic strategies for fibrosis.

COMMENTS

Background

Glycyrrhizic acid (GA), a major active component of *Glycyrrhiza glabra* roots, is commonly used in Asia to treat patients with chronic hepatitis. Liver fibrosis is a common outcome in many chronic liver diseases. Necrosis and apoptosis are involved in the development of liver fibrosis. In a previous study, the authors found that hepatocyte apoptosis could induce liver fibrosis. In this study, the authors investigated the mechanism how GA exerts its antiapoptotic effect against fibrosis in carbon tetrachloride (CCl_4)-induced liver injury, and its contributing factors in this process.

Research frontiers

In the present study, the authors found that GA treatment significantly ameliorated CCl_4-induced liver fibrosis by inhibiting hepatocyte apoptosis and hepatic stellate cell (HSC) activation, which may provide potential therapeutic strategies for fibrosis.

Innovations and breakthroughs

This study investigated the mechanism how GA exerts its anti-apoptotic effect against fibrosis in CCl_4-induced liver injury, and its contributing factors in this process. The results of this study demonstrated that GA treatment significantly ameliorated CCl_4-induced liver fibrosis by inhibiting hepatocyte apoptosis and HSC activation.

Applications

The results of this study may provide potential therapeutic strategies for fibrosis.

Terminology

Hepatocyte apoptosis, a cardinal feature of many liver diseases, is considered to play a part in initiating and maintaining HSC activation. Apoptosis of parenchymal cells, an important inflammatory stimulus, activates HSCs, which display a surprising capacity to phagocytize apoptotic bodies, rather than being a silent consequence of liver injury. Thus, factors which affect apoptosis may be used to modulate liver fibrosis.

Peer-review

In this study, the authors investigated how GA exerted its anti-apoptotic effect against fibrosis in CCl_4-induced liver injury and its contributing factors. The study is well designed and presented. The results shown in the manuscript is interesting and GA is a potential therapeutic agent for liver cirrhosis.

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TOPIC HIGHLIGHT

2015 Advances in Alcoholic Liver Disease

Binge drinking: Burden of liver disease and beyond

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Abstract

The consumption of alcoholic beverages is harmful to human health. In recent years, consumption patterns of alcoholic beverages have changed in our society, and binge drinking has generalized. It is considered to be a socio-sanitary problem with few known consequences in terms of individual and third-party social impacts (in the form of violence or traffic accidents) and its organic impact (affects the liver and other organs and systems, such as the nervous and cardiovascular systems) and represents an important financial burden due to its increasing economic impact. This review provides a global approach to binge drinking and emphasizes its epidemiological character, the effect of this type of consumption and the possible management of a problem with an increasing tendency in our society.

Key words: Binge drinking; Binge drinking adolescent; Binge drinking brain; Binge drinking cardiovascular; Alcohol binge; Binge drinking liver

Core tip: Binge drinking is an alcohol consumption conduct that is primarily performed during the weekend by 24% of teenagers and young adults. Although the consequences of this habit are not well known, they have a social and organic impact on individuals. Binge drinking is considered to be a public health issue that should be addressed with primary prevention programs and a comprehensive intervention of the problem.

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INTRODUCTION

The consumption of alcoholic beverages is harmful to human health. Excessive alcohol intake is a major global and public health challenge that has been

identified as one of the main determinants of a variety of noncommunicable diseases[1]. The excessive consumption of alcohol is the leading global cause of preventable morbidity and mortality and a major problem in Western countries. According to the World Health Organization (WHO), it is the cause of 4.5% of the diseases in the world and 4% of the deaths in the world and is considered the main cause of death among men between 15 and 59 years of age, especially in Eastern Europe countries[2,3]. In the United States, this excessive alcohol consumption causes 75000 deaths each year and is the third leading preventable cause of death[4]. Alcohol is the main cause of cirrhosis and indication for liver transplants in Europe, and accounts for 1.8% of all deaths caused by liver disease[5]. When the data are adjusted by age, alcohol is the main risk factor for impairment (*i.e.*, life-years lost at early ages) in young populations between 10-24 years of age[6].

In recent years, consumption patterns of alcoholic beverages have changed in our society, and binge drinking has generalized. The reason for this change and its implications for the individual and the society are not well known. For this reason, we present this review using a comprehensive approach to the binge-drinking problem.

DEFINITION OF BINGE DRINKING

A unified definition of binge drinking is necessary to effectively approach this subject and to analyze the risk factors of binge drinking, its socio-sanitary implications and its relation to alcohol dependence. We review the controversial term of binge drinking, which lacks a consensus among the different studies. The controversy stems from the following items: (1) its inadequate definition; (2) the minimum amount of consumption that is considered to be a problem has not been established; (3) a standard drinking unit (SDU) that is common to all countries has not been established; and (4) the unspecified period of time that is considered to be "a single event".

Consequently, epidemiological studies describe important methodological problems; the prevalence of this type of consumption in young populations varies between 7% and 40% due to the lack of uniform cut-off points[7]. This variability is attributed to the lack of consensus in determining the harmful consumption levels of alcohol and the differences in pure ethanol in an SDU for each country. Therefore, the cut-off points for the number of SDUs ingested in each event (*i.e.*, five alcoholic beverages and six alcoholic beverages) and the frequency intervals (*i.e.*, in the last week, 15 d, and 30 d) in which the episodes of heavy consumption occur vary in the different studies[8]. Regarding the term binge drinking, several authors suggest that this definition traditionally refers to a pattern of consuming large amounts of alcohol in a few hours and primarily during weekend nights that is conducted by younger age groups without a differentiation of gender[8]; they primarily correlate it with clinical definitions of abuse or dependence[9-11].

To prevent confusion, alternate terms have been suggested, such as heavy drinking[12-16], heavy episodic drinking[17-22], heavy sessional drinking, risky single-occasion drinking[23], dangerous drinking[24], or high-risk drinking[25]. In Spain, the First Conference in Health Prevention and Promotion in the Clinical Practice in 2007 proposed the term heavy episodic drinking of alcohol.

Although binge drinking cannot be identified with the common criteria for the harmful consumption of alcohol, many authors have stressed its social and health consequences, which may exceed the social and health consequences of regular alcohol consumption[26-29].

In the 1990s, the effect of alcohol consumption regarding the sex of the patient was determined in the Harvard School of Public Health College Alcohol Study[30,31]. Wechsler's group employed a questionnaire to evaluate the habits of alcohol consumption. The group discovered that significant problems of alcohol consumption occur in men after the intake of five beverages in one event, whereas similar problems occur after the intake of four beverages by women. The term heavy alcohol consumption (HAC) evolved and was understood as the consumption of five or more drinks by men and four or more drinks by women in a single occasion, at least once in the last two weeks[31].

Regarding the "single-event" discussion that is referenced in the binge drinking definition, several authors consider including the concentration of alcohol in the blood to determine the adequate threshold for the binge-drinking pattern. This threshold is explained by the difference in the effect of the intake of one alcoholic beverage in one hour during five continuous hours in an adult with an average body weight and the intake of the same amount of alcohol (five beverages) over a shorter period, for example, two hours.

Accordingly, the National Institute on Alcohol Abuse and Alcoholism (NIAAA)[32] redefined the term HAC by considering the level of concentration of alcohol in the blood. HAC considers minimum levels of 0.08 g/L of alcohol in the blood when determining the pattern of alcohol consumption. In adults, this level would correspond to the intake of five or more beverages by men and four or more beverages by women in approximately two hours. The NIAAA considers duration (two hours) in the HAC definition.

To consolidate a definition that includes alcohol levels in the blood, several studies have employed different variants of Widmark's formula, which was developed in the 1930s and has proven adequate reliability[33]. This equation establishes that the maximum concentration of alcohol in the blood is $A/(p \times r)$, where A = amount of alcohol consumed (in grams); p = body weight and r = fat/water ratio (0.7 for men and 0.6 for women).

Recently, another study[34] determined that the definition by Wechsler's group[30,31] and the NIAAA proposal[33] are strongly correlated with a similar pattern of association among the variables of sex, race and

Table 1 Prevalence of binge drinking

Ref.	Prevalence
Galán *et al*[41]	13.35%
Slutske *et al*[150]	19.50%
Bartoli *et al*[42]	37.85%
Delegación del Gobierno para el Plan Nacional sobre Drogas[40]	18%
CDC[44]	17.10%
Grucza *et al*[45]	50%
Hanewinkel *et al*[151]	27.00%
Lee *et al*[152]	46.30%

Table 2 Factors associated with binge drinking[42]

	OR (95%CI)
Female gender	1.57
Living with parents	0.57
High financial availability for each weekend	1.33
Cannabis use	1.61
Smoking e-cigarettes	2.49
Positive alcohol expectancies	1.11
Peer influence	2.4
Interest for discos and parties	1.53
High educational level	3.63

age and the initiation of consumption. These authors believe that quantity and duration should be considered, as suggested by the NIAAA, because the sole inclusion of the quantity variable underestimates the HAC prevalence and is insufficiently sensitive in discriminating between problematic and nonproblematic patterns of consumption.

An additional and more adequate definition for the clinical environment may be the consumption of six or more alcoholic beverages by men (60 g) and five or more alcoholic beverages by women (50 g) in a single occasion (in a two-hour period) at least once in the last 30 d. This definition is similar to the approach by the NIAAA[32] and Wechsler's group[30,31] and gathers all proven relevant variables of quantity and frequency but requires customization to the country in which the research will be conducted.

EPIDEMIOLOGY

Since Strauss and Bacon's epidemiological study, which was performed in the United States during the 1950s[30], several authors have reported an alarming increase in alcohol consumption among young global populations and consider it to be a risk pattern of consumption in this population (Table 1)[35-38]. At the European level (Eurobarometer, 2007), approximately 80 million Europeans who are aged 15 years or older [over one-fifth of the adult European Union (EU) population] reported binge drinking at least once a week in 2006; this proportion has increased since 2003 in the adult population of the EU 15 (Austria, Belgium, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, The Netherlands, Portugal, Spain, Sweden, and the United Kingdom). Binge drinking is not the prerogative of the young. Eighteen percent of persons aged 55 years of age and older reported binge drinking at least once a week in 2006 compared with 24% of persons aged between 15 and 24 years. Eastern Europe had the highest pattern of drinking score of 4.9, which indicated that people in this region frequently consumed large quantities of alcohol and frequently drank to intoxication, engaged in prolonged binges, and primarily consumed alcohol outside of mealtime[39]. Traditionally, alcohol consumption in Spain has been associated with the adult population; its regular consumption was primarily linked with gastronomy and social events. In the last 20 years in the remaining Mediterranean countries, important changes have occurred regarding the quantity, patterns and meaning of consumption that are similar to the increased binge drinking in the rest of the world[8]. One of the most recent household surveys on alcohol and drug use in Spain showed that 18% of the population between 15 and 34 years of age (with a mean age of first contact with alcohol at the age of 16.8 years) indicated that they consumed five or more alcoholic beverages in one single occasion (occasion refers to the intake of several glasses in a couple of hours) in the last 30 d[40]. A cross-sectional study[41] with a significant number of participants (n = 20608) of 15 years of age and older that employed the 2011-2012 National Health Survey as a source of information (Servicio Nacional de Salud, by its initials in Spanish) considered the intake of $\geqslant$ 40 g/d of alcohol in men or $\geqslant$ 24 g/d in women to be high-risk consumption. Binge drinking was defined as the consumption of $\geqslant$ 6 (men) and $\geqslant$ 5 (women) standard beverages of alcohol in 4-6 h in the last 12 mo. A total of 1.3% of the surveyed subjects were average high-risk drinkers; 19.6% of the men and 7.1% of the women had performed binge drinking in the last year. This pattern decreased with age but increased with educational level in both sexes, with beer as the most-consumed beverage. A study in Italy[42] of 654 individuals with a mean age of 20.6 years showed that 38% of the subjects had recently engaged in binge drinking. By performing a multivariate analysis, a relation was observed between this type of consumption and higher educational expectations, a larger amount of money available to spend during the weekends, interests in parties and discos, a higher prevalence in women (despite the reports from Anglo-Saxon countries), the use of cannabis, a greater influence of friends and the use of electronic cigarettes. Conversely, living with parents produced a protective factor (Table 2). In another Italian study based on the CAGE questionnaire or Alcohol Use Disorders Identification Test showed that 19.5% of the 1520 patients who attended an emergency service during the five months of the study had problems with alcoholism; the most frequent attendees were young males (18-20 years of age), divorced or single patients, and unemployed, homeless or immigrant patients[43].

In the United States, approximately 38 million adults binge drink, according to a 2010 survey by the Centers for Disease Control. The total prevalence of binge drinking among adults in the 48 states and the District of Columbia was 17.1%[44]. Epidemiological studies have identified that binge drinking is prevalent on college campuses; some studies indicate that approximately 50% of students reported binge drinking in recent weeks[45]. A recent study noted that approximately 500000 college students are injured and 1700 college students die each year from alcohol-related injuries[46].

Binge drinkers have a greater risk for developing alcohol dependence[47]. In addition, binge drinking has been associated with unplanned and unsafe sexual activity, assaults, falls, injuries, criminal violations, automobile crashes, and total poor neuropsychological functioning[48]. Each year, two thousand homicides are registered in Europe due to excessive alcohol consumption.

The importance of this problem translates to high healthcare costs. In the United States, the estimated annual expenditure for binge drinking is 168 billion dollars[44]. The estimated cost of binge drinking for the English Public Health System was £1.7 billion in 2003, which reflects the physical and psychological health problems that are associated with this type of excessive drinking[49].

ALCOHOL AND TOBACCO

Similar to alcohol, tobacco is considered to be a major cause of morbidity and mortality[50,51]. Tobacco has been directly responsible for 100 million deaths in the XX century[50,51]. Cigarette smoking is strongly associated with alcohol consumption[52-58]. Conversely, drinkers, especially binge drinkers, are more likely to smoke than nondrinkers[52,55]. This tobacco-alcohol relationship involves the pleasure-reward dopamine brain systems, as evidenced in murine models. In a recent study of mice, the rodents that were exposed to nicotine tended to ingest alcohol more frequently than rodents that were not administered nicotine due to a reduction in the dopamine response of the reward-response system in the brain, which decreased the pleasurable response to alcohol[59].

Young adults perceive an increased enjoyment of and desire for cigarettes while drinking alcohol[53,60], which may explain why smokers smoke more cigarettes while under the influence of alcohol[53,61-63], especially during binge drinking episodes[53,61]. If the frequency of alcohol consumption, binge drinking and being a smoker are associated, we are experiencing a global health issue with an early beginning in adolescence because both substances synergically increase the future risk to a level that exceeds the usual risk for liver, cardiovascular and neoplastic diseases posed by the individual use of either of these substances[64]. Young adults smoke cigarettes at rates that are higher than any other age group. According to the 2010 National Survey on Drug Use and Health survey, 34.2% of young adults aged 18 to 26 are current smokers, compared with 22.8% of adults aged 26 or older. In a recent study[65], teenagers who attend bars and discos showed a higher rate of tobacco consumption; this consumption was highly associated with the intake of alcoholic beverages.

TOXICITY OF ALCOHOL

The factors that affect the susceptibility to alcohol toxicity include genetics, gender, lifestyle/nutrition, exposure to environmental chemicals and drugs, and comorbidities. Toxic and other adverse effects of alcohol on organs and tissues in humans are a consequence of its metabolism to acetaldehyde, the associated formation of reactive oxygen and nitrogen species, the depletion of co-factors (*e.g.*, NAD+), and the impairment in energy homeostasis[66]. Due to the considerable redundancy in the oxidative enzymatic pathways (alcohol dehydrogenases, CYP2E1 and catalase) that can convert alcohol to acetaldehyde, the majority of tissues are capable of alcohol metabolism even though the liver is the primary site. Similarly, acetaldehyde dehydrogenases are ubiquitous in mitochondria. A minor and non-oxidative pathway of alcohol metabolism is *via* fatty acid ethyl ester (*via* fatty acid ethyl ester synthase) and phosphatidyl ethanol (*via* phospholipase D). Alcohol impacts the integrity of the gastrointestinal mucosal barrier, resulting in the translocation of the gut bacteria-derived lipopolysaccharide (endotoxin) and other molecules to the liver *via* the portal blood flow and the activation of the innate immune response. The molecular and cellular sequelae of the toxic mediators of alcoholic injury assume many forms. Acetaldehyde and oxidants are highly reactive molecules that can damage deoxyribonucleic acid (DNA), proteins and lipids. Changes in hepatic respiration and lipid metabolism can cause tissue hypoxia and impairment in the mitochondrial function. Secondary effects include the disruption of signaling pathways and ion channel function, the unfolded-protein response and oxidative stress as well as the activation of adaptive immune response that is significantly triggered by acetaldehyde protein adducts. Cell death triggers additional innate immune response, activation of fibrogenesis, and tissue repair. In addition to pro-inflammatory mediators, other signaling molecules, such as neurotransmitters, are affected by alcohol. Depending on the affected tissue, gross pathological changes that are associated with alcohol drinking include most or all of the following conditions: Fat accumulation (steatosis), inflammation, necrosis and fibrosis and functional deterioration[67]. Alcohol *via* acetaldehyde also favors carcinogenesis and has been considered to be a class 1 carcinogen by the WHO[68].

CONSEQUENCES

Compared with nonbinge drinkers, frequent binge drinkers were more likely to report fair or poor health and experience a greater number of sick days. These findings appear to reflect the generally negative consequences

Table 3 Summary of the organic effects of alcohol-binge drinking

Hepatic	Neurocognitive	Renal
Steatosis	Impaired verbal memory	Glomerulonephritis
Steatohepatitis	Impaired episodic memory	Acute nephropathy
Fibrosis	Deficits language and attentional tasks	Kidney graft failure
Cirrhosis	Prospective memory	
Hepatocellular carcinoma	Executive functions	
Oncogenic	Cardiovascular	Others
Oral cavity	Hypertension	Acute pancreatitis
Pharynx	Ischemic heart disease	Chronic pancreatitis
Larynx	Stroke	Major depression
Esophagus	Cardiomyopathy	Impaired fertility
Colorectum	Myocarditis	Premature and low weigh births
Breast	Arrhythmias	Fetal alcohol syndrome
Pancreas	Atherosclerosis	

of alcohol abuse but at an earlier stage in poor health development[69]. Binge drinking is associated with the deterioration of work performance, brain damage, alcohol dependence, stroke, heart rhythm disturbances, coronary disease, sexually transmitted diseases and premature death[35]. Table 3 summarized the organic effects of binge drinking on different organs and systems.

Effect on the liver

The epidemiological evidence demonstrates that binge drinking in chronic alcoholics augments liver injury[70]. A recent study showed that frequent consumers (5-7 d/wk) have a higher mortality rate compared with persons with lower rates of consumption (1-4 d/wk)[71]. A heavy binge drinking episode in patients who chronically consume alcohol is the most common trigger for the admission of patients with steatohepatitis[72]. A study of a large cohort of drinkers with consecutive biopsies suggested the concept of multiple hits of alcoholic hepatitis in the same patients as the prime determinant in the progression of alcoholic liver injury[73]. Mathews *et al*[74] have recently developed a chronic plus binge alcohol feeding model in mice, which is similar to the drinking patterns of many alcoholic hepatitis patients: A history of chronic drinking and recent excessive alcohol consumption have begun to identify novel mechanisms that participate in the pathogenesis of alcoholic liver injury. Chronic binge ethanol feeding induces higher levels of steatosis, serum alanine transaminase, and liver inflammation[74]. Binge alcohol consumption aggravates oxidative stress and promotes the pathogenesis of nonalcoholic steatohepatitis from obesity-induced simple steatosis. Alcohol and high fat diets synergistically induce nitrosative, endoplasmic reticulum, and mitochondrial stress and an up-regulation of hepatic toll-like receptor 4 (TLR4), thereby contributing to steatohepatitis[75,76]. Additionally, high fat diet plus binge ethanol synergistically exacerbates acute steatohepatitis through the induction of CXCL1 and subsequent hepatic neutrophil infiltration[77]. Moderate ethanol binges induce significant liver damage (hepatocyte apoptosis) in genetically obese (*ob*/*ob*) mice by increasing tumor necrosis factor α and decreasing nuclear factor κB activity[78]. Individuals with fatty liver are predisposed to increased liver injury by chronic binge alcohol drinking. This finding has been proven in studies involving rats, where repeated alcohol binges in the context of mild steatosis may promote the activation of stellate cells and contribute to liver injury[79].

Despite these findings, note that the majority of experimental data concerning the impact of binge drinking on the pathogenesis of a liver injury may not be completely extrapolated to humans because the majority of the studies are based on animal models that do not completely mimic liver injury in humans. Note that ethanol sensitivity in human, rat, mice, and other animal models (*e.g.*, drosophila, zebrafish) can also vary due to differences in populations, species, and strains. In animal models, several approaches have been considered to examine the effect of binge ethanol, including the single binge, the intermittent repeat binge, and chronic ethanol exposure followed by episodes of binging. Evidence from these animal studies provide mechanistic information on the binge ethanol effect relevant to alcoholic liver disease. For example, the cellular effects of ethanol are increasingly attributed to the modulation of immunological, metabolic, signaling, and epigenetic pathways[80-82]. Binge alcohol alters the levels of several cellular components and dramatically amplifies liver injury in chronically alcohol exposed liver. Evidence exists that acute alcohol exposure inhibits hepatic mitochondrial DNA synthesis and also impairs mitochondrial metabolism and dynamics. Alcohol intoxication inhibits the inflammatory response by inhibiting signaling through TLRs when a potent external TLR stimulus is provided during alcohol intoxication[83]. As previously reported, binge drinking promotes the activation of stellate cells and contributes to liver injury *via* a pro-fibrogenic response[79].

Other factors involved in the toxicity of alcohol to the liver are obesity, resistance to insulin, chronic infection with hepatitis C virus, being female, and tobacco consumption[84]. A priori, tobacco appeared to have a minor role in fibrosis and chronic liver disease; however, additional studies have suggested its deleterious role in the course of chronic liver disease. Tobacco and alcohol may have a synergic and deleterious impact on chronic liver disease[85]. Tobacco may accelerate the progression to cirrhosis in patients with alcoholic chronic liver disease and increase liver decompensations in individuals with established cirrhosis[86]. Approximately 90% of the patients with advanced alcoholic chronic liver disease are smokers[87]. Tobacco seems to be involved in the risk of developing hepatocarcinoma[88] by increasing aflatoxin B1, which is a known hepatic carcinogen[89].

The mechanisms by which smoking promotes the progression of chronic diseases are substantially unknown. Smoking may accelerate the progression of "fibrogenic" conditions, such as chronic renal, cardiac or pancreatic diseases[90,91]. Cigarette smoke induces an

array of pathogenic effects that are potentially involved in tissue fibrogenesis, including systemic inflammation, thrombogenesis and oxidative stress[92]. Smoking exerts powerful immunoregulatory actions that can produce an impaired wound healing response to injury. These effects may be more pronounced in susceptible individuals as suggested by genetic epidemiological studies[93]. The strongest evidence to support a fibrogenic effect of smoking is the fact that smoking cessation has beneficial effects on the progression of chronic renal diseases[94,95].

Smoking increases the production of pro-inflammatory cytokines (interleukin 1, 6 and 13) and tumor necrosis factor α, angiogenic factors (vascular endothelial growth factor-A) and fibrogenic mediators (leptin, transforming growth factor β1 and angiotensin Ⅱ) also induces oxidative stress by stimulating nicotinamide adenine dinucleotide phosphate oxidase and decreasing antioxidant defenses, which cause lipid peroxidation[92]. These effects can cause an increase in hepatocellular damage and the subsequent activation of resident hepatic stellate cells, which comprise a major fibrogenic cell type. Another potential mechanism by which smoking causes liver fibrogenesis may be iron deposition. Smoking also induces profound changes in the microvasculature, such as endothelial dysfunction, smooth muscle cell proliferation and vasoconstriction, which cause impaired delivery of nitric oxide and tissue hypoxia[96]. These events are potentially implicated in the wound healing response of the liver to chronic injury. Heavy smokers commonly exhibit several features of the insulin resistance syndrome and develop an increased risk for type 2 diabetes[97]. Because insulin resistance promotes liver fibrogenesis, it can participate in the fibrogenic effect of tobacco in the liver. Therefore, we can conclude that the interaction between alcohol and tobacco synergistically elevates the disease risk to a level above the risk posed by the individual use of either of these substances[64].

Oncogenic effects

Alcoholic beverages and ethanol in alcoholic beverages are classified by the WHO International Agency for Research on Cancer as "carcinogenic to humans" (group 1)[68]. Probable mechanisms for the association between alcohol drinking and upper digestive tract cancer have been presented in several studies[98,99]. The carcinogen of esophageal cancer, with regard to alcohol consumption, is acetaldehyde[100,101], which is a highly reactive and toxic alcohol metabolite. Acetaldehyde interferes with DNA repair machinery and directly inhibits O6 methylguanyltransferase, which is an enzyme that is deemed important for the repair[102]. The inhalation of acetaldehyde has been known to cause bronchial cancer and esophageal cancer. Several studies have reported the hazards of binge drinking using experiments. After *in vivo* administration of ethanol in the stomach of rats, which is analogous to the binge drinking condition, histone H3 modification, which primarily affects histone methylation in the liver, lung and spleen, was detected in the histone of rat tissues[103]. A study in a South Korean population that included 2677 men of 55 years of age, with a follow-up of 20.8 years, associated severe binge drinking and its frequency with mortality due to oral and esophageal cancer. A higher mortality was observed in these cancers for patients with a daily binge drinking habit compared with nondrinkers. The alcohol dose and mortality due to esophageal cancer and the mortality and the frequency of alcohol consumption are highly associated, whereas the volume of consumed alcohol is not highly associated. Note that tobacco consumption was an important confounding factor in this study[104]. Binge alcohol consumption seems to be a risk factor for pancreatic cancer. After adjusting for sex and age in a case-control population study[105] in San Francisco (United States) with 532 cases and 1701 controls, the risk of pancreatic cancer was determined to be higher in binge drinking patients when a higher amount of units were consumed and a longer consumption had occurred. This finding supports the notion that a high consumption of alcohol, including binge drinking, is a risk factor for the development of pancreatic cancer. Alcohol is also involved in the development of a hepatocarcinoma. Acetaldehyde, a reactive metabolite of ethanol, binds to nucleic acids, proteins such as enzymes, microsomal proteins and microtubules. The generated reactive oxygen species can also activate or repress the epigenetic elements such as chromatin remodeling, non-coding RNAs (micro-RNAs), DNA (de) methylation and histone modification that affect gene expression, hence leading to hepatocarcinoma[106].

We should consider that smoking is a known risk factor for upper digestive tract cancer, including oral cavity, pharynx and esophagus cancers. Therefore, the interaction of alcohol and tobacco synergistically elevates the disease risk to a level above the risk posed by the individual use of either of these substances.

Neurocognitive effects

The consequences on the memory of alcoholic beverage binge drinking have been explored in animal models. The results show that binge doses of alcohol cause a disruption in the growth of new brain cells; this lack of new growth may cause the long-term deficits detected in key areas of the brain (such as hippocampal structure and function) that are induced by binge drinking[107,108]. The increasing interest in performing studies to analyze the neurotoxic effect of alcohol due to the increased practice of binge drinking in adolescence is not surprising. A "safe" alcohol dose for the developing brain of an adolescent is unknown. The prefrontal cortex and limbic system, which includes the hippocampus, undergoes prominent reorganization during the late teenage years[109,110]; these cognitive processes, which are dependent on these areas of the brain, such as memorial processes, are very sensitive to any damage caused by excessive alcohol ingestion. Different studies have reported poorer performance in neurocognitive tests with the worst verbal memory and poorer episodic memory[111]. Binge

drinking affects the executive functions and the working memory from the Brodmann areas 46 and 9 of the dorsomedial prefrontal cortex. Studies of neurocognitive function in teenagers aged 15-19 years with a history of alcohol abuse have revealed deficits for a range of language and attentional tasks, verbal and non-verbal memory tasks, and specific working memory impairments[112,113]. Compared with nonalcohol drinkers, binge drinkers evinced cognitive impairments in the Paced Auditory Serial Addition Test regarding executive planning function and episodic memory tasks-these findings were similar to frontal function deficits observed in Korsakoff alcoholics[105]. Using magnetic resonance, several studies[114] have correlated binge drinking in post-adolescence and early adulthood with brain structural alterations. These results showed a greater decrease in the gray matter of the dorsomedial prefrontal cortex in binge drinking subjects compared with the control subjects. A positive correlation between the increased gray matter in binge drinkers and the results from the Self-Ordered Pointing Test (SOPT), which is an error test, was observed[109]. The measure of the prefrontal cortex was also correlated with the volume and the rate of alcohol intake[109].

A Spanish cohort study[115] evaluated the binge drinking habits of 89 university students with a two-year follow-up. The neuropsychological performance was measured using several scales; binge drinkers yielded the worst scores in the Wechsler Memory Scale-Ⅲ and the SOPT and demonstrated a worse verbal memory compared with nonbinge drinkers. Another study, with a longer follow-up of ten years, of adolescents with abusive alcohol consumption revealed that verbal memory deteriorated with time for adolescents who presented the habit to a young adult age[116]. At a neuropsychological level, binge drinking subjects show deficiencies in the assessment tests for the frontal executing functions of attention, planning, cognitive flexibility, work memory, decision-making, verbal fluency, decision-changing and inhibitory control tasks[117].

Regarding the effect of prospective memory, a study[118] showed similar results on the Prospective and Retrospective Memory Questionnaire test for binge drinkers and nonbinge drinkers. These findings contrast with another study by the same author[119]. A higher number of short- and long-term prospective memory lapses were observed in this group. This study excluded consumers of other substances and lacked the control of the age, type of alcohol consumption, hours after the last intake or period of consumption. However, a lower score in the prospective remembering video procedure (PRVP) was observed in binge drinkers, which revealed differences when consumers of other substances and consumers who had drank alcohol in the last 48 h were excluded. Nonsignificant differences were observed between the groups regarding age, anxiety or depression levels, and years of alcohol consumption. Subjects with a higher intake of alcohol units per week demonstrated lower results in the PRVP test.

Effect on the cardiovascular system

Approximately 10% of cardiovascular disease-related deaths are attributable to alcohol[120]. The probability of coronary heart disease and cardiovascular mortality increases with heavy consumption[121]. Studies suggest that a binge pattern of drinking may precipitate myocardial ischemia or infarction[122], and evidence of an association between binge alcohol consumption and a two-fold greater mortality after acute myocardial infarction also exists[123]. In addition to the volume of consumption, the *pattern* of drinking must be considered. Recently, Liu *et al*[124] demonstrated that binge patterns in mice increase the development of atherosclerosis compared with no alcohol controls. The results from retrospective studies of adults who range in age between 40 and 60 years have indicated that binge drinking is associated with a heightened risk of cardiovascular (CV) events, such as stroke, sudden death, myocardial infarction, and increased mortality after myocardial infarction[123,125-127]. In addition, an alcohol binge drinking pattern is associated with the progression of carotid atherosclerosis[128]. Endothelial dysfunction is an early indicator of blood vessel damage and atherosclerosis and a strong prognostic factor for future CV events[129-131]. In binge drinkers, cardioprotective changes in high density lipoproteins are not observed, and adverse changes in low-density lipoproteins are acquired. Binge drinking seems capable of predisposing the heart to arrhythmia by reducing the threshold for ventricular fibrillation and by causing scarring of the myocardium. The myocardium may be especially sensitive during withdrawal, as will occur after weekend binges. In addition, irregular drinking is associated with an increased risk of thrombosis, which is most likely to occur after heavy drinking stops. These physiological mechanisms may explain the observed increase in cardiovascular events during the weekend and on Mondays. In countries with known weekend binge drinking, the Monday peak is pronounced and is accompanied by slight increases in mortality on Saturdays and Sundays. This finding has been observed in countries of the former Soviet Union and in Scotland[132-134]. Chenet *et al*[135] hypothesize that alcohol, particularly when drunk in binges, serves as a catalyst in acute ischemic heart diseases by being synergetic to other triggering factors.

In an experimental animal model in which binge alcohol was administered after chronic alcohol treatment, binges caused a decrease in the messenger ribonucleic acid (mRNA) of low-density lipoprotein-receptor (LDL-R) and increased mRNA levels of the angiotensinogen gene in the liver. Binge ethanol intake in chronically exposed rat liver decreased LDL-R and increased angiotensinogen gene expression[136]. Note that increases in plasma LDL cholesterol and angiotensin are cardiovascular risk factors in human alcoholics. In a recent study performed in ApoE KO mice, the arterial lumen was reduced and the deposits of macrophages were more evident, which confirms the aterogenic capacity of alcohol binge drinking[124]. These results imply that binge alcohol-

induced alterations in liver have consequences on the cardiovascular system. Thus, binge drinking affects interorgan cross-talk. This finding is further supported by increases in the plasminogen activator inhibitor (PAI). PAI-1 serves a major role in fibrin metabolism by blocking fibrinolysis. The role of PAI-1 in fibrin accumulation in vascular disease is well understood to contribute to endothelial dysfunction and inflammation.

Thus, these findings provide strong evidence to support a health message that discourages binge drinking. The provision to healthcare professionals of scientific evidence that binge drinking can accelerate atherosclerosis may encourage them to perform brief interventions for individuals with at-risk drinking behaviors.

Other effects

The effect of alcohol on other organs and systems varies. Binge drinking is one of the main causes of pancreatitis[137] and is involved in a higher mortality from a duodenal ulcer[138]. It is also the cause of neuropsychiatric conditions, such as depression[139]. In the kidney[140], binge drinking has been correlated with glomerulonephritis, acute nephropathies, and the loss of kidney transplants. It is the cause of fertility disorders, prematurity, low weight and newborn alcoholic syndrome[141].

APPROACHING THE PROBLEM

Numerous social and political interventions are available to decrease this type of consumption, such as laws against driving under the effect of alcohol, increased taxes, restriction of access and availability of alcohol, and brief interventions, such as medical advice and control *via* publicity.

Our main weapon against this problem is primary prevention, which is difficult to develop due to established alcohol consumption among different cultures, which is primarily associated with social events. In this manner, the WHO has developed a strategic plan to approach the harmful consumption of alcohol, based on preventive interventions[142,143] with the help of health services, to reduce access to alcoholic beverages and prohibit its marketing and by increasing prices.

A substantial amount of evidence across different countries to support making alcohol more expensive, primarily *via* taxation, and to reduces the extensive range of harm due to intoxication and binge drinking, including road traffic accidents and fatalities, intentional and unintentional injuries, rapes and robberies, homicides, crime, and violence[144]. Another issue in this plan is the marketing control of the illicit production of beverages with regulation systems.

Similarly, a substantial amount of evidence to support raising the minimum purchasing age to reduce alcohol-related road traffic accidents and to reduce the density of alcohol outlets to reduce drunkenness, assaults, and road traffic fatalities. However, these strategies will only be effective if it is not backed up with a credible threat to remove the licenses of outlets that repeatedly sell to under-aged customers. These strategies are also more effective when supported by community-based prevention programs. Some of these measures are effective in decreasing the damage caused by alcohol but are also cost-effective from a revenue point of view due to increases in the price and taxes of alcoholic beverages[143,145].

Preliminary data support the intriguing possibility that integrated intervention may enhance smoking cessation and reduce binge drinking[146].

Decreased smoking and improved maintenance of abstinence may result from a behavioral intervention to reduce binge drinking. This hypothesis is supported by several lines of evidence, including conditioning mechanisms in which the craving to smoke is elicited by higher levels of alcohol consumption[147,148], and environmental factors, such as parental and peer influence for concurrent use of cigarettes while engaging in binge drinking[149].

Smoke-free bar policies may not be sufficient to influence the association between smoking and drinking, particularly if tobacco marketing continues in these venues or in the absence of programs that specifically address the co-use of tobacco and alcohol. Tobacco interventions should prioritize bars and other social venues that are popular among young adults to reach persons who are at greatest risk. The strong and consistent association between smoking and drinking indicates that public health efforts and clinical cessation programs need to address the paired use of tobacco and alcohol among the young adult bar-going population.

CONCLUSION

Binge drinking is an increasing public health issue that affects teenagers and young adults. Although its consequences are not well known, relevant hepatic, cardiovascular, neurocognitive and oncogenic effects may be present. Binge drinking also has a significant social and economic impact. Interventions should be globally approached to address the consumption of alcohol and tobacco.

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·病毒性肝炎·

甘草酸二铵脂质复合物肠溶胶囊与甘草酸二铵胶囊对照治疗慢性病毒性肝炎687例荟萃分析

凌青霞 金宏慧 郑建铭 施光峰

【摘要】 目的 根据现有临床研究系统评价甘草酸二铵脂质复合物肠溶胶囊与传统甘草酸制剂甘草酸二铵胶囊治疗慢性病毒性肝炎的疗效及安全性。 **方法** 检索中国生物医学光盘数据库和中国期刊网数据库，纳入自2005年至2012年比较甘草酸二铵脂质复合物肠溶胶囊与甘草酸二铵胶囊治疗慢性病毒性肝炎的疗效及安全性的随机对照试验。并对相关文献进行荟萃分析。 **结果** 共9项随机对照试验入选，共纳入687例患者。荟萃分析结果显示与传统甘草酸二铵胶囊相比，甘草酸二铵脂质体复合物肠溶胶囊可提高慢性病毒性肝炎患者ALT的复常率[相对危险度（RR）=4.15，95%置信区间（*CI*）为1.55～11.15，$P<0.01$）]。甘草酸二铵脂质复合物肠溶胶囊降低血清ALT的效率高于甘草酸二铵胶囊[加权平衡差（WMD）=−32.75，95% *CI*为−46.67～−18.83，$P<0.01$）]。降低血清AST的效率高于甘草酸二铵胶囊（WMD=−12.70，95% *CI*为−21.13～−4.27，$P<0.01$）。在改善血清胆红素方面有优于甘草酸二铵胶囊的倾向，但差异无统计学意义（WMD=−0.74，95% *CI*为3.98～2.49，$P>0.05$）。在改善血清白蛋白方面有优于甘草酸二铵胶囊的倾向，但两组间差异无统计学意义（WMD=1.03，95% *CI*为−1.03～3.09，$P>0.05$）。 **结论** 与传统口服甘草酸制剂相比，甘草酸二铵脂质复合物肠溶胶囊具有更强的抗炎保肝作用。两者均未发现明显不良反应。

【关键词】 肝炎，病毒性，人； 甘草酸二铵； Meta分析

A meta-analysis of diammonium glycyrrhizinate enteric-coated capsules versus diammonium glycyrrihizinate in patients with chronic viral hepatitis *Ling Qingxia*, Jin Honghui, Zheng Jianming, Shi Guangfeng. *Department of Infectious Diseases, Huashan Hospital, Fudan University, Shanghai 200040, China*

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【Abstract】 Objective To systematically evaluate the efficacy and safety of diammonium glycyrrhizinate enteric-coated capsules versus diammonium glycyrrihizinate in patients with chronic viral hepatitis. **Methods** The Chinese Biomedical Literature Database (CBM on CD-ROM) and the China Academic Journals Full-Text Database (Chinese National Knowledge Infrastructure, CNKI) were searched for randomized controlled trials (RCTs) that compared the efficacy and safety of diammonium glycyrrhizinate enteric-coated capsules versus diammonium glycyrrihizinate in treatment (>2 months) of chronic viral hepatitis published between 2005 and 2012. A meta-analysis was performed on the selected RCTs to determine the effects on alanine aminotransferase (ALT) normalization, serum levels of ALT, aspartate aminotransferase (AST), total bilirubin (TBil) and albumin, as well as rates of adverse reactions. **Results** Nine RCTs, involving 687 patients, were included in the meta-analysis. Compared to the patients treated with diammonium glycyrrihizinate, the patient treated with diammonium glycyrrhizinate enteric-coated capsules had a significantly better recovery rate of ALT (relative

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risk (RR) = 4.15, 95% confidence interval (*CI*): 1.55 to 11.15, $P < 0.01$) and significantly more robust decreases in ALT (weighted mean difference (WMD) = -32.75, 95% *CI*: -46.67 to -18.83, $P < 0.01$) and AST (WMD = -12.70, 95% *CI*: -21.13 to -4.27, $P < 0.01$). In contrast, the patients treated with diammonium glycyrrhizinate showed more robust improvements in the TBil level (WMD = -0.74, 95% *CI*: 3.98 to 2.49, $P = 0.653$) and albumin (WMD = 1.03, 95% *CI*: -1.03 to 3.09, $P = 0.326$), but the differences did not reach the threshold for statistical significance ($P > 0.05$). Only four adverse reactions were reported, all of which were related to the lipid complex nature of the diammonium glycyrrhizin enteric-coated capsules and were mild, including dry mouth, dizziness and mild gastrointestinal discomfort and reactions. **Conclusion** Diammonium glycyrrhizinate enteric-coated capsules elicited superior anti-inflammatory and liver protection effects than diammonium glycyrrihizinate, and produced only mild side effects that are tolerable to the patients.

【Key words】 Hepatitis, viral, human; Diammonium glycyrrihizinate; Meta-analysis

传统甘草酸制剂包括复方甘草酸苷、甘草酸二铵等药物，均为大分子物质，极性较大，口服不易吸收。甘草酸二铵胶囊是目前临床最常用的保肝、抗炎药物之一。多年的临床应用结果显示其具有较强的抗炎、免疫调节、保护肝细胞膜、改善胆红素代谢、抗肝纤维化等方面的作用。慢性病毒性肝炎患者因肝脏的持续炎症和破坏，其肝脏长期处于受损状态，ALT及AST反复升高，致使患者需长期用药。目前，甘草酸二铵胶囊已广泛用于慢性病毒性肝炎治疗，不仅能降低ALT和AST，并能改善肝功能。本研究应用Meta分析的方法，荟萃多项随机对照试验，定量分析各研究结果数据，以期更加客观地评价甘草酸二铵脂质复合物肠溶胶囊与甘草酸二铵胶囊治疗慢性病毒性肝炎的作用。

资料与方法

1. 资料：文献来源于2005年至2012年中国生物医学文献数据库(CBM)光盘、中国期刊网数据库(CNKI)。

2. 文献检索方法：采用关键词和主题词(全部扩展树)检索“病毒性肝炎”；自由词检索“甘草酸二铵”、“甘草酸二铵胶囊”、“甘草酸二铵脂质复合物肠溶胶囊”、“对照研究”、“对比研究”、“分组研究”；特征词检索，查找了2项数据库，即中国生物医学文献光盘(CBM)和中国期刊网数据库(CNKI，网上检索)，检索年限为2005年至2012年。并根据查阅文献的参考文献查缺补漏。

3. 文献纳入标准：(1) 研究设计：随机对照试验；(2) 研究对象：慢性病毒性肝炎；(3) 干预措施：试验组给予甘草酸二铵脂质复合物肠溶胶囊，对照组给予甘草酸二铵胶囊，治疗时间均＞2个月；(4) 研究结果：ALT复常率，血清ALT、AST、总胆红素、白蛋白的变化，不良反应。

4. 统计学方法：两名研究者独立进行文献选择和资料提取工作，而后进行交叉核对。对入选研究用Stata8.2进行统计分析。观察指标为计量资料选用加权均数差值(weighted mean differences，WMD)。计数资料时采用Peto方法，计算相对危险度(RR)，两者均计算95%的可信区间(*CI*)，而后进行异质性检验，当试验结果的异质性无统计学意义($P > 0.05$)时，选择固定效应模型Meta分析，当试验结果的异质性有统计学意义($P < 0.05$)时，选择随机效应模型。

结　果

1. 文献入选情况：共9项随机对照试验研究入选，纳入了687例慢性病毒性肝炎患者[1-9]。所有病例的诊断均依据1995年北京第5次全国传染病和寄生虫学术会议修订的标准[10]，或2000年西安第10次全国传染病和寄生虫病学术会议修订的《病毒性肝炎防治方案》诊断标准[11]。各组在性别、年龄、病程及病变程度上具有可比性。

2. 甘草酸二铵脂质复合物肠溶胶囊治疗组与甘草酸二铵胶囊对照组治疗后ALT复常率的比较：7项研究比较了两种药物治疗后ALT的复常率，甘草酸二铵脂质复合物肠溶胶囊治疗组ALT的复常率高于甘草酸二铵胶囊对照组(RR = 4.15，95% *CI* 为1.55～11.15)，两组间的差异有统计学意义($P < 0.01$)，见图1。

3. 甘草酸二铵脂质复合物肠溶胶囊治疗组与甘草酸二铵胶囊对照组治疗前后ALT变化的比较：6项研究比较了两种药物的治疗前后ALT的变化，甘草酸二铵脂质复合物肠溶胶囊降低ALT效率高于甘草酸二铵胶囊，两组间差异有统计学意义，见图2。Meta分析结果为WMD = −32.75，其95% *CI* 为(−46.67，−18.83)，$P < 0.01$。

4. 甘草酸二铵脂质复合物肠溶胶囊治疗组与甘

草酸二铵胶囊对照组治疗前后 AST 变化的比较：5 项研究比较了两种药物的治疗前后 AST 的变化，甘草酸二铵脂质复合物肠溶胶囊降低 AST 效率高于甘草酸二铵胶囊，两组间差异有统计学意义，见图 3。Meta 分析结果为 WMD = −12.70，其 95% *CI* 为 (−21.13，−4.27)，$P < 0.05$。

5. 甘草酸二铵脂质复合物肠溶胶囊治疗组与甘草酸二铵胶囊对照组治疗前后血清总胆红素 (TBil) 变化的比较：3 项研究比较了两种药物治疗前后 TBil 的变化，甘草酸二铵脂质复合物肠溶胶囊降低 TBil 效率有高于甘草酸二铵胶囊的倾向，但两组间差异无统计学意义，见图 4。Meta 分析结果为 WMD = −0.74，其 95% *CI* 为 (−3.98，2.49)，$P = 0.653$。

6. 甘草酸二铵脂质复合物肠溶胶囊治疗组与甘草酸二铵胶囊对照组治疗前后白蛋白变化的比较：2 项研究比较了两种药物治疗前后白蛋白的变化，两组间差异无统计学意义，见图 5。Meta 分析结果为 WMD = 1.03，其 95% *CI* 为 (−1.03 ~ 3.09)，$P = 0.326$。

7. 不良反应：4 项临床研究对不良反应进行了观察和具体说明，其结论为甘草酸二铵脂质复合物肠溶胶囊在临床试验中不良反应轻微，少数病例有口干、头晕等感觉或轻微胃肠道反应，无需治疗，均可耐受。

讨　论

甘草酸是甘草的提取物，经药理临床研究确认为安全有效的治疗肝炎的药物[12]。甘草酸二铵具有控制肝脏炎症、降低血清转氨酶的作用，并能抑制肝脏对类固醇激素的还原代谢，从而使类固醇激素的作用增强，显示其抗炎作用，但无类固醇激素样的不良反应。甘草酸二铵注射液现已广泛应用于临床治疗，但由于给药途径的限制，存在疗程短、停药后病情反复现象。甘草酸二铵胶囊自应用以来，弥补了静脉给药的不足，经临床观察其疗效良好，但与静脉制剂间仍有一定差距[13]。甘草酸为高亲水性、高极性的大分子物质，经肠道吸收率低，血药浓度无法达到相应水

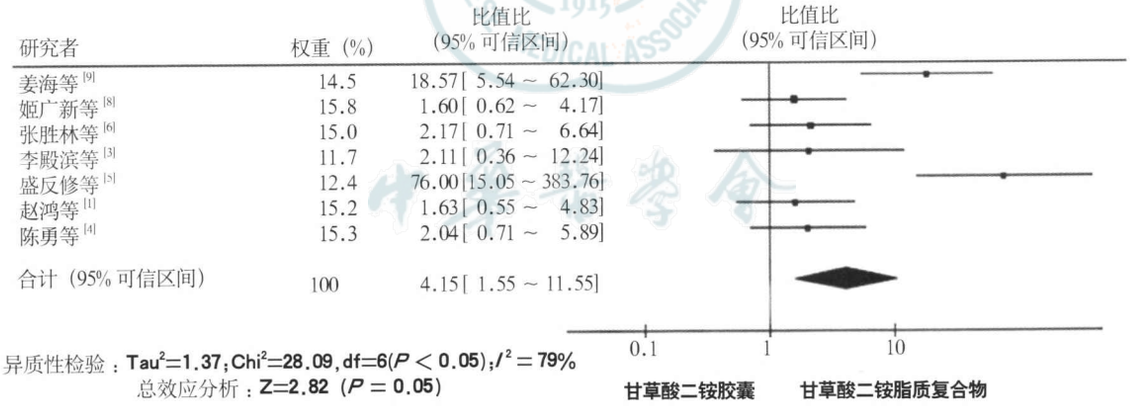

图 1 甘草酸二铵脂质复合物肠溶胶囊与甘草酸二铵胶囊治疗后 ALT 复常率的比较

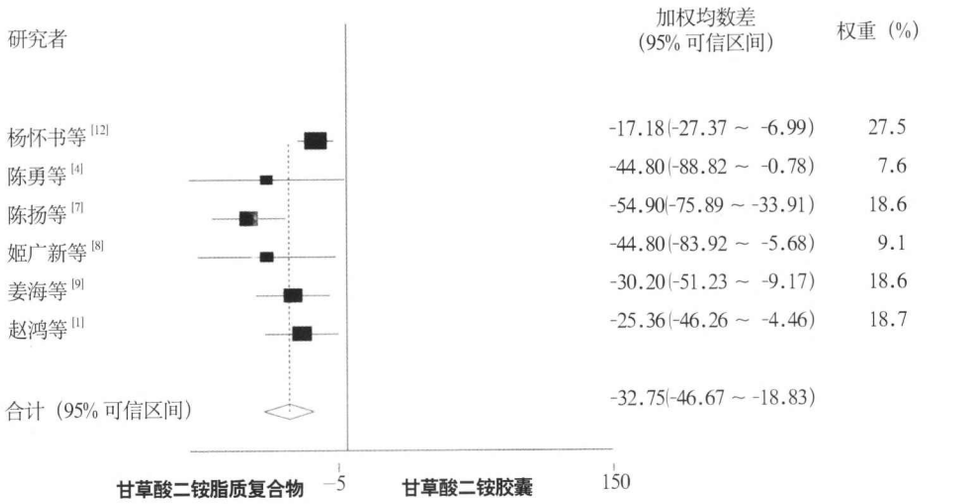

图 2 甘草酸二铵脂质复合物肠溶胶囊与甘草酸二铵胶囊治疗前后 ALT 变化的比较

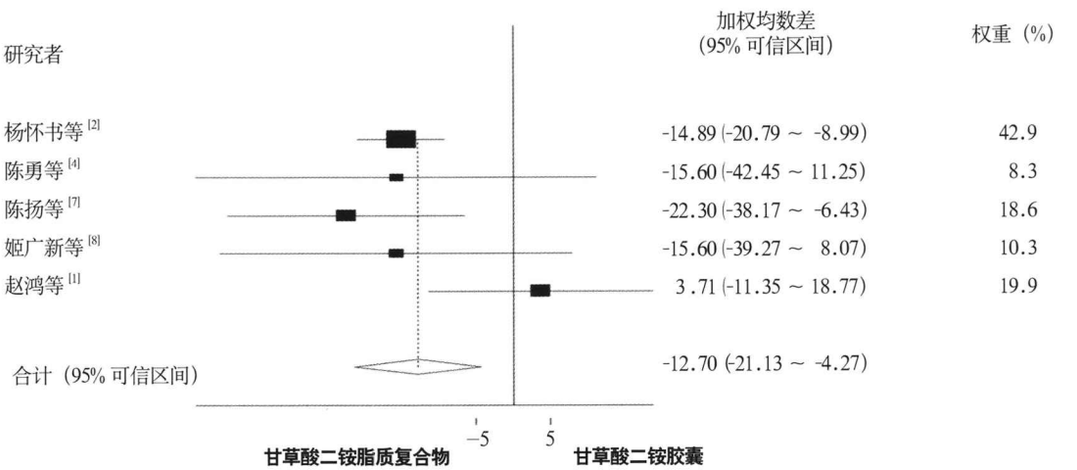

图3　甘草酸二铵脂质复合物肠溶胶囊与甘草酸二铵胶囊患者治疗前后 AST 情况的比较

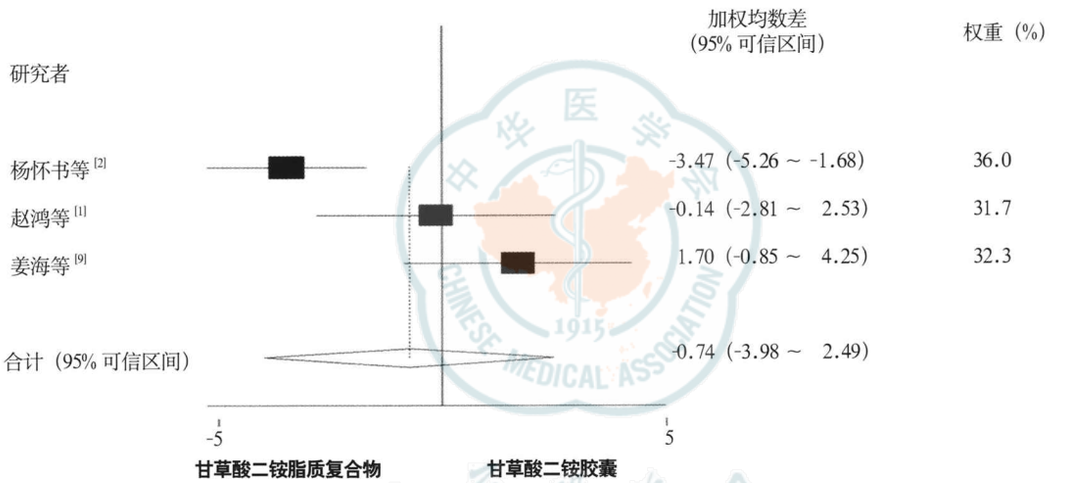

图4　甘草酸二铵脂质复合物肠溶胶囊与甘草酸二铵胶囊患者治疗前后血清总胆红素变化的比较

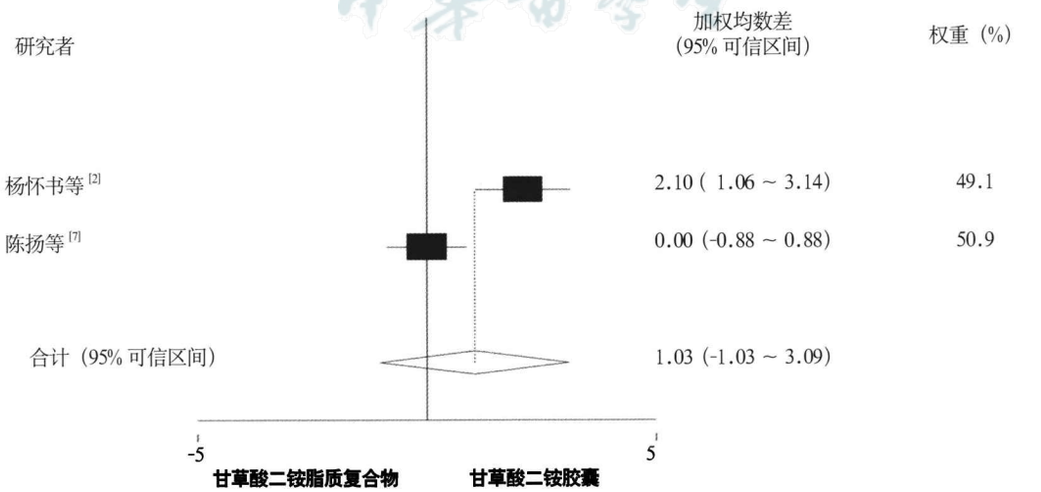

图5　甘草酸二铵脂质复合物肠溶胶囊与甘草酸二铵胶囊患者治疗前后白蛋白变化的比较

平。甘草酸二铵脂质复合物肠溶胶囊为甘草酸与磷脂结合形成的脂质复合体，增加了其水溶性，并且利用口服甘草酸主要在肠道吸收的特点，制成肠溶胶囊从而使药物定点在肠道释放，从而促进药物的吸收，增强药物的疗效。

本研究结果显示，甘草酸二铵脂质复合物肠溶胶囊治疗慢性病毒性肝炎的有效率高于甘草酸二铵胶囊，体现为临床症状改善明显,降酶作用可靠、迅速，能有效控制肝脏炎症。因此，甘草酸二铵脂质复合物肠溶胶囊治疗慢性病毒性肝炎的疗效优于甘草酸二铵胶囊。二者的不良反应均较少，但是由于多数文献无随访报告，有待于在今后进一步的临床研究

中加以完善。

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· 读者 · 作者 · 编者 ·

本刊对论文中实验动物描述的要求

根据国家科学技术部 1988 年颁布的《实验动物管理条例》和卫生部 1998 年颁布的《医学实验动物管理实施细则》，本刊对论文中有关实验动物的描述，要求写清楚以下事项：(1) 品种、品系及亚系的确切名称；(2) 遗传背景或其来源；(3) 微生物检测状况；(4) 性别、年龄、体质量；(5) 质量等级及合格证书编号；(6) 饲养环境和实验环境；(7) 健康状况；(8) 对实验动物的处理方式。

医学实验动物分为四级：一级为普通级；二级为清洁级；三级为无特定病原体（SPF）级；四级为无菌级（包括悉生动物）。卫生部级课题及研究生毕业论文等科研实验必须应用二级以上的实验动物。

异甘草酸镁对大鼠非酒精性脂肪性肝炎的防治作用

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[摘要] **目的**：研究异甘草酸镁对大鼠非酒精性脂肪性肝炎（NASH）的防治作用。**方法**：SD 大鼠 40 只，随机分为 4 组（n=10）：对照组、模型组、烟酸组及异甘草酸镁组。除对照组外，其余各组每天给予脂肪乳剂和烟酸，异甘草酸镁。实验开始 1 周后断尾取血，测定血清脂质；3 周后处死动物，测定血清三酰甘油（TG）、总胆固醇（TC）、丙氨酸氨基转移酶（ALT）、天门冬氨酸氨基转移酶（AST）、游离脂肪酸（FFA）、肿瘤坏死因子-α（TNF-α）和肝匀浆 TG、TC、FFA，并行肝脏病理学检查。**结果**：模型组大鼠出现 NASH，异甘草酸镁组能降低大鼠血清 TG（$P<0.05$）、ALT（$P<0.01$）、FFA（$P<0.05$）、TNF-α（$P<0.01$），同时也能降低肝匀浆 TG（$P<0.01$）、FFA（$P<0.05$），肝脂肪变性程度和炎症均明显减轻（$P<0.05$）。**结论**：异甘草酸镁对脂肪乳剂诱导的 NASH 大鼠有较好的防治作用。

[关键词] 异甘草酸镁；非酒精性脂肪性肝炎；大鼠；防治作用
[中图分类号] R963 [文献标识码] A [文章编号] 1001-5213(2010)18-1587-03

The protective and therapeutic effects of magnesium isoglycyrrhizinate on nonalcoholic steatohepatitis in rats

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ABSTRACT: OBJECTIVE To study the protective and therapeutic effects of magnesium isoglycyrrhizinate(MgIG) on nonalcoholic steatohepatitis in rats. **METHODS** 40 SD rats were randomized into control, model, nicotinic acid, MgIG group(n=10). The control group were only fed with a standard diet and the other groups were fed with a standard diet and fat emulsion. At the same time, the control and model groups were fed with sodium carboxymethylcellulose, the other groups were fed with relative medicine. The serum TG, TC, ALT, AST, FFA, TNF-αand hepatic TG, TC, FFA were detected and with histologically. **RESULTS** The model group developed NASH after 3 weeks. MgIG significantly reduced TG($P<0.05$), ALT($P<0.01$), FFA($P<0.05$)and TNF-α($P<0.01$)of the serum and TG($P<0.05$), FFA($P<0.05$) of the liver tissue, and ameliorated hepatocellular macrovesicular steatosis($P<0.05$). **CONCLUSION** MgIG has protective and therapeutic effects on fat emulsion-induced NASH in rats.

KEY WORDS: magnesium isoglycyrrhizinate; nonalcoholic steatohepatitis; rat; protective and therapeutic effects

非酒精性脂肪性肝炎（nonalcoholic steatohepatitis，NASH）是以无过量饮酒史及肝细胞脂肪变性、气球样变、弥漫性肝小叶炎症和（或）肝中央静脉、肝窦周围胶原沉积为特征的慢性肝脏疾病，可发展为肝纤维化和肝硬化，甚至导致死亡，其发病率也在逐年升高，但其发病机制至今尚未明确，也缺乏有效的防治手段。异甘草酸镁（magnesium isoglycyrrhizinate，MgIG）是第四代甘草酸制剂，具有降酶，减轻肝细胞变性、坏死及炎症细胞浸润的作用[1]，但其是否具有防治 NASH 的作用尚无人探讨。本研究旨在观察异甘草酸镁对 NASH 的防治作用，为其扩大临床适应证提供实验依据。

1 材料与方法

1.1 药物与试剂 异甘草酸镁（江苏正大天晴制药公司，批号 070503）；丙赛优（上海复星朝晖药业有限公司）；聚山梨酯 80（蚌埠化学试剂厂）；1-2 丙二醇（淮南市化学试剂厂）；烟酸片（上海九福药业有限公司）；胆固醇（中国医药（集团）上海化学试剂公司）；脱氧胆酸钠（北京双旋微生物培养基制品厂）。三酰甘油（TG）、总胆固醇（TC）测定试剂盒购自北京福瑞生物工程公司；TNF-α 放免试剂盒（北京东亚免疫技术研究所）；天门冬氨基转移酶（AST）、丙氨酸氨基转移酶（ALT）、游离脂肪酸（FFA）、考马斯亮兰蛋白测定试剂盒购自南京建成生物工程研究所。

1.2 仪器 电子天平 FA2004、79HW-1 恒温磁力搅拌器、722 紫外分光光度计，医学图像分析系统等。

1.3 实验动物 Spague-Dawlay（SD）大鼠 40 只，雌雄各半，体质量 180～200 g，由安徽医科大学实验

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动物中心提供。

1.4　动物实验　按体质量随机分为 4 组（每组 10 只）：对照组、模型组、烟酸组（120 mg·kg^{-1}·d^{-1}），异甘草酸镁（120 mg·kg^{-1}·d^{-1}，由成人剂量1.5 g·d^{-1}折算，羧甲基纤维素钠溶解，配制成质量浓度为 15 g·L^{-1}的溶液）组，脂肪乳剂灌胃复制动物模型[2]。除对照组外，各组每天上午灌脂肪乳剂（10 mL·kg^{-1}·d^{-1}），下午灌胃给予相应的药物，对照组和模型组予以等量的羧甲基纤维素钠。于给药 1 周后，断尾取血，测定血清脂质；3 周后，放血处死动物，迅速取出肝脏备用。

1.5　观察指标及测定方法

1.5.1　一般情况　实验前后每周称体质量 1 次，观察食欲行为、粪便及动物死亡情况；实验结束时处死动物并称量肝脏湿重。

1.5.2　血清生化　血清 TC、TG、ALT、AST、FFA、TNF-α 测定，按试剂盒规范操作。

1.5.3　肝脏生化　在相同部位精确称取肝脏 0.4 g，加入预冷的生理盐水，在冰水中制成 10%的匀浆，4 ℃，3 000 r·min^{-1}离心 10 min，提取上清液，测定 TC、TG、FFA 含量。

1.5.4　组织学病检　石蜡切片进行 HE 染色，在光镜下观察肝脂肪变性和炎症活动情况。肝细胞脂肪变性程度判断标准参照文献[3]。炎症活动度计分标准参考 Knodell 提出慢性肝炎组织学活动指数，并结合文献[4]提出的慢性肝炎炎症活动度计分方案。

1.6　统计学处理　计量资料 $x \pm s$ 表示，采用方差分析；等级资料采用秩和检验，$P<0.05$有显著性差异。

2　结果

2.1　一般情况　实验期间各组体质量无明显差异。与对照组相比，模型组肝指数（肝湿重/体质量×100%）显著性增加；与模型组相比，烟酸组及异甘草酸镁组肝指数则明显降低，见表 1。

表 1　各组体重、肝指数的变化（$x \pm s$, $n=10$）

Tab 1　Changes of body weight, liver index ($x \pm s$, $n=10$)

组别	剂量/mg·kg^{-1}	体质量/g				肝指数/%
		0 周	第 1 周	第 2 周	第 3 周	
对照组	-	192±8	199±5	209±6	218±8	2.75±0.14
模型组	-	193±7	202±7	210±9	225±7	3.13±0.15[a]
烟酸组	120	196±4	199±8	222±5	229±11	2.71±0.23[b]
异甘草酸镁组	120	195±6	204±6	216±7	226±6	2.62±0.25[b]

注：与对照组比较，[a] $P<0.01$；与模型组比较，[b] $P<0.01$

2.2　血清脂质变化　与对照组相比，模型组 1 周和 3 周时 TC、TG 均显著升高；与模型组相比，烟酸组、异甘草酸镁组 TG、TC 则明显降低，见表 2。

2.3　血清 ALT、AST、FFA、TNF-α 的变化　与对照组相比，模型组 ALT、FFA、TNF-α 明显升高；与模型组相比，异甘草酸镁组 ALT、FFA、TNF-α 明显下降，见表 3。

表 2　各组血清脂质动态变化（$x \pm s$, $n=10$）

Tab 2　Dynamically changes of TG, TC of the serum ($x \pm s$, $n=10$)

组别	剂量/mg·kg^{-1}	TG/mmol·L^{-1}		TC/mmol·L^{-1}	
		第 1 周	第 3 周	第 1 周	第 3 周
对照组	-	0.49±0.13	0.48±0.12	1.43±0.25	1.47±0.56
模型组	-	0.65±0.16[a]	0.69±0.15[a]	2.18±0.37[a]	2.81±0.52[a]
烟酸组	120	0.53±0.09[b]	0.55±0.09[b]	1.78±0.29[b]	2.35±0.71[b]
异甘草酸镁组	120	0.54±0.12[b]	0.52±0.11[b]	1.83±0.24[b]	2.43±0.45[b]

注：与对照组比较，[a] $P<0.01$；与模型组比较，[b] $P<0.05$

表 3　各组血清 ALT、AST、FFA、TNF-α 的变化（$x \pm s$, $n=10$）

Tab 3　Changes of ALT, AST, FFA and TNF-α of the serum ($x \pm s$, $n=10$)

组别	剂量/mg·kg^{-1}	ALT/U·L^{-1}	AST/U·L^{-1}	FFA/mmol·L^{-1}	TNF-α/nmol·L^{-1}
对照组	-	10.14±3.90	42.23±19.02	445.07±116.6	45.85±1.42
模型组	-	59.14±20.42[a]	60.12±20.72	748.02±160.51[a]	13.92±2.85[a]
烟酸组	120	38.14±16.40	53.35±14.66	623.56±173.29	11.13±1.93[b]
异甘草酸镁组	120	33.90±14.16[c]	48.35±24.42	583.01±165.39[b]	8.83±1.32[c]

注：与对照组比较，[a] $P<0.01$；与模型组比较，[b] $P<0.05$，[c] $P<0.01$

2.4　肝脏脂质变化　与对照组相比，模型组 TG、FFA 显著升高；与模型组相比，异甘草酸镁能 TG 和 FFA 明显降低，但烟酸组对 FFA 无明显影响，见表 4。

表 4　各组肝匀浆 TG、TC、FFA 的变化（$x \pm s$, $n=10$）

Tab 4　Changes of TG, TC and FFA of the liver ($x \pm s$, $n=10$)

组别	剂量/mg·kg^{-1}	TG/mmol·l^{-1}	TC/mmol·l^{-1}	FFA/μmol·g^{-1}
对照组	-	0.47±0.08	1.32±0.16	4.5±0.5
模型组	-	1.69±0.34[a]	1.38±0.28	5.6±1.0[a]
烟酸组	120	0.9±0.4[b]	1.13±0.17	5.3±1.3
异甘草酸镁组	120	0.93±0.30[c]	1.18±0.24	4.8±1.1[b]

注：与对照组比较，[a] $p<0.01$；与模型组比较，[b] $p<0.05$，[c] $p<0.01$

2.5　肝脏病理学变化　光镜下，对照组大鼠肝脏无异常。模型组动物均出现程度不同的弥漫性肝细胞脂肪变性，脂变肝细胞极度肿胀，成圆形，胞浆内充满大量脂肪空泡，界限不清；并出现程度不同炎症，但未见纤维化。与模型组相比，异甘草酸镁组脂肪变性及炎症活动度明显减轻，见表 5 和图 1。

3　讨论

最近研究显示，异甘草酸镁不仅具有降低丙二醛 MDA、升高超氧化物歧化酶等抗脂质过氧化、抑制肝星状细胞（HSC）增殖作用[5]，还具有很强抗脂肪变性、防止肝细胞坏死的作用[1]。

本实验在用脂肪乳剂喂养大鼠同时给予异甘草酸镁，结果显示异甘草酸镁组防治组大鼠血清 TG、

表 5 各组肝脂肪变性及炎症活动度结果比较($\bar{x} \pm s$, $n=10$)

Tab 5 Degree of the hepatic steatosis and inflammation score ($\bar{x} \pm s$, $n=10$)

组别	剂量 /mg·kg^{-1}	脂变程度					炎症积分
		−	+	++	+++	++++	
对照组	−	10	0	−	−	−	0.5 ± 0.73
模型组	−	−	4	6	−	−[a]	3.54 ± 0.78[a]
烟酸组	120	3	6	1	−	−[b]	2.89 ± 0.94[b]
异甘草酸镁组	120	5	4	1	−	−[b]	2.41 ± 1.07[b]

注：与对照组比较，[a]$P<0.01$；与模型组比较，[b]$P<0.05$

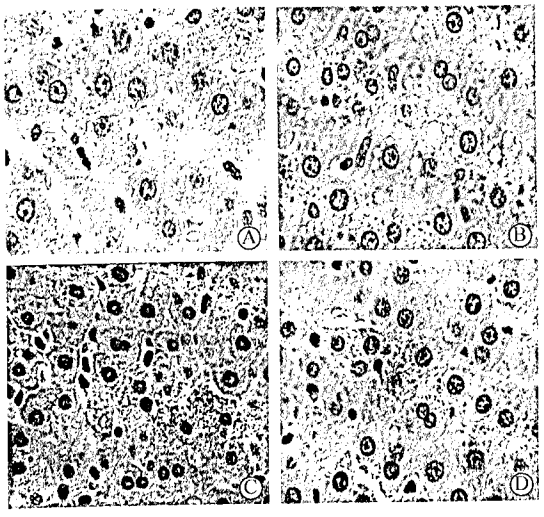

图 1 各组肝脏病理图（HE×400）

A. 对照组；B. 模型组；C. 烟酸组；D. 异甘草酸镁组

Fig 1 Liver tissue histology of rats (HE×400)

A. control group; B. model group; C. nicotinic acid group; D. MgIG group

ALT 均显著降低，病理检查显示，肝脏脂质变性与炎症明显改善，提示异甘草酸镁对大鼠 NASH 有一定的防治作用，降低 FFA 方面优于烟酸组，但其他方面未显示更好疗效。

目前研究表明，TNF-α 和 FFA 在 NASH 的发病过程中起着十分重要的作用，TNF-α 和 FFA 水平与肝损伤程度成正相关，而且两者具有协同作用。FFA 可以通过肝细胞溶酶体途径刺激 TNF-α 表达，造成肝脏脂毒性。经 FFA 刺激后，首先 B 细胞淋巴瘤/白血病-2 相关的 X 蛋白向溶酶体内转位，促使 Ctsb（溶酶体内一种半胱氨酸蛋白酶）释放到胞浆，激活 I-κB 激酶 β（IKK-β）使 I-κB 磷酸化降解，导致 NF-κB（核转录因子-κB）的活化，使之向细胞核内移位，启动炎症因子如 TNF-α 的基因转录，大量表达引起 TG 的蓄积，发生脂肪变性；TNF-α 又能进一步促进溶酶体渗透，激活 NF-κB 这样形成一个加重肝损伤的循环。本实验发现模型组大鼠 TNF-α 和 FFA 均明显升高，而异甘草酸镁能够显著降低 TNF-α 和 FFA 水平，这可能是异甘草酸镁防治 NASH 的分子作用机制[6]。

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Serum Gamma-Glutamyl Transpeptidase Activity as an Indicator of Disease of Liver, Pancreas, or Bone

Gifford Lum and S. Raymond Gambino

Serum γ-glutamyl transpeptidase (GGT), leucine aminopeptidase, alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase activities were assayed in controls and in patients with liver, pancreatic, or bone disease. GGT activity was above normal in all forms of liver disease studied (viral hepatitis, cirrhosis, cholecystitis, metastatic carcinoma to liver, pancreatic carcinoma, liver granuloma, and acute pancreatitis). GGT more sensitively indicated hepatic disease than did alkaline phosphatase, much more so than did leucine aminopeptidase. GGT was disproportionately more active in relation to the transaminases in cases of intra- or extrahepatic biliary obstruction; the reverse was true in cases of viral hepatitis. GGT activity was normal in children, adolescents, and pregnant women, and in cases of bone disease and renal failure. Kinetic measurement of GGT activity offers a simple, sensitive, and direct means for distinguishing whether bone or liver is the source of increased serum alkaline phosphatase activity. Activity was highest in obstructive liver disease.

Additional Keyphrases *pancreatic, hepatic, and hepatobiliary disease • diagnostic aid • relative usefulness of various enzyme assays for differential diagnosis • chronic alcoholism • heart disease • normal values for adults, children, pregnant women • reticulum cell sarcoma*

Abnormally high activity of serum GGT[1] appears to be specific for diseases of the liver, biliary tract, and pancreas. Interest in GGT has focused on its value in the diagnosis of various liver diseases. GGT activity is increased in cases of viral hepatitis, cholecystitis, chronic hepatitis, fatty liver, cholangitis, cholelithiasis, metastatic carcinoma to the liver, congestive heart failure, chronic alcoholism, post-myocardial infarction, epilepsy, and brain tumors (*1–8*).

We became interested in GGT because we were concerned with the general problem of elucidating which tissue is the source of increased serum ALP activity. The conventional means of separating ALP isoenzymes—by electrophoresis, heat inactivation, or chemical inhibition—were unattractive to us because they often are insensitive and give equivocal results. In an effort to differentiate between the isoenzymes of ALP originating in bone and liver by the use of a single enzyme assay, we undertook a study of GGT and LAS. LAS and GGT do not suffer from the drawbacks of ALP because LAS and GGT appear to be more nearly specific for hepatobiliary disease. In addition, LAS and GGT activity parallels that of ALP in hepatobiliary disease. We also studied AST and ALT in an attempt to find patterns of increased enzyme activity that would aid in the differential diagnosis of hepatic disease.

Materials and Methods

The patient population consisted of 230 clinic and inpatients at the Columbia–Presbyterian Medical Center, New York, N. Y.

GGT and LAS were measured kinetically at 25 °C on the Model 2000 spectrophotometer (Gilford Instrument Labs., Inc., Oberlin, Ohio 44074). GGT was measured by the method of Szasz, with L-γ-glutamyl-*p*-nitroanilide as substrate (*9*), and LAS was assayed by using leucine-*p*-nitroanilide as substrate (*10*). ALP activity was determined at 37 °C on the SMA 12/60 (Technicon Corp., Tarrytown, N. Y. 10591), with *p*-nitrophenol phosphate substrate (*11*). AST (uv assay) was measured at 37 °C on the SMA 12/60, and AAT was assayed kinetically at 340 nm at 35 °C with the Model 8600 Reaction Rate Analyzer (LKB Instrument Corp., Inc., Rockville, Md. 20852) (*12*). GGT, LAS, and AAT were assayed within 48–60 h after the serum sample was collected, and ALP and AST within 24 h. All specimens were stored at 4 °C before

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[1] Nonstandard abbreviations used: GGT, γ-glutamyl transpeptidase (no EC number yet assigned). Other abbreviations were editorially changed in manuscript to conform with recently recommended standard abbreviations [*J. Clin. Pathol.* **24**, 656 (1971)]. In the past GOT has often been used as an abbreviation for AST, as has GPT instead of ALT. AST, aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1); ALT, alanine aminotransferase (L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2); LAS, leucine aminopeptidase (L-leucyl-peptide hydrolase, EC 3.4.1.1); and ALP, alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1).

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assay if they were not assayed within 3 h of collection.

Results

GGT activity was measured in a total of 80 sera from hospital patients (half were women and half men) who were free of known liver, renal, or pancreatic disease. The mean for men was 13.6 ± 6.9 U/liter (range 2–39) and for women 10.9 ± 6.4 U/liter (range 2–36). No significant difference was found between the mean value of GGT activity between males and females. The mean activity of LAS in the same group was 10.4 ± 4.3 U/liter.

The upper limit of normal for GGT was set at 30 U/liter, for LAS 22 U/liter, for AST and ALT 50 U/liter, and for ALP 85 U/liter. We have summarized all of our data in Table 1. In addition to the absolute activities of all five enzymes studied, we have also chosen to express our results (the mean change in enzyme activity) as "times (×) upper limit of normal." Hence an absolute value of 120 U/liter for GGT would be expressed as 4.0 × the upper limit of normal, i.e., 120 divided by 30. By reporting our results in this way, it is easier to compare relative changes among a variety of enzyme assays.

Table 1 summarizes the data for patients with diseases of the liver, pancreas, and (or) bile duct; diseases of bone; and for patients with isolated elevation of ALP.

Discussion

The data presented in Table 1 make it readily apparent that serum GGT activity is increased in all the forms of liver disease we studied, with the highest activities being seen in extra- or intrahepatic obstructive disease. In viral hepatitis, whether antigen-positive or -negative, activities of the transaminases were disproportionately increased as compared to that of GGT, indicating acute hepatic cellular necrosis. There is suggestive evidence that the ALT/GGT ratio may best discriminate between obstructive hepatic disease and viral hepatitis (*13*). Our data indicate that the disproportionate increase in transaminase activity as compared to GGT activity is characteristic of viral hepatitis, whereas the disproportionate increase in GGT activity as compared to transaminase activity (with a ALT/GGT ratio of less than 1.8) suggests obstructive hepatobiliary disease rather than hepatitis.

In cases of cholecystitis or cholelithiasis, GGT was more sensitive than either the ALP or LAS, showing a mean increase of sevenfold the upper limit of normal. The increase in GGT often preceded the rise of ALP in cases of surgically proven biliary tract obstruction. GGT was normal in cases of acute cholecystitis without biliary obstruction.

In 13 cases of cholangitis and hepatic abscess, where high ALP activities are found, GGT was increased to the same extent as was ALP activity, while LAS was only moderately increased.

In our series of 12 patients with metastatic carcinoma to the liver with primary sites located in the lung, ovary, stomach, and skin, GGT activity was extremely high as compared to that of the transaminases. This confirms reports in the literature in which GGT assay has been suggested as being useful in detection of hepatic metastases (*1*, *3*). We found normal GGT activity in several patients with cancer in which the liver was not involved. In patients with hematologic disorders (leukemia) or lymphomas, GGT activity was increased if there was evidence of hepatic infiltration by leukemic cells or liver involvement by lymphoma.

GGT activity was greatest in cases of primary carcinoma of the head of the pancreas and adenocarcinoma of the bile duct. LAS was far less sensitive for detecting obstructive pancreatic neoplasm.

In chronic alcoholics, GGT appears to be a sensitive indicator of hepatic damage, with the highest activities seen in the sickest patient. The greatest increase in GGT was found in a case of a chronic alcoholic with delirium tremens and pneumonia. As have others, we have seen several patients with chronic alcoholism whose GGT activity was increased but whose other liver enzyme activities were normal (*2*, *5*).

In cirrhosis of the liver, GGT and ALP were both increased, whereas AST activity was moderately abnormal and that of ALT was normal, a transaminase pattern commonly seen in cirrhosis.

In patients with liver granulomas, including miliary tuberculosis and sarcoidosis, we found both GGT and ALP to be elevated, with borderline elevations of the transaminases. If there was no liver involvement by granuloma, then GGT was normal.

In acute pancreatitis, GGT activity was increased, often concomitantly with normal ALP activity. Although GGT activity is increased in cases of acute pancreatitis, the increase is not diagnostic of, or specific for, pancreatitis because its elevation may reflect concomitant hepatic damage in an alcoholic.

In the patients with congestive heart failure, increased ALP and GGT activities were secondary to hepatic damage.

The data in Table 1 show that if an increased ALP was of osseous origin, GGT activity was always normal—this was true of Paget's disease, hyperthyroidism (*14*), metastatic cancer to bone, and hyperparathyroidism. In Table 1 we have not

Table 1. Comparison of Enzyme Patterns in Indicated Diseases

Disease (No. cases)	Enzyme	Mean change U/liter	Mean change times normal	Range U/liter	No. elev., %
Diseases of liver, pancreas, and bile duct					
Hepatitis (17)	GGT	156	5.2	23–388	94
	ALP	213	2.5	107–575	100
	LAS	35	1.6	18–52	88
	AST	1820	36.5	186–5040	100
	ALT	1580	31.0	208–5320	100
Cholecystitis or cholelithiasis (8)	GGT	210	7.0	47–420	100
	ALP	255	3.0	79–625	75
	LAS	26	1.2	15–58	50
	AST	78	1.5	38–173	50
	ALT	91	1.8	16–320	50
Cholangitis (13)	GGT	249	8.3	71–520	100
	ALP	593	7.0	170–975	100
	LAS	46	2.1	19–97	77
	AST	100	2.0	31–325	77
	ALT	95	1.9	23–220	77
Carcinoma metastatic to liver (12)	GGT	396	13.2	208–820	100
	ALP	560	6.6	240–925	100
	LAS	35	1.6	19–58	75
	AST	220	4.4	57–900	100
	ALT	150	3.0	14–720	83
Alcoholics (5)	GGT	99	3.3	27–850	80
	ALP	110	1.3	75–140	80
	LAS	22	1.0	15–35	20
	AST	80	1.6	9–212	60
	ALT	35	0.7	9–57	20
Cirrhosis of liver (6)	GGT	132	4.4	26–256	83
	ALP	298	3.5	93–700	100
	LAS	31	1.4	15–44	67
	AST	100	2.0	36–237	83
	ALT	45	0.9	11–77	33
Granuloma of liver (5)	GGT	303	10.1	116–740	100
	ALP	425	5.0	191–700	100
	LAS	33	1.5	19–44	60
	AST	75	1.5	59–97	100
	ALT	70	1.4	50–115	80
Pancreatitis acute (10)	GGT	300	10.0	165–520	100
	ALP	440	5.2	35–1227	80
	LAS	42	1.9	12–68	80
	AST	75	1.5	38–134	80
	ALT	55	1.1	20–105	40
Primary carcinoma of pancreas or bile duct (4)	GGT	590	19.6	264–1040	100
	ALP	1200	14.2	700–2250	100
	LAS	51	2.3	35–68	100
	AST	55	1.1	26–93	50
	ALT	55	1.1	22–105	50
Congestive heart failure (9)	GGT	180	6.0	58–568	100
	ALP	170	2.0	131–294	100
	LAS	33	1.5	16–62	78
	AST	40	0.8	23–81	33
	ALT	40	0.8	13–159	33

(cont'd)

Table 1. (Continued)

Disease (No. cases)	Enzyme	Mean change U/liter	Mean change times normal	Range U/liter	No. elev., %
Diseases of bone					
Paget's disease (7)	GGT	18	0.6	5–32	14
	ALP	780	9.2	475–1175	100
	LAS	13	0.6	8–26	14
	AST	30	0.6	24–42	0
	ALT	15	0.3	4–30	0
Hyperthyroid (10)	GGT	15	0.5	6–24	0
	ALP	110	1.3	47–222	70
	LAS	15	0.7	5–29	10
	AST	25	0.5	14–40	0
	ALT	30	0.6	14–81	10
Carcinoma, metastatic to bone (4)	GGT	30	1.0	13–54	25
	ALP	560	6.6	167–730	100
	LAS	13	0.6	7–15	0
	AST	40	0.8	25–60	25
	ALT	35	0.7	14–52	25
Hyperparathyroid (3)	GGT	15	0.5	11–18	0
	ALP	194	2.3	75–450	67
	LAS	13	0.6	7–16	0
	AST	40	0.8	20–54	33
	ALT	30	0.6	22–49	0
Normal adolescents (10)	GGT	18	0.6	5–45	10
	ALP	305	3.6	157–450	100
	LAS	18	0.8	5–61	10
	AST	35	0.7	23–58	20
	ALT	15	0.3	9–31	0
Diseases accompanied by increased alkaline phosphatase activity					
Pregnant patients at term (14)	GGT	9	0.3	2–21	0
	ALP	136	1.6	105–179	100
	LAS	146	6.6	80–312	100
	AST	55	1.1	29–87	21
	ALT	15	0.3	2–29	0
Renal failure (9)	GGT	18	0.6	6–52	11
	ALP	102	1.2	51–164	67
	LAS	11	0.5	7–19	0
	AST	75	1.5	2–325	45
	ALT	40	0.8	8–137	22
Gilbert's disease (4)	GGT	12	0.4	2–23	0
	ALP	76	0.9	58–150	25
	LAS	11	0.5	8–18	0
	AST	25	0.5	23–28	0
	ALT	15	0.3	14–25	0

included one patient who had one of the highest ALP activities ever measured at the Presbyterian Hospital (3800 U/liter, or 45-fold increase above normal). This patient, whose GGT activity was normal, was found at autopsy to have reticulum cell sarcoma of the bone, with no liver involvement.

In children and adolescents, who normally have an increased ALP activity (as compared to adults) secondary to bone growth, GGT activity was normal. In pregnant patients at term, serum placental ALP and LAS activities were increased, but GGT activity was normal, suggesting that in pregnant patients with liver disease, GGT would be helpful in making an accurate differential diagnosis.

Although the highest GGT activities are found in the brush border of the proximal convoluted

tubules of the kidney (*1*), we found serum GGT activity to be normal in patients with renal failure. Slightly increased or normal GGT activity has been reported in a number of patients with renal disease (*1*). Much lower GGT activities are found in the liver, spleen, pancreas, and intestine. In the hepatic parenchyma, GGT activity appears to be concentrated in the microsomes (*15*), which suggests that the increased GGT activity seen in epileptic patients may be secondary to induction of microsomal enzymes by various drugs such as diphenylhydantoin ("Dilantin") and phenobarbital (*16*).

In patients with Gilbert's disease (chronic unconjugated hyperbilirubinemia), GGT activity was normal, as were the activities of all the other hepatic enzymes, indicating the benign nature of this syndrome.

In a patient with pulmonary infarction (our Autopsy No. 24220) GGT activity was normal, but ALP activity was increased to 550 U/liter (6.5 × normal). An increase in ALP after pulmonary infarction has been reported (*17*, *18*).

Finally, we mention the increased ALP and GGT activities seen in cases of myocardial infarction, not related to congestive heart failure (*7*). Thus, GGT is not absolutely specific for disease in the right upper quadrant, and it cannot be used for differential diagnosis during recovery from a myocardial infarction or in epilepsy (*7*, *8*). Ewen and Griffiths' work (*8*) also suggests that we should study ALP and GGT in patients with a variety of healing lesions, because young fibroblasts and budding capillaries contain increased amounts of ALP and GGT.

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Glycyrrhizin in patients who failed previous interferon alpha-based therapies: biochemical and histological effects after 52 weeks

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SUMMARY. Chronic hepatitis C patients often fail to respond to interferon-based therapies. This phase III study aimed at confirming the efficacy and safety of glycyrrhizin in interferon + ribavirin-based therapy non-responders. A randomised, double-blind, placebo-controlled, comparison of glycyrrhizin, administered intravenously 5×/or 3×/week, and 5×/week placebo for 12 weeks to 379 patients, was followed by a randomised, open comparison of glycyrrhizin i.v. 5×/versus 3×/week for 40 weeks. Primary endpoints were: (1) the proportion of patients with ≥50% ALT (alanine aminotransferase) reduction after 12 weeks double-blind phase, and (2) the proportion of patients with improvement of necro-inflammation after 52 weeks as compared with baseline. The proportion of patients with ALT reduction ≥50% after 12 weeks was significantly higher with 5×/week glycyrrhizin (28.7%, $P < 0.0001$) and 3×/week glycyrrhizin (29.0%, $P < 0.0001$) compared with placebo (7.0%). The proportion of patients with improvement in necro-inflammation after 52 weeks was 44.9% with 5×/week and 46.0% with 3×/week, respectively. Glycyrrhizin exhibited a significantly higher ALT reduction compared to placebo after 12 weeks of therapy and an improvement of necro-inflammation and fibrosis after 52-weeks treatment. Generally, glycyrrhizin treatment was well tolerated.

Keywords: ALT, chronic hepatitis C, fibrosis, glycyrrhizin, hepatocellular carcinoma, IFN non-responders, necro-inflammation.

INTRODUCTION

Treatment of hepatitis C virus (HCV) infection has significantly improved over the past 2 decades. However, the current standard of care (SOC), pegylated interferon alpha (peg-IFNα) plus ribavirin (RBV), has its limitations. Sustained virological response (SVR) rates are less than 50% for genotype 1 patients and there is no SOC for non-responders to date [1,2]. In addition, contraindications as well as intolerance to IFN based therapies leave a large number of patients ineligible for this medication. These patients are at risk to develop cirrhosis and its complications, including hepatocellular carcinoma (HCC) [3].

Studies with long-term application of low dose peg-IFNα failed to achieve reduced progression of liver disease. These studies showed that inflammation and fibrosis progression could not be suppressed [4,5]. However, the COPILOT study demonstrated positive effects on portal hypertension

Abbreviations: HCV, hepatitis C virus; SVR, sustained virological response; HCC, hepatocellular carcinoma; IFNα, interferon alpha; peg-IFNα, pegylated interferon alpha; RBV, ribavirin; RNA, ribonucleic acid; DAA, direct antiviral agents; GL, glycyrrhizin; CHC, chronic hepatitis C; ALT, alanine aminotransferase; ULN, upper limit of normal; HAI, histology activity index; PCR, polymerase chain reaction; SNMC, Stronger Neo-Minophagen® C; QOL, quality of life; FAS, full analysis set; PPS, per protocol set; LOCF, last-observation-carried-forward; RAND, Research and Development Corp. (Santa Monica/USA); SAF, safety analysis set; SD, standard deviation; HRQOL, Health-related QOL.

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[5]. To what extent new direct acting antivirals (DAAs) will meet the expectations for IFN failure or IFN intolerant patients remains uncertain. Response rates to triple therapy with HCV protease inhibitors, peg-IFNα and ribavirin in previous nonresponder patients with liver cirrhosis are still below 20% [6]. Therefore it is legitimate to search for alternative treatment strategies to suppress inflammatory activity and fibrosis progression in non-responders to IFN-based therapies.

Intravenous glycyrrhizin (GL) has been used for more than 30 years in the treatment of liver diseases in Asian countries, mainly in Japan. Studies performed in Asia and Europe showed that administration of GL in patients with chronic hepatitis C (CHC) leads to an improvement of necro-inflammation and liver function tests in a significant proportion of patients. Such effects were also observed in IFN non-responders. It has been demonstrated that there was a dose-dependent effect of GL on elevated ALT levels. The observed decrease of ALT level was rapid, linear and could be maintained in CHC patients receiving at least three injections weekly for varying duration of treatment [7–10]. ALT is a marker of biochemical necro-inflammatory activity of the liver. Persistently high ALT levels are correlated with disease progression leading to complications such as cirrhosis and HCC [11]. The effect of GL is mediated primarily through a suppression of inflammation and through a decreased liver cell injury. The anti-inflammatory activity of GL may be mediated by the direct binding of the molecule to cell membrane components, especially to lipocortin I or to enzymes like phospholipase-A2, which is the initial enzyme in the arachidonic acid metabolic system. GL also directly binds to lipoxygenase, an enzyme to produce the inflammatory chemical mediators. GL selectively inhibits the activation by phosphorylation of these enzymes [12,13]. Furthermore GL and its derivatives are able to inhibit the production of inflammatory chemokines IL-8 and eotaxin 1, which are both potent chemo-attractants to leukocytes during inflammation, and may counteract the expression of those pro-inflammatory chemokines [14]. Long term treatment with GL reduced the incidence of HCC in some studies [7,15–17].

Most studies with GL therapy were performed in Asia so far. In this study the efficacy and safety of GL was evaluated in a 52-weeks treatment of European chronic hepatitis C patients not responding or having contraindications to standard therapy (IFN + RBV or peg-IFNα + RBV).

PATIENTS AND METHODS

Patients and study design

This Phase III study was conducted at 73 centres in 11 European countries from October 2002 to April 2006. Study duration was 52 weeks per patient and consisted of two phases: a 12 weeks double-blind phase followed by an open phase of 40 weeks. Two different regimens of GL were compared to placebo during the double-blind phase (Fig. 1). After completing this phase, all patients were randomised again into the subsequent open phase. The study used a four-stage group sequential adaptive design with sample size adjustments after the planned interim analysis [18,19].

Out of 603 screened patients 379 were randomised for the first phase. While 16 patients (4.2%) dropped out from the study during the double-blind phase 363 patients (95.8%) were randomised into the subsequent open phase from Week 13 to Week 52. Another 24 patients (6.6%) terminated the study prematurely, thus 339 patients (93.4%) completed the open phase (Fig. 2).

Demographic data are shown in Table 1. Male and female patients fulfilling the following main inclusion criteria were enrolled: age 18–65 years, positive serum HCV-RNA and documented non response or un-sustained response to treatment with IFN + RBV or peg-IFNα + RBV therapy for at least 3 months. Non-response was defined as positive serum HCV-RNA and abnormal ALT values, with ALT value >1.5× upper limit of normal (ULN). Non response or intolerance was determined by the individual investigator

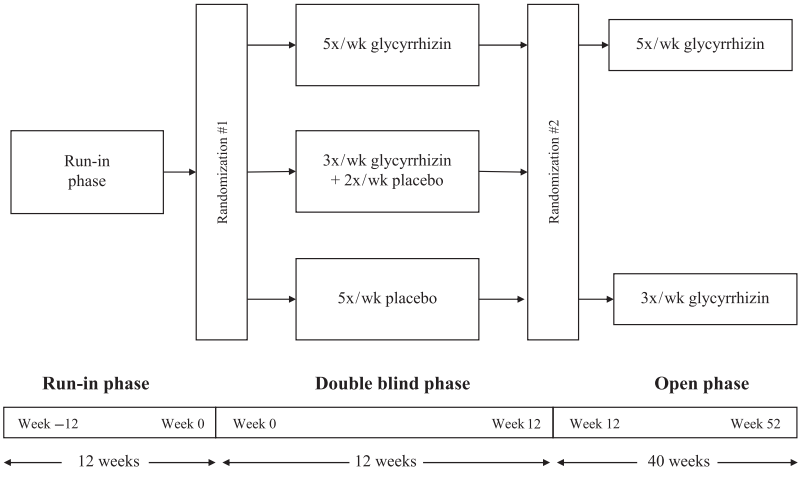

Fig. 1 Study flow chart. In this randomised, double-blind, placebo-controlled study, glycyrrhizin was administered intravenously 5×/or 3×/week, or placebo was injected 5×/week for 12 weeks to 379 patients. This double-blind phase was followed by a randomised, open comparison of glycyrrhizin i.v. 5×/versus 3×/week for 40 weeks.

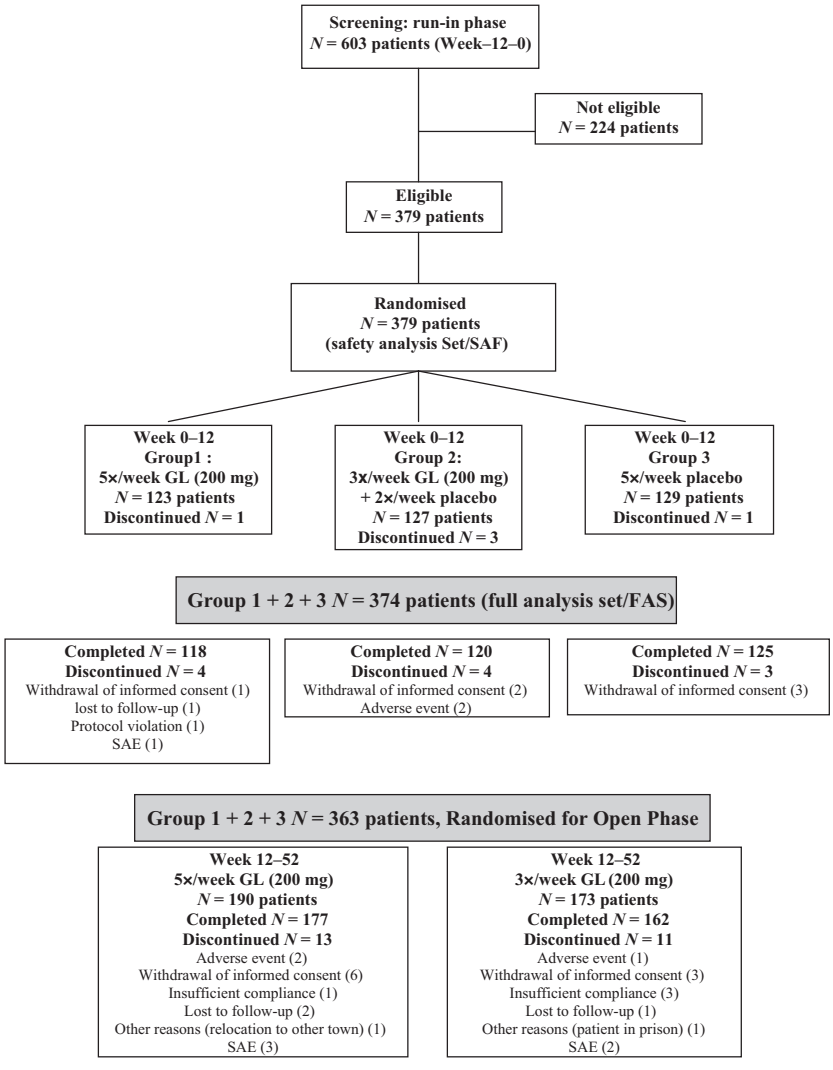

Fig. 2 Study design and patient disposition.

according to standard criteria. Further inclusion criteria were contraindications to IFN therapy, ALT > 2× ULN (22 U/L for male, 17 U/L for female) on at least two occasions within 12 weeks before randomisation, necro-inflammation and fibrosis score positive (score ≥1). Exclusion criteria were: chronic hepatitis B, auto-immune hepatitis, haemochromatosis, Wilson disease, α-1-antitrypsin deficiency, decompensated liver cirrhosis, HCC, fatty liver, cholestasis, serious concomitant disease, antiviral treatment, contraindications to GL or liquorice, alcohol or drug abuse, and female patients with absence of adequate contraceptive measures, pregnancy or breast-feeding.

The study was performed in compliance with the Declaration of Helsinki, ICH-Guidelines for Good Clinical Practice, and the applicable regional regulatory requirements of all countries involved in this study. Approval by the respective Ethics Committees and signed Informed Consent of each patient were obtained before starting the study. This study is referenced in the EudraCT database with the EudraCT number 2004-000773-60.

Study endpoints

The primary efficacy endpoints were the proportion of patients with ≥50% ALT reduction after 12-weeks double-blind treatment and the proportion of patients with improvement of modified Histology Activity Index (modified HAI) [19], necro-inflammation score (decrease of ≥1) after 52 weeks compared to baseline. Secondary efficacy endpoints were mean change of ALT from baseline at the end of week 12, proportion of patients with ALT reduction below 1.5× ULN within the 12-weeks double-blind treatment period and after 52-weeks of treatment, proportion of patients with improvement in total HAI (decrease ≥1) and in fibrosis score (decrease ≥1), change in viral load determined by HCV-RNA-activity and change in quality of life (QOL) assessed through a validated local translation of the 36-item Health Survey developed at RAND (Research and Development Corp.) [20].

Laboratory tests were performed centrally by LKF (Laboratorium für Klinische Forschung GmbH, Schwentinental/

Table 1 Patient characteristics (FAS)

Characteristics	All patients	5×/week GL	3×/week GL + 2×/week Placebo	5×/week Placebo
Total number of patients	*n* = 374	*n* = 122	*n* = 124	*n* = 128
Male (%)	67.9	64.8	63.7	75.0
Age (year) (mean ± sd)	41.8 ± 11.4	42.2 ± 11.4	41.6 ± 11.3	41.7 ± 11.6
Body weight (kg) (mean ± SD)	78.1 ± 15.2	78.3 ± 15.6	76.2 ± 15.9	79.8 ± 14.1
BMI (kg/m^2) (mean ± sd)	26.05 ± 4.37	26.24 ± 4.37	25.59 ± 4.44	26.33 ± 4.31
Viral load (copies/mL):*				
$<10^6$	50.5%	52.5%	47.6%	51.6%
10^6 to 2×10^6	19.0%	20.5%	21.0%	15.6%
$\geq 2 \times 10^6$	30.2%	26.2%	31.5%	32.8%
HCV genotype 1 (%)†	6.7	4.9	8.9	6.3
HCV genotype 1a (%)†	7.8	10.7	7.3	5.5
HCV genotype 1b (%)†	58.6	58.2	59.7	57.8
HCV genotype 2 (%)†	2.4	3.3	2.4	1.6
HCV genotype 2a (%)†	1.9	2.5	2.4	0.8
HCV genotype 3 (%)†	20.9	16.4	18.5	27.3
Median ALT (U/L)	76.8 ± 49.0	80.7 ± 50.5	75.2 ± 49.3	74.5 ± 47.2
Necro-inflammation score (mean ± SD)	7.6 ± 2.5 (*n* = 326)	7.9 ± 2.5 (*n* = 109)	7.5 ± 2.5 (*n* = 107)	7.3 ± 2.6 (*n* = 110)
Fibrosis score (mean ± sd)	3.1 ± 1.8 (*n* = 324)	3.3 ± 1.7 (*n* = 108)	2.8 ± 1.8 (*n* = 107)	3.1 ± 1.8 (*n* = 109)

FAS: full analysis set, GL: glycyrrhizin
*HCV viral load was assessed by real-time PCR with the COBAS Ampliprep/Taqman-Systems (Roche Diagnostics) (detection limit: 15 IU/mL). †HCV genotype was assessed by VERSANT HCV Genotype 2.0 Assay (Siemens Diagnostics).

Germany). Determination of HCV-RNA-activity and HCV-genotype was done by Labor Lademannbogen, Hamburg/Germany (formerly Labor Prof. Arndt & Partner). HCV viral load was assessed by real-time PCR with the COBAS Ampliprep/Taqman-Systems (Roche Diagnostics) (detection limit: 15 IU/mL). VERSANT HCV Genotype 2.0 Assay (Siemens Diagnostics) was used to determine HCV-genotype.

Liver histology

Liver biopsy was taken from all patients during the 2 months screening period who gave informed consent. A second biopsy was done at the end of treatment (Week 52). All samples were assessed by three pathologists blinded to all clinical information (R.G., London/UK; T.R. Leuven/Belgium; H.D., Cologne/Germany).

A decrease of ≥1 was defined as improvement in the modified HAI, while an increase of ≥1 was defined as deterioration. No change in the HAI scores was defined as no further deterioration (see suppl. Data).

Study drug and randomisation

The study drug used was Stronger Neo-Minophagen® C (SNMC, Minophagen Pharmaceutical Co. Ltd., Tokyo/Japan) in 20 mL ampoules each containing monoammonium glycyrrhizinate equivalent to 40 mg GL. At each administration, subjects received 5 ampoules of 20 mL of either study drug or placebo, or a combination of the two. Active study medication and placebo were not distinguishable.

Statistical analysis

The detailed statistical analysis plans are provided in the online supplementary information.

RESULTS

Biochemical response after 12 weeks double-blind phase

For the first primary endpoint the proportion of patients with ALT reduction ≥50% was significantly higher with 5×/week GL (28.7%, $P < 0.0001$) and 3×/week GL + 2×/week placebo (29.0%, $P < 0.0001$) compared with 5×/week placebo (7.0%) after 12 weeks of treatment. Under active treatment, mean ± SD change from baseline in ALT values (95% confidence interval) was −32.8 ± 41.2 U/L (−40.2 to −25.4) and −26.9 ± 31.5 U/L (−32.5 to −21.2), respectively. The decrease occurred within 2 weeks. In contrast, under placebo mean ± sd ALT levels increased slightly by 0.6 U/L

Table 2 Improvement of ALT levels after double-blind treatment (after 12 weeks) with glycyrrhizin (FAS)

Regimen	5×/week GL *N* = 122	3×/week GL + 2×/week Placebo *N* = 124	5×/week Placebo *N* = 128
Proportion of ALT reduction ≥50% (*N*, %)	35 (28.7%)* (*P* < 0.0001)	36 (29.0%)* (*P* < 0.0001)	9 (7.0%)
ALT mean change from baseline (U/L) (mean ± SD)	−32.8 ± 41.2* (*P* < 0.0001)	−26.9 ± 31.5* (*P* < 0.0001)	0.6 ± 51.0
<1.5× ULN† (*N*, %)	35 (28.7%)* (*P* < 0.0001)	23 (18.5%)* (*P* = 0.0031)	8 (6.3%)

FAS: full analysis set, GL: glycyrrhizin
*Significant difference. †ULN = Upper Limit of Normal (normal range: male 0–22 U/L, female 0–17 U/L).

(−8.4 to 9.5). Detailed data on biochemical response is given in Table 2.

Histological results after 52 weeks of treatment

Concerning the second primary endpoint improvement in modified HAI necro-inflammation score (decrease ≥1), the proportion of patients with an improvement (necrosis and inflammation) was 44.9% with 5×/week GL and 46.0% with 3×/week GL after 52 weeks of treatment. A significant number of patients showed no further deterioration in modified HAI necro-inflammation scores (18.4% with 5×/week GL; 15.0% 3×/week GL). Combined results for improved modified HAI necro-inflammation and/or no further deterioration are 63.3% (5×/week GL) and 61.0% (3×/week GL), respectively. Data on modified HAI necro-inflammation score after 52 weeks is given in Table 3 and suppl. data.

Regarding fibrosis score, improvement (decrease ≥1) was higher with 5×/week GL than with 3×/week GL (37.2% vs. 28.6%, respectively). However, this did not reach statistical significance ($P > 0.05$). No further deterioration was seen in 31.4% (5×/week GL) and 36.6% (3×/week GL) of patients, respectively. Combined results for improved HAI fibrosis and/or no further deterioration were 67.0% [33.3% with improvement and 33.7% with no further deterioration (see Table 3)].

Secondary endpoint

Predefined secondary endpoint of the study was mean change of ALT from baseline at week 12. After the double-blind treatment phase ALT values were significantly improved in comparison with placebo, reaching one of the additional endpoints of this study. The proportion of patients with ALT ≥1.5× ULN at baseline and decrease in values <1.5× ULN after 12 weeks was significantly higher with 5×/week GL (28.7%) and with 3×/week GL + 2×/week placebo (18.5%) than with placebo (6.3%). An overview on changes in ALT levels after 12 weeks of treatment is given in Table 2.

Further decrease of ALT was observed during the open phase with the mean values −14.2 ± 33.2 U/L and −8.2 ± 37.5 U/L in the group treated with 5×/week and 3×/week GL, respectively.

ALT values for total dose were also analysed by stratification of six sub-groups of all study regimens. The mean ALT levels of the six treatment groups are demonstrated in Fig. 3.

Table 3 Changes of necro-inflammation and fibrosis score after 52 weeks of treatment with glycyrrhizin in evaluable patients (*N*, %) (FAS, open phase treated patients only)

	Population (%)	5×/week GL	3×/week GL
Necro-inflammation	*N* = 249 (363)*	*N* = 136 (190)*	*N* = 113 (173)*
Improvement	113 (45.4%)	61 (44.9%)	52 (46.0%)
No change	42 (16.9%)	25 (18.4%)	17 (15.0%)
Deterioration	94 (37.8%)	50 (36.8%)	44 (38.9%)
Fibrosis	*N* = 249 (363)*	*N* = 137 (190)*	*N* = 112 (173)*
Improvement	83 (33.3%)	51 (37.2%)	32 (28.6%)
No change	84 (33.7%)	43 (31.4%)	41 (36.6%)
Deterioration	82 (33.0%)	43 (31.4%)	39 (34.8%)

FAS: full analysis set, GL: glycyrrhizin
()* Number of patients participating in the study
A decrease of ≥1 was defined as improvement in modified HAI, while an increase of ≥1 was defined as deterioration.

Four sub-groups receiving GL throughout the study from week 0 showed an immediate decrease in ALT values compared to the two placebo arms. Obviously, there was no dose dependent effect among those groups on ALT reduction assessed at week 12 and week 52 (reduction range: 76–96%). The two placebo arms showed subsequently a decrease in mean ALT levels once they were switched to either 5× GL/week or 3× GL/week in the open phase. After switching to verum, the response rate became similar to the other four sub-groups (81% under GL, 48% under placebo) (Fig. 3).

Serum HCV-RNA

No changes in serum HCV-RNA as measured by real-time PCR (i.e. in median HCV serum virus load) during the double-blind or open phase were observed. There were no differences among treatment groups (data not shown).

Safety

Treatment with GL was well tolerated. Adverse events, laboratory parameters and vital signs congruently showed changes in line with pseudoaldosteronism and thus can be explained by the mechanism of action of GL. The most frequent adverse events possibly or probably related to the study drug were hypertension (including aggravated pre-existing hypertension), hypokalaemia, headache, paraesthesia, peripheral oedema, upper abdominal pain, increased blood creatine phosphokinase and nausea. A total of 4.2% patients in the double-blind phase and 6.6% patients in the open phase of patients dropped out due to treatment related adverse events, respectively. Table 4 summarises the most frequent adverse events related to GL observed during the double-blind phase. They appeared to be dose-dependent. Hypertension and hypokalaemia were more frequent during the open phase in patients receiving 5×/week GL than in patients receiving 3×/week GL. Paraesthesia occurred during the double-blind phase in the GL groups only. Systolic blood pressure (mean ± SD) changed in the three treatment groups (i.e. 5×/week GL, 3×/week GL and 5×/week placebo) during the double-blind phase by 3.5 ± 16.2 mmHg, 2.8 ± 14.7 mmHg and −0.5 ± 11.1 mmHg, respectively. The diastolic pressure increased by 0.8 ± 8.0 mmHg, 3.1 ± 9.1 and 0.6 ± 7.6 mmHg, respectively.

Quality of life (QOL)

Slight improvements in QOL including health change assessments were observed during both treatment phases and in all treatment groups. In the double-blind phase, a more pronounced improvement was observed in patients receiving GL compared to placebo. In the open phase, patients treated more frequently with GL (5×/week) showed more distinct improvement in QOL than patients receiving 3×/week GL. However, no significant changes could be observed in either treatment phase. Details can be obtained from the online Table S1 in the supplementary file.

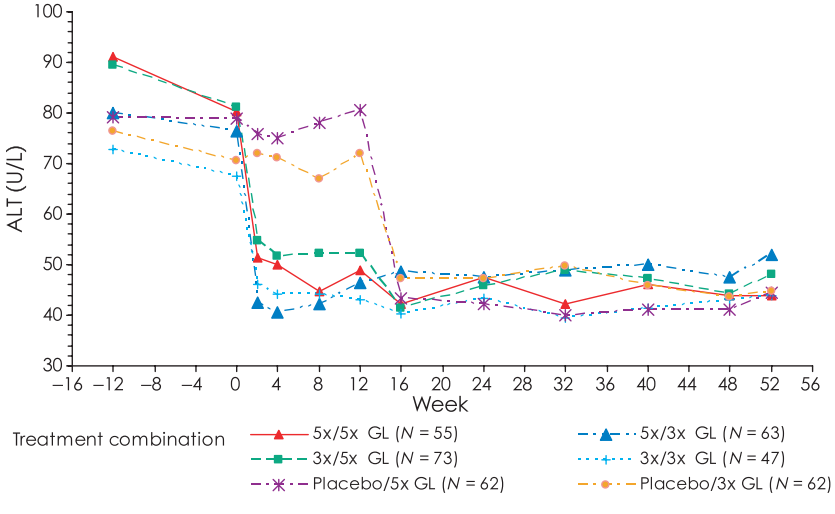

Fig. 3 Time course of serum ALT-levels during the study. *N* Number of patients; *ALT* alanine aminotransferase, *GL* glycyrrhizin. ALT values for total dose were analysed by stratification of 6 sub-groups of all study regimens. Four sub-groups receiving GL from week 0 (5×/5× GL, 5×/3× GL, 3×/5× GL, 3×/3× GL) showed an immediate decrease in ALT values (mean) compared to the two placebo arms. There was no dose dependent effect on ALT reduction among the four groups receiving GL throughout the study assessed at week 12 and week 52 (reduction range: 76–96%). The two placebo arms showed subsequently a decrease in mean ALT levels once they were switched to either 5× GL/week or 3× GL/week in the open phase. After switching to GL, the response rate became similar to the other 4 sub-groups (81% under GL, 48% under placebo).

Table 4 Most frequent adverse events (AEs) possibly/probably related to glycyrrhizin during the 12 week double-blind treatment (*N*, %) (SAF)

	5×/week GL *N* = 123	3×/week GL *N* = 127	Placebo *N* = 129
Number of subjects	123	127	129
Number of subjects with AEs	57	48	35
Number of AEs	138	101	82
Relationship possible (*N*, %)			
Hypertension aggravated	12 (8.7%)	1 (1.0%)	4 (4.9%)
Hypertension NOS*	7 (5.1%)	4 (4.0%)	–
Headache	5 (3.6%)	6 (5.9%)	1 (1.2%)
Abdominal pain, upper	1 (0.7%)	2 (2.0%)	6 (7.3%)
Paraesthesia	2 (1.4%)	4 (4.0%)	–
Blood pressure increased	1 (0.7%)	3 (3.0%)	2 (2.4%)
Relationship probable (*N*, %)			
Hypertension NOS	6 (4.3%)	5 (5.0%)	–
Paraesthesia	5 (3.6%)	5 (5.0%)	–
Hypokalaemia	5 (3.6%)	3 (3.0%)	–

SAF: safety analysis set, GL: glycyrrhizin
*NOS: Not otherwise specified.

DISCUSSION

This study demonstrates that treatment with GL reduces ALT levels. After the 12 week double-blind treatment phase ALT values were significantly improved in comparison to placebo. Suppression of necro-inflammation as well as improvement of fibrosis was observed in patients treated with GL either 5×/week or 3×/week for 52 weeks. Despite the fact that no changes in serum HCV-RNA levels were seen during the double-blind or open phase, GL leads to a decrease in ALT values compared to the two placebo arms.

The combination of peg-IFN and RBV has been the standard of care (SOC) for patients with chronic hepatitis C since 2001. However, certain patient populations in particular non-responders to prior IFN-based therapies show very poor response rates to these therapies with less than 16% of patients clearing HCV RNA [21,22]. Thus, alternative options are needed. Treatment that could halt or diminish the progression of fibrosis would be beneficial. However, three major studies, HALT-C, EPIC-3 and COPILOT, failed to show efficacy of low dose pegylated interferon on fibrosis progression in patients who previously failed to IFN based therapies [5,23].

Innovative agents that are in clinical development include HCV protease, HCV polymerase and HCV NS 5A inhibitors as well as the host targeting agent (HTA) alisporivir [24]. Currently, phase III study results of two HCV protease inhibitors are available [25,26]. They have to be combined with peg-IFN + RBV and therefore there are no solutions for IFN intolerant patients at the moment. Two phase III studies, REALIZE and RESPOND-2, explored the benefits of telaprevir and boceprevir in previous peg-IFN + RBV failure patients, respectively [6,25]. Response rates were higher among patients who had previously relapsed than those who were non-responders, partial as well as null responders [27]. Still more than half of non-responder patients will fail to upcoming triple therapies with first generation HCV protease inhibitors and response rates are particular poor for subjects with advanced fibrosis or cirrhosis, HCV genotype 1a and African Americans. Treatment regimens with all oral anti-HCV therapies combining protease inhibitors, polymerase inhibitors as well as NS 5A inhibitors are in earlier stages of clinical development [28]. As a remaining alternative out of the currently available therapies, GL is considered worth re-evaluating its utility in the treatment of IFN non-responder or IFN non-tolerant chronic hepatitis patients.

The result of this study suggests that GL may be helpful in patients who did not respond to previous IFN based therapies or who cannot tolerate interferon. GL led to a significant reduction of ALT levels in comparison to placebo after 12 weeks of treatment. Further decrease of ALT was observed during the 40 weeks of open phase. First experience with GL in IFN non-responders in European chronic hepatitis C patients was reported by Schalm *et al.* (2003) [29]. The recent study was performed to provide data from a larger European patient population and to assess the effect of GL on ALT and on liver histology. ALT is a marker of inflammatory activity in the liver [30]. Moriyama *et al.* (2005) reported that patients achieving ALT levels less than twice the ULN after IFN therapy had a reduced risk of progression to HCC [31]. Several studies have shown that long-term treatment with GL decreases elevated ALT levels in chronic hepatitis, while development of HCC was reduced [15]. Rino *et al.* (2006) demonstrated that consistent reduction of elevated ALT level with GL significantly decreased the risk of developing HCC from 66% to 41% [32].

Necro-inflammation and fibrosis scores improved significantly after GL treatment. Combining the results for improved modified HAI grade and no further deterioration, histological efficacy of GL reaches 63.3% (5×/week GL) and 61% (3×/week GL), respectively. In regard to fibrosis scores, the combined results reached 67%. However, a control group has not been part of the study design since two biopsies 52 weeks apart in peg-IFN plus ribavirin non-responders receiving placebo for 52 weeks was not regarded ethical.

Necro-inflammation scores were improved although they did not reach the targeted endpoints of ≥60% improvement. A closer look at the results of necro-inflammation sub-scores confirmed the positive effects of GL. In particular, the results of periportal or periseptal interface hepatitis scores and the focal (spotty) lytic necrosis, apoptosis, and focal inflammation indicate a favorable inhibition. No such effects have been demonstrated in IFN non-responders before [3]. When this study was planned, there were no reliable histological data available on non-responders. An improvement rate of 60% was considered feasible (by assumption) based on a limited source of information available, but in hindsight this proved too optimistic [33], particularly in IFN non-responding, difficult-to-treat patients.

The treatment rationale for hepatitis C should include not only reduction in liver disease progression and HCC development but also an improvement in health-related quality of life (HRQOL). HRQOL was significantly decreased in patients with chronic hepatitis C with advanced fibrosis and cirrhosis and treatment with peg-IFN + RBV improved HRQOL as assessed by SF-36 questionnaire [34]. In our study, HRQOL-improvements were observed in both study phases, exceeded placebo effect in patients receiving GL and appeared to be dose dependent. Despite the reported negative impact of long-term intravenous application on QOL, a good compliance of GL therapy was confirmed by low drop-out rates. Based on the mode of action of GL, adverse events, laboratory parameters and vital signs were compatible with symptoms of pseudoaldosteronism. While the incidence of AEs was slightly higher than that observed in the past clinical studies [10,11] treatment with GL was found generally well tolerated and this study confirmed the safety profile of GL.

Limitations of this study need to be considered. No long-term follow-up of patients was performed to investigate if the potential beneficial effects on disease activity had also a long-term impact on progression of liver disease. This information would be required to determine the optimal treatment duration of GL also considering cost-benefit ratios. Moreover, treatment with GL required frequent intravenous injections over a prolonged period of time and thus might be feasible in clinical practice only in some countries.

In conclusion, it was shown that GL reduces ALT and prevents disease progression in a proportion of chronic hepatitis C patients who did not respond to or tolerate previous IFN based therapies. Improvement of ALT levels in IFN non-responders and IFN intolerant patients irrespective of HCV-RNA can be achieved in a proportion of patients. Since frequent i.v. injection (5×/week or 3×/week) required for a GL therapy can possibly be a burden for chronic hepatitis patients, development of a formulation which allows less frequent administration or non-parenteral application would be a significant step forward.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Table S1: Patient characteristics (FAS).

Table S3.1: Periportal or periseptal interface hepatitis (piecemeal necrosis) score in patients (*N*, %) after 52 weeks of treatment (all patients treated during the open phase).

Table S3.2: Confluent necrosis score in patients (*N*, %) after 52 weeks of treatment (all patients treated during the open phase).

Table S3.3: Focal (spotty) lytic necrosis, apoptosis, and focal inflammation score in in patients (*N*, %) after 52 weeks of treatment (all patients treated during the open phase).

Table S3.4: Portal inflammation score in in patients (*N*, %) after 52 weeks of treatment (all patients treated during the open phase).

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REVIEW

Molecular Links between Alcohol and Tobacco Induced DNA Damage, Gene Polymorphisms and Patho-physiological Consequences: A Systematic Review of Hepatic Carcinogenesis

Abdul Anvesh Mansoori, Subodh Kumar Jain*

Abstract

Chronic alcohol and tobacco abuse plays a crucial role in the development of different liver associated disorders. Intake promotes the generation of reactive oxygen species within hepatic cells exposing their DNA to continuous oxidative stress which finally leads to DNA damage. However in response to such damage an entangled protective repair machinery comprising different repair proteins like ATM, ATR, H2AX, MRN complex becomes activated. Under abnormal conditions the excessive reactive oxygen species generation results in genetic predisposition of various genes (as ADH, ALDH, CYP2E1, GSTT1, GSTP1 and GSTM1) involved in xenobiotic metabolic pathways, associated with susceptibility to different liver related diseases such as fibrosis, cirrhosis and hepatocellular carcinoma. There is increasing evidence that the inflammatory process is inherently associated with many different cancer types, including hepatocellular carcinomas. The generated reactive oxygen species can also activate or repress epigenetic elements such as chromatin remodeling, non-coding RNAs (micro-RNAs), DNA (de) methylation and histone modification that affect gene expression, hence leading to various disorders. The present review provides comprehensive knowledge of different molecular mechanisms involved in gene polymorphism and their possible association with alcohol and tobacco consumption. The article also showcases the necessity of identifying novel diagnostic biomarkers for early cancer risk assessment among alcohol and tobacco users.

Keywords: Chronic alcohol consumption - reactive oxygen species - DNA damage - inflammatory cytokines

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Introduction

The graph of Gastrointestinal (GI) tract malignancies increasing day by day in the world and are increasingly reported in the Indian subcontinent (Ferlay et al., 2010). In the last 5 years, about 1 million new cases of cancers were registered in India reported by International Agency for Research on Cancer (Park et al., 2008; Jemal et al., 2011). The most widespread form of GI cancer in India is of liver origin, which accounts for about 3% of the total cancer deaths. Out of the total diagnosed cases, 20144 incidences were of liver cancers only. (Bhargava et al., 2012; Bray et al., 2012). Liver carcinoma, an extremely severe condition with underprivileged diagnosis and poor survival is most widespread in central Indian population (Ferlay et al., 2010; Bray et al., 2012). The epidemiological statistics signifies the global rise in the Liver cancer cases particularly in a developing country like India.

Alcohol, mainly ethanol, is by far the most abused drug for centuries globally. The types of alcohol consumed in alcoholic beverages include wine, spirits, liquors, beers and as traditional brew especially in developing countries (Al-Azri et al., 2014). According to the WHO 2014 report, around 30% of the total population of India consumed alcohol in the year 2010. About 93% of alcohol was consumed in the form of spirits, followed by beer with 7% and less than 1% of the population consumed wine (WHO, 2014). In addition to alcohol, tobacco is also included in carcinogen that causes dysfunction of various genes as well as enzymes involved in detoxification of alcohol and nicotine, consequences in generating various types of liver related diseases (LRD) such as fibrosis, alcoholic hepatitis, cirrhosis, hepatocellular carcinoma (HCC) (Koh et al., 2011; Su et al., 2013).

Epidemiologic studies of the last decades have unequivocally acknowledged chronic alcohol consumption as an important risk factor for the development of various types of cancers, including cancers of the organs and tissues of the respiratory tract and the upper digestive tract (i.e. upper aerodigestive tract), liver, colon or rectum (i.e.

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colorectum), and breast (de Menezes et al., 2013; Kar, 2014). Heavy drinking (i.e. consumption of more than 80 g alcohol, or more than five to six drinks per day), especially combined with smoking, enhances the risk of developing these cancers by a factor of 50 or more, depending on the population studied (Tong et al., 2014). But some people develop cancer even at relative moderate daily alcohol consumption. These observations suggest that a genetic predisposition may influence cancer risk. At least part of this genetic predisposition may be related to alcohol metabolism because the rate of alcohol metabolism is genetically determined.

According to meta-analysis of sub-population 85%-90% of primary liver cirrhosis causes cancers (El-Serag and Rudolph, 2007) accounting for 3.5% and 7.5% of all cancers among women and men, respectively (Poustchi et al., 2010; Tong et al., 2014) and accounts for half a million deaths per year (Kirk et al., 2006). Multiple non-viral factors have been concerned with the development of liver cancer include iron overload syndromes, alcohol use, tobacco, oral contraceptive, aflatoxin, pesticides exposure and betel quid chewing, a prevalent habit in the developing world (Gao et al., 2012; Hamed and Ali, 2013; Arora et al., 2015).

Alcohol metabolism through xenobiotics

A Cancer Preventive Pathway: The xenobiotic-metabolizing machinery contains two main types of enzymes: Phase I - mediating oxidative metabolism, and Phase II - conjugating enzymes. Many compounds are transformed to reactive electrophilic metabolites (i.e. ROS) by the oxidative Phase I enzymes, which are mainly alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and cytochrome P-450 enzymes (CYPs). Phase II conjugating enzymes, such as glutathione-S-transferase (GST), act usually as inactivating enzymes that inactivate carcinogens (Zakhari, 2013, Yamada et al., 2014).

Ethanol is metabolized by various enzymes including the ADH enzyme isoforms, ALDH and cytochrome P450 enzyme system. CYP2E1 produces reactive oxygen species (ROS), which increase the risk of tissue damage especially to the liver hepatocytes. ROS includes hydroxyethyl, superoxide anion, hydroxyl radicals and number of free radicals (Zakhari, 2013). CYP2E1 which exacerbates some of the toxic effects of acetaldehyde, leads to a harmful condition called oxidative stress in the cells that is characterized by excess levels of ROS (shown in Figure 1). Glutathione S-transferases (GSTs) are a multi-gene family of phase-II metabolic enzymes. The conjugation of reduced glutathione is catalyzed by GSTs enzymes with a variety of endogenous and exogenous electrophilic compounds, including several potentially toxic carcinogens and chemotherapeutic drugs (Yamada et al., 2014), thereby reducing the reactivity of the compounds by making them water soluble and favouring their elimination from the body.

In the body, particularly in the liver cells xenobiotic pathways engender a number of potentially harmful by-products that cause deleterious possessions on the body tissues and organs. Acetaldehyde, a reactive metabolite of ethanol, binds to nucleic acids, proteins such as enzymes, microsomal proteins and microtubules (Gupta et al., 2014). The generated ROS can also activate or repress the epigenetic mechanisms such as chromatin remodeling, non-coding RNAs (microRNAs), DNA (de) methylation and histone modification that affect gene expression, hence leading to causes various liver diseases and many other disorders (Day and Sweatt, 2011; Gao et al., 2012). It damages DNA leading to chromosome-breakage disease that causes developmental defects, sterility, bone-marrow failure and a highly elevated risk of cancer and other body defects (Nourazarian et al., 2014; Orsetti et al., 2014).

Molecular Regulation of Tobacco Metabolism & Cancer Prevention

Tobacco exposure while smoking includes major classes of carcinogenic compounds such as polycyclic aromatic hydrocarbons (PAHs), aromatic amines, nitrosamines, and heterocyclic amines (HCAs); these carcinogenic compounds can enter the alimentary tract or the circulatory systems (Senthilkumar and Thirumurugan, 2012). These carcinogenic compounds are then catalized by CYPs (CYP1A1, CYP1A2, CYP2E1, CYP2A6), leading to DNA-adduct formation or by GSTs (GSTM1, GSTT1, GSTP1) leading to excretion (Koh et al., 2011; Gao et al., 2014).

Nicotine contents of the tobacco leaves can be absorbed in body tissues including skin, respiratory epithelium, and mucous membrane of the mouth. The carcinogenetic potential of tobacco is well-known. Exposure of Nicotine leads to the activation of nicotinic acetylcholine receptors (nAChRs) and may contribute to cancer succession (shown in Figure 1); coupled with the fact that as tumors development there is an increased expression of nAChRs, further representing the connection of this pathway during carcinogenesis (Russo et al., 2012;

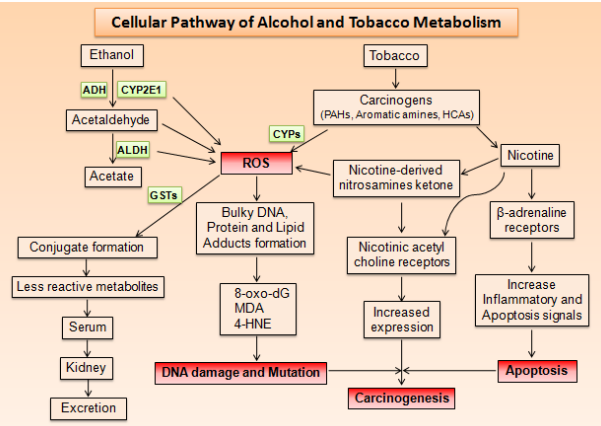

Figure 1. Cellular Pathway of Alcohol and Tobacco Metabolism. Short summary about the link between alcohol and tobacco metabolism by xenobiotic enzymes such as alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), cytochrome P-450 enzyme (CYP2E1) and glutathione-S-transferase enzymes (GSTs) for the generation of ROS that induced adducts formation(i.e. 8-Oxo-2'-deoxyguanosine (8-oxo-dg), malondialdehyde (MDA), 4-Hydroxynonenal (4-HNE), etc.) DNA damage, and apoptosis, which finally results in Cancer

Senthilkumar and Thirumurugan, 2012). Nitrosamines, such as nicotine-derived nitrosamine ketone (NNK), are high affinity ligands for the nAChR signalling and have been shown to enhance intracellular ROS levels in liver cells (Ye et al., 2004).

Identifying diverse form of Tobacco Exposures

The exposures of Tobacco are of diverse forms. Some people smoked tobacco in the form of cigarette or Bidi, while other chewed in the form of gutkha.

Cigarette Smoke (CS): CS contains p-benzoquinone (p-BQ) which is derived from p-benzosemiquinone (p-BSQ), produces ROS that is accompanied by inflammation and apoptosis (Panda and Chatterjee, 2007, Banerjee et al., 2008). ROS produced by p-BQ is a redox cycling agent that leading to oxidative damage (Margaret et al., 2011). Since CYP2E1 induction is associated with ROS generation and lipid peroxidation, this may be a mechanism whereby tobacco smoke may contribute to HCC. Several studies have suggested that tobacco smoking is a significant risk factor for the development of HCC (Lee et al., 2009; Zygogianni et al., 2011; Purohit et al., 2013). Gajalakshmi and Kanimozhi, (2015) has done a case control study that showed a synergistic interaction between heavy alcohol consumption and tobacco smoking in the progression of HCC.

Tobacco and Gutkha Chewer: About forty percent of the tobacco consumed in India is in the smokeless form such as Pan, Pan masala, Zarda and Gutkha (Gajalakshmi and Kanimozhi, 2015). Almost 70-80 percent of gutkha contains some specific alkaloids named arecoline present in areca nut. According to International Agency for Research on Cancer (IARC 2004), areca nut chewing has been classified to be carcinogenic. Patel et al., (2009) studied harmful effects of tobacco chewing on chromosome integrity in healthy tobacco chewers and found a significant increase in frequency of genetic toxicity in cell (as DNA damage, chromosomal aberration (CA), sister chromatid exchanges, micronucleated cells (SCE) etc. of chewers over controls. A large number of studies related to genotoxic effects of pan masala (Gandhi and Kaur, 2000; Fareed et al., 2011) and gutkha (Jyoti et al., 2011; Chadha and Yadav, 2011) has been reported in literature.

Tobacco Dust Inhale by Bidi Roller: Bidi (the Indian equivalent of a cheap cigarette) rolling is a common cottage industry in India. The bidi rollers are from low socio-economic strata and often work in ill-ventilated confined environments in their dwellings. So they inhale a lot of tobacco dust which leads to many systemic diseases and this is also harmful for the integrity of their chromosomes and DNA (Umadevi et al., 2003, Shukla et al., 2011). Bidi rollers (mostly women) are exposed to tobacco constituents mainly nicotine through the cutaneous route or through inhalation of tobacco dust (Poonam et al., 2010). The amount of tobacco dust may be having cumulative effects on their genetic material with the years of exposure and will cause oxidative damage on lipid, protein, carbohydrate, and nucleic acid molecules (Margaret et al., 2011, Sundaramoorthy et al., 2013). Among the most exploited working groups in the country are Bidi rollers and Tendu pluckers comprising women and children earning bare minimum wages and pathetic to access healthcare facilities (Karabi and Bhavna, 2010). Khanna et al. (2014) investigated that bidi rollers seem to be facing the occupational hazard of genotoxicity due to handling bidi tobacco and inhalation of tobacco dust. They should be advised to work under well-ventilated conditions. In this study the selected bidi rollers and controls were neither smoker nor did they indulge in tobacco chewing.

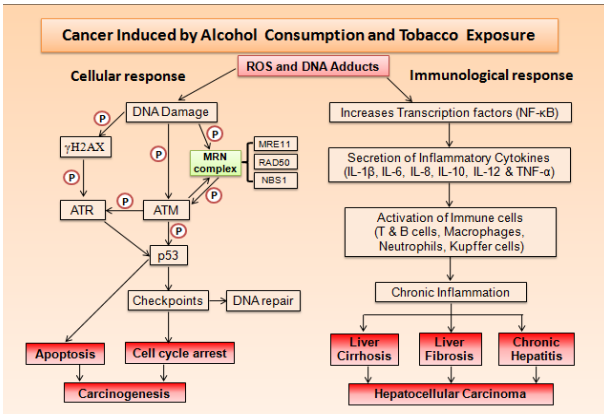

Figure 2. Cancer Induced by Alcohol Consumption and Tobacco Exposure. Mechanism underlying activation of Cellular and Immunological responses which includes activation of DNA damage checkpoints and repair proteins (such as Ataxia-telangiectasia mutated (ATM), Ataxia-telangiectasia Rad 3-related (ATR), MRN complex (Mre11, RAD50 and NBS1 proteins complex), Histone H2A isoform γ protein (γH2AX), p53 protein), activation of immune cells for the secretion of various class of Interleukins (ILs) & Tumor necrosis factors (TNF-α) which induces chronic inflammation in liver that causes cancer in later stages

Cancer Induced by Alcohol Consumption and Tobacco Exposure

Generated ROS and adducts formation causes structural and functional alternations to DNA that causes cell cycle arrest or apoptosis. This damage severely affects the gene function, such as replication and transcription, and plays a major role in age-related diseases and cancer (Goldar et al., 2015). Continuous oxidative stress induced production of various cytokines, activation of immune cells and finally results in inflammation that become chronic in later stage (shown in Figure 2).

Cellular Responses

Upshot of Cancer: DNA damage generate a cellular response which is a complex process that includes detection of the DNA damage, activation of signaling pathways including cell cycle checkpoints, and repair of the damage (Niida and Nakanishi, 2006; Seitz and Mueller, 2015). A complex and entangled network of DNA damage response (DDR) mechanisms act as cellular defence against DNA injuries and are able to remove the vast majority of injuries from the genome (Shin et al., 2008; Areeshi, 2013). Each DNA repair mechanism work with

its own damage specificity. The biological significance of a functional DDR for human health is clearly illustrated by the severe outcomes of inherited defects in DDR factors resulting in various diseases (Shin et al., 2008; Hoeijmakers, 2009).

MRN Complex Association: The MRN complex, whose core comprises of Mre11, RAD50 and NBS1 proteins and is involved in the initial processing of DNA double-strands breaks (DSBS) due to its nuclease activity and DNA binding capability (Uziel et al., 2003). This complex acts as a break sensor and recruits the protein kinase, ataxia telangiectasia mutated (ATM), to DSB sites, facilitating the subsequent processes DNA repair (Lavin, 2007; Lee and Paull, 2007).

The MRN complex adheres to the sites of DSBs instantly following their induction, and this process is independent of ATM (Mirzoeva and Petrini, 2001). It enhances the accumulation of ATM at these sites. NBS1 is important for regulation of the Mre11 complex, influencing DNA binding as well as Mre11 nuclease activity. The activities of the MRN complex are in the very early stage of the DSB response, between damage induction and ATM activation (Stracker et al., 2011).

ATM (Ataxia-Telangiectasia Mutated)

An important protein play a crucial role in the cellular response to DNA damage is the ataxia telangiectasia mutated (ATM) protein. Conformational changes occur due to DSBs in the ATM protein that stimulates the kinase to phosphorylate Ser1981 by intermolecular autophosphorylation (Shiloh, 2003; Farooqi et al., 2014). After activation, the phosphorylated ATM monomers accumulates at the DNA damage sites and phosphorylates a number of proteins involved in cell cycle checkpoint control, apoptotic responses and DNA repair, including p53, H2AX, Rad17, Nbs1 as well as itself (Shiloh, 2006; Farooqi et al., 2014).

ATM kinase activity is also governed by binding to MRE11 that enhances its ability to phosphorylate itself. A recent study also evidence that autophosphorylation at serine 1981 is essential for monomerization and chromatin association of ATM (Berkovich et al., 2007). Phosphorylation of these and other substrates by ATM initiates cell-cycle arrest at G1/S, intra-S and G2/M checkpoints and also promotes DNA repair (Lavin and Kozlov, 2007). ATM is also known as the caretaker of the genome in humans and act as a central mediator of responses to DNA DSBs in cells. Mutations in ATM cause genomic instability syndrome termed ataxia telangiectesia, a rare autosomal recessive disease characterized by immunodeficiency, genome instability and predisposition to cancer in humans (Niida and Nakanishi, 2006; Chaudhary et al., 2013).

ATR (Ataxia-Telangiectasia Rad 3-related)

Like ATM, ATR kinase activity is responsible for phosphorylation of substrates but its functions may be largely dependent on its subcellular localization. ATR exists in a stable complex with ATR-interacting protein (ATRIP) in human cell, a potential regulatory partner (Unsal-Kacmaz and Sancar, 2004). ATRIP protein that is phosphorylated by ATR, determines ATR expression, and is a critical component of the DNA damage checkpoint pathway. ATR and ATRIP both confine to intra-nuclear foci after DNA damage or inhibition of replication (Wu et al., 2014).

Once ATR is activated then it is translocated to DNA replication foci where activated ATR phosphorylates Chk1, initiating a signal transduction cascade that finally results in cell cycle arrest. Therefore, ATR appears to be a multi-functional kinase that regulates several distinct events from S phase to M phase. Another function of ATR is thought to function in unperturbed DNA replication after activating the DNA damage checkpoint (Chaudhary et al., 2013). ATR is considered as a second checkpoint-activating kinase, after ATM, which is activated by double strand breaks in DNA or chromatin disruption (Ohashi et al., 2014).

γH2AX (Histone H2A Isoform γ Protein)

Phosphorylated H2AX is also known as γ-H2AX. Falck et al. (2005) evicted that at the sites of DNA-DSBs, DDR mechanism responsible for the phosphorylation of the histone variant H2AX by all three phosphoinositide 3-kinases (PIKs)-ATM, ATR and DNA-PK (DNA-dependent protein kinase) (Sharma et al., 2015). The phosphorylation of H2AX at C-terminal Serine residues (Ser136 and Ser139) is a relatively early event immediately occurs after DNA damage. γ-H2AX is also responsible for MRN complex binding and ATM activation (Fernandez-Capetillo et al., 2004). It also promote efficient repair (Hu et al., 2015) by facilitating the accumulation of components of DNA repair, including NBS1/Mre11/RAD50 and 53BP1 at damage loci to ensure proper cell cycle arrest or DNA repair.

After DNA DSB damage, γ-H2AX is mainly mediated by ATM, evoking that H2AX is a downstream mediator of ATM function (Guo et al., 2015). But H2AX is also essential for the recruitment of 53BP1 to sites of DNA DSB damage, and 53BP1 appears to be an upstream activator of ATM (Hu et al., 2015). Therefore, it remains a question whether H2AX plays any role in activating ATM after DNA DSB damage.

p53 Binding Protein

The p53 tumor suppressor protein is also known as guardian of the genome, integrates various physiological signals in mammalian cells (Charni et al., 2014). In reverberation to DNA damage, p53 becomes functionally operative and take action either a transient cell cycle arrest, cell death (apoptosis) or permanent cell cycle arrest (cellular senescence). Both apoptosis and cellular senescence are robust tumor suppressor mechanisms that irreversibly forestall damaged cells from undergoing neoplastic transformation (Rodier et al., 2007; Callen et al., 2013). The amount p53 protein and its transcriptional activity is determined by post-translational modification, such as phosphorylation, sumorylation, neddation and acetylation (Charni et al., 2014).

DNA damage causes phosphorylation of p53 at several sites in its transactivation domain, including at Ser15 and Ser20. Activated ATM and ATR phosphorylate p53

protein at Ser15 residue, resulting in p53 stabilization (Choi et al., 2012; Callen et al., 2013). Various recent studies reported that 53BP1 has been implicated in DNA damage responses and plays a central to both the S and G2 checkpoints and also responsible for G1 arrest after encounter of DNA damages. The main transcriptional target of p53 is the p21 Cdk inhibitor (p21CKI), which inhibits cyclin E-Cdk2 activity, thereby inhibiting G1/S transition (Lee et al., 2014; Malakar et al., 2014). However, accentuated apoptosis may ultimately lead to liver pathologies, primarily steatosis, which can advance into a more severe disease such as steatohepatitis, fibrosis, and cirrhosis (Charni et al., 2014).

Patho-Physiological Responses

Causes Cancer progression: Diverse population-based studies suggested that individuals who are prone to chronic inflammatory disorders in liver have an increased risk of liver related diseases development (Gao and Bataller, 2011). In response to a varied range of cellular stresses cytokines are released including infection, inflammation, and carcinogen-induced injury. Host responses that are stimulated by cytokines aimed at controlling cellular stress and minimizing cellular damage (Chung and Lim, 2015). An injury which fails to resolve can elicit excessive immune cell infiltration and lead to persistent cytokine production. Therefore, the host response to stress provokes changes in cytokine expression, which can impact several stages of cancer formation and progression.

Nuclear Factor-κB (NF-κB)

In the past decade, a number of inflammatory mediators have been shown to contribute to the progression of CLD, many of which are either targets or activators of nuclear factor-κB (NF-κB). NF-kB is maintained in an inactivated or resting state by the inhibitory kappa B (IkB) in the cytoplasm. After the phosphorylation of IkB by inhibitor kappa kinase (IkK), NF-kB is activated and free to translocate the nucleus where it can perform its function (Zhang et al., 2015). NF-κB is a main transcriptional regulator of the inflammatory response, and plays an important role in the regulation of inflammatory signalling pathways in the liver.

In almost every chronic liver disease NF-κB is activated, including alcoholic liver disease, viral hepatitis and biliary liver disease (Mandrekar and Szabo, 2009; Yin et al., 2013). NF-κB regulates multiple important functions in hepatocytes, Kupffer cells and hepatic stellate cells (HSCs). Genetic inactivation of different NFκB signalling components results in liver phenotypes that include spontaneous injury, fibrosis and carcinogenesis suggesting that NF-κB makes an essential contribution to liver homeostasis and wound-healing processes (Yao et al., 2014). Activated NF-κB stimulates Toll like receptors (TLRs), as well as inflammatory cytokines such as tumor necrosis factor (TNF) or interleukin (IL)-1 (Yin et al., 2013).

Tumor Necrosis Factor alpha (TNF-α)

TNF-α is pleiotropic growth factor produced by Kupffer cells and other immune cells in response to chronic inflammation and tissue injury. Pro-inflammatory TNF-alpha is connected with an increase in cell cycle progression and oxidative stress through the formation of 8-oxo-deoxyguanosine, a well-known marker of DNA damage associated with chronic hepatitis in human liver (Wang et al., 2012).

Afshar et al. (2015) studied the effect of chronic alcohol consumption in HCV core-expressing transgenic mice and observed that hepatic expression of TGF-β and TNF-α has been raised. In another study, higher levels of TNF-α was detected in the tissue surrounding HCC and hepatic metastasis than in the tumor (Park et al., 2010; Chung and Lim, 2015). TNF-α polymorphism produces the TNF-α (-308bp) SNP (single nucleotide polymorphisms) in the promoter region of the gene, which includes TNF-α1 (-308G) and TNF-α2 (-308A) alleles, is linked with cancer susceptibility and induced expression of TNF-α (Talaat et al., 2012; Cen and Wu, 2013).

Pro-inflammatory Cytokines

Many scientists and researchers all over the world reviewed that cytokines play crucial role in liver carcinoma (Bei et al., 2014; Chung and Lim, 2015). They suggested that cytokines are concerned in liver development and regeneration but their serum level may also contribute to the potential biomarker for pathogenesis of liver-related diseases such as cirrhosis, fibrosis, hepatitis, cancer etc. (Table 1) caused by chronic consumption of alcohol and tobacco abuses. So, one can explore the correlation between expression of cytokines and the different phases of alcoholic liver disease.

Genetic Polymorphism

Susceptibility to Cancer*:* Many of the enzymes (such as ADH, ALDH, CYP2E1, GSTM1, GSTT1 and GSTP1) tangled in metabolic xenobiotic pathways have recently been shown to evince genetic polymorphism in the population. ROS and adducts formation is responsible for the predisposition of gene that causes genetic polymorphism (Munaka et al., 2003; Heit et al., 2015).

Table 1. Increased Level of Inflammatory ammatory Cytokines in Serum Associated with Several Chronic Liver Diseases

Cytokine	HCV/HBV	Carcinoma	Other Liver
Interleukin-1β (IL-1β)	Bortolami et al., 2008	Kanwal et al., 2011	Kamo et al., 2013
Interleukin 6 (IL-6)	Andrade et al., 2013	Yin et al., 2013	Coskun et al., 2004
Interleukin-8 (IL-8)	Langhans et al., 2013	Lee et al., 2013	El-Tayeh et al., 2012
Interleukin 10 (IL-10)	da Silva et al., 2015	Hsia et al., 2007	Kitaoka et al., 2003
Interleukin 12 (IL-12)	Zhu et al., 2015	Lo et al., 2010	Tung et al., 2010

Alcohol Dehydrogenase (ADH) and Aldehyde Dehydrogenase (ALDH)

Lee et al. (2001) determined genotype and allele frequencies of ALDH2, CYP2E1, ADH2 and ADH3 in Korean male patients with alcoholic cirrhosis, without evidence of liver disease, and nondrinkers by using PCR or PCR-directed mutagenesis followed by restriction enzyme digestion and confirmed the observation that the ALDH2 gene protects against the development of alcoholism.

In a population based study done by Zhang et al. (2007) evaluated the effect of polymorphisms in alcohol metabolizing genes, including ADH1B, ADH1C and ALDH2, on levels of alcohol drinking and susceptibility of stomach cancer in Poland. They suggested that the ALDH2 allele may be functionally deficient in eliminating acetaldehyde and discourage alcohol drinking (Eng et al., 2007; Cichoz-Lach et al., 2010; Gubergrits et al., 2014). Furthermore, heavy drinkers of alcohol who were genetically prone to accumulate acetaldehyde may face an increased risk of gastric cancer (Cao et al., 2010; Ferrari et al., 2012).

Cytochromes P450 2E1 (CYP2E1)

Bennett et al. (1999) investigated that never-smoking women who are exposed to environmental tobacco smoke (ETS) and develop lung cancer are a genetically susceptible population. They analyzed germline polymorphisms in genes that have been associated with cancer susceptibility and whose products activate (CYP2A1) and detoxify (GSTM1 and GSTT1) chemical carcinogens found in tobacco smoke (Yu et al., 2012).

In Brazil, Rossini et al. (2007) analyzed the risk of esophageal squamous cell carcinoma (ESCC) associated with tobacco and alcohol consumption and with polymorphisms of wild type alleles i.e. CYP2A6 (CYP2A6*2), CYP2E1 (CYP2E1*5B, CYP2E1*6), GSTP1 (Ile105Val), GSTM1 and GSTT1 null genotypes in cases and age and gender-matched controls. There was no risk associated with CYP2A6, CYP2E1 and GSTM1 polymorphisms. In conclusion, this study suggests an opposite role of GSTP1 and GSTT1 polymorphisms for the risk for ESCC. Kury et al. (2007) examined the influence on sporadic colorectal cancers risk of environmental factors co-analyzed with combinations of six single nucleotide polymorphisms located in CYP450 genes mainly related to red meat consumption in France. To investigate whether GSTM1, GSTT1, GSTP1, CYP1A1 and CYP2E1 gene polymorphisms represent risk-modifying factors for ethanol related diseases, a study was conducted involving Brazilian alcoholics and controls with similar ethnic backgrounds (Burim et al., 2004). Results were indicating that persons with these genotypes are genetically more prone to the development of alcoholic pancreatitis and alcoholic cirrhosis, respectively (Lakkakula et al., 2013; Malakar et al., 2014; Yu et al., 2015).

Glutathione S-Transferases (GST)

Among all classes of GSTs, GSTM1, GSTT1 and GSTP1 polymorphisms are extensively studied worldwide. Homozygous deletions of GSTM1 and GSTT1 genes are common and result in a complete loss of enzyme activity. The frequencies of GSTM1 null alleles display race and ethnic variations, being highest in Europeans (42-60%) & Asians (41-63%) compared with that of Africans (16-36%) (Cotton et al., 2000; Gao et al., 2010). However, the frequency of GSTT1 null genotypes is somewhat less in Europeans (13.31%) compared with that of Africans (14-57%) and in Asians (35-48%) (Sobti et al., 2005; Sharma et al., 2012).

Comparison of the frequencies of GST polymorphisms in South Indian population was done by Vettriselvi et al. (2006). He examined that the GSTM1 and GSTT1 null genotype frequencies were found to be 22.4% and 17.6% respectively. Allelic variants of GSTM1, GSTT1, and GSTP1 have been associated with increased risk of various cancers like colorectal, lung, breast, prostate and others (Kiyohara et al., 2000; Vijayalakshmi et al., 2005; Malakar et al., 2012; Senthilkumar and Thirumurugan, 2012; Liu et al., 2013; Shen et al., 2014). Susceptibility to oral cancer by genetic polymorphisms among Indians through tobacco exposure as a risk modulator has been studied. The role of polymorphisms at CYP1A1, GSTM1 and GSTT1 to oral squamous cell carcinoma (OSCC) in a case-control study involving patients with precancerous lesions, cancer patients and age and habit-matched controls have been investigated. Finding shows increased susceptibility to buccal mucosa cancer among individuals carrying these genetic markers. These results support the finding that GSTM1 null genotype is a risk factor to OSCC among Indian tobacco habits; GSTT1 null genotype, however, emerged as a protective factor (Anantharaman et al., 2007; Senthilkumar and Thirumurugan, 2012; Dunna et al., 2013;).

Role of Epigenetic Alternations in Cancer Induction

Epigenetic alteration includes assortment of chemical modification at molecular level that involve alteration of the DNA (e.g. methylation) and the histone proteins (e.g. methylation, acetylation, phosphorylation, ubiquitinylation, ADP-ribosylation, and sumoylation) (Curtis et al., 2013). The epigenetic profile of the organism directly depends on varied aspects including age, environment, and exposure to toxins (e.g., alcohol and tobacco) (Feil and Fraga, 2012). Oxidative metabolites such as acetaldehyde, acetate, acetyl-CoA, and reactive oxygen species (ROS) are generated through alcohol metabolism under the oxidative pathway can induce tissue-specific epigenetic changes (Shukla and Aroor, 2006; Choudhury and Shukla, 2008; Kang et al., 2015). Ethanol metabolism increases ROS level enhances histone H3 acetylation in hepatocytes (Choudhury et al., 2010) and also increases production of acetyl-CoA significantly, which is used in histone acetylation by histone acetyl transferases (HATs) (Yamashita et al., 2001). Continuous exposure of high dose of alcohol to liver cells noticeably amplifies pro-inflammatory cytokine (such as IL-6 and TNFα) responses and was also associated with increased acetylation of histone H3 and H4 demonstrated by several

studies (Kendrick et al., 2010). Finally alcohol exposure has tissue specific and immunological responses, such as influencing cell recruitment to infect or inflamed tissue, varying cytokines production level, impairing antigen presentation, hampering with phagocytosis, or inducing apoptosis and finally causes cancer.

Conclusions for preventing cancer

Prospective studies have indicated a very strong link between the alcohol consumption and tobacco exposure. This link can be assessed by correlating the activation of DNA damage repair proteins, analysing secretion levels of inflammatory cytokines and status of xenobiotic genes polymorphism. Usually people considered alcohol intake as the major risk factor for the development of liver related disorders. But evidence suggested that both alcohol intake and tobacco exposure equally contribute to liver diseases and also enhances the toxic potency of each other. This review focuses on the DNA damage caused by alcohol and tobacco consumption, risk stratification, professional, educational and public awareness connected with genetic and environmental factors. These are the important components of a new strategic approach to the prevention of alcohol related cirrhosis and cancers.

In recent years, due to advancement in molecular epidemiology, an emerging new field that combines extremely sensitive and specific techniques for detecting early damage associated with cancer, it has been possible to identify risks and thwart adverse health consequences related to environmental exposures by exogenous and endogenous compound. Therefore, this review focuses on the possible relationship between inter-individual differences in gene structure and inducibility of enzymes responsible for xenobiotic metabolism. This information has been used for the evaluation of increased susceptibility for cancer. Furthermore, this review will represent a crucial step to understand the susceptibility to develop cirrhosis and cancer in alcoholics and will be of importance in the social awareness to people and prevention of alcohol related diseases and/or the development of better therapies. It will be also be important to identify new diagnostic biomarkers in future that might allow more straightforward treatment and for identifying high risk individuals at an early stage. Hopefully, this review will implement the researchers and clinicians to foster, explore and authenticate the impacts and underlying mechanisms of DNA damage response (DDR) pathways in liver cirrhosis and cancer.

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Management of alcoholic hepatitis

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Therapeutic issues in alcoholic hepatitis

Introduction

Alcoholic hepatitis (AH) is a clinical syndrome of liver inflammation, hepatocyte injury, and fibrosis that occurs in the setting of recent consumption of large amounts of alcohol. The clinical presentation and mechanisms underlying AH have recently been reviewed [1]. Prominent among these mechanisms is the notion that alcohol affects the barrier between the small bowel lumen and the *milieu intérieur*, and, as a result, there is translocation of lipopolysaccharides (LPS) from the gut into the portal blood stream. From there, LPS gain access to the liver sinusoids and interact with Kupffer cells to release cytokines and reactive oxygen species, which in turn mediate the inflammatory response in the liver [1]. Tumor necrosis factor alpha (TNF-α) is considered to be the principal cytokine in animal models but the importance of its role in humans has been recently called into question. Treatments focused on reducing inflammation, and abrogating cytokines and reactive oxygen species are among the therapies that will be discussed below.

AH usually arises in patients who meet criteria for diagnosis of abusive or addictive drinking. AH presents in a spectrum, from mild abnormalities of liver chemistry tests to life threatening liver failure. In the most acute clinical presentation of AH, in which serum bilirubin levels are markedly elevated along with leukocytosis, death is common despite stopping to drink. Consequently, we will concentrate on the management of severe AH.

Clinical presentation

The clinical syndrome of AH consists of jaundice and right upper quadrant discomfort. The liver is enlarged and tender on palpation. Often AH occurs against a background of established cirrhosis, and patients may also have features of chronic liver injury and portal hypertension, such as ascites, variceal hemorrhage, and encephalopathy. Severe AH may progress to multisystem organ failure. The advent of acute kidney injury is a particularly worrisome development. We shall discuss, from the point of view of the physician, the management of extrahepatic organ failure and systemic inflammatory response syndrome (SIRS).

AH continues to be a cause of considerable mortality and morbidity in Europe and North America [2,3]. There were 56,809 hospital admissions for AH in the US in 2007, which amounted to 0.71% of all admissions for that year [2]. In this dataset, the average length of stay was 6.5 days, and the in-hospital mortality was 6.8%. A Danish study indicated that the 28-day mortality of patients hospitalized for AH was 15% in 2008, the most recent year for which data were reported [3]. Data from combined treatment studies of severe AH have shown 28-day mortality of 34% in patients not receiving corticosteroids. These data emphasize the high short-term mortality of patients admitted to hospital with AH.

AH is associated with a histologic picture consisting of ballooned hepatocytes, Mallory bodies, lobular neutrophils, and lattice-like fibrosis surrounding hepatocytes in the centrizonal area. These histopathological features can persist for months after the patient has stopped drinking [4]. Opinions are divided on the role of liver biopsy in making the diagnosis of AH, since coagulopathy and thrombocytopenia are common in this population and increase the risk of bleeding following a standard percutaneous approach. This risk is reduced by transjugular approaches, although this is not available in all centers.

Assessment of prognosis in alcoholic hepatitis

There are several scoring systems available to assess severity and prognosis of AH (see Tables 1 and 2). The relative characteristics and utility of three of these scores – the modified Maddrey Discriminant Function (DF), the MELD score, and the Glasgow Alcoholic Hepatitis Score – have been reviewed in two recent publications [1,5]. Another score from the Barcelona group, which they have entitled ABIC, can be added to these [6]. The purpose of these scoring systems is twofold: first to enable the managing physician to estimate the likelihood of short-term survival, and second to determine whether the patient should be treated with corticosteroids. A fifth score, the Lille score, is somewhat different, enabling the physician to decide whether corticosteroid therapy should be stopped after a week, or continued for 28 days [7]. Table 1 shows the components of each score. As it can be seen, there is considerable overlap. All scores use total bilirubin. The MELD score, Glasgow, ABIC, and Lille score all incorporate a measure of kidney function, underscoring the

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Table 1. Comparison of the elements that constitute 5 prognostic instruments in alcoholic hepatitis.

	Bilirubin	PT/INR	Creatinine/ Urea	Leucocytes	Age	Albumin	Change in bilirubin from day 0 to day 7
Maddrey score	+	+	-	-	-	-	-
MELD score	+	+	+	-	-	-	-
GAHS score	+	+	+	+	+	-	-
ABIC score	+	+	+	-	+	+	-
Lille score	+	+	+	-	+	+	+

Maddrey score, Maddrey discriminant function; GAHS, Glasgow Alcoholic Hepatitis Score; ABIC score, Age, serum Bilirubin, INR, and serum Creatinine score; MELD score, Model-For-End-Stage-Liver-Disease score; PT/INR, Prothrombin Time/International Normalized Ratio.

Table 2. Advantages of the available prognostic scores.

	Pros	Cons
Maddrey score	Verified by 20+ years experience Identifies patients who do not need corticosteroids	Admits patients who may not need corticosteroids (see Glasgow score) Requires PT and control
MELD score	Ease of use Verified in acute/chronic liver failure	Uncertainty about the threshold for initiating corticosteroids
GAHS score	Stratifies DF >32 patients in need of corticosteroids	Not verified outside UK
ABIC score	Stratifies patients into high, moderate, and low risk	Uncertainty about the threshold for initiating corticosteroids Not verified outside Spain
Lille score	Allows stopping corticosteroids at day 7 Verified in retrospective, multi-national dataset	No clear alternatives to corticosteroids in treatment failures

prognostic significance of impaired kidney function in patients with AH. A brief statement of pros and cons is given in Table 2. We lack studies of adequate power that consider the relative utility of all of these scores and it is inconclusive as to which is best. In the review on behalf of the American Association for the Study of Liver Diseases (AASLD), O'Shea *et al.* advocate for using the Maddrey Discriminant Function, along with other clinical data [5]. We would advocate that physicians use this score, particularly when determining that the clinical severity is not sufficiently severe to initiate therapy. The authors of the Glasgow score have shown that their score may identify a subgroup of high-DF patients who will recover without steroids, and it may have utility in this regard [8]. The ABIC has the advantage in indicating high, intermediate, and low urgency [6]. However, since it was derived among patients who were treated with corticosteroids, it is potentially compromised as a tool to identify patients best suited to corticosteroids. We advocate the use of the Lille score at day 7 to plan stopping corticosteroids or completing a 28-day course.

Management of extrahepatic manifestations of alcoholic hepatitis

Cessation of alcohol consumption is the *sine qua non* of therapy for AH, and in the milder forms is sufficient for clinical recovery. On the other hand, patients who continue to drink are likely to die, and relapse to alcohol use is a common reason for exclusion from consideration for liver transplantation [9]. There are no studies of strategies to encourage or maintain abstinence in patients with AH. Thus, it is not possible to say whether agents directed to initiate abstinence, or discourage continued drinking, such as disulfiram, naltrexone, acamprosate or topiramate, are efficacious or safe in patients with AH. A small randomized controlled trial of baclofen administered to patients with alcoholic cirrhosis, showed a significant increase in patients maintaining abstinence for 12 weeks in the baclofen group compared to subjects who received placebo [10]. Whether baclofen would have a salutary effect in AH is unknown. We therefore avoid pharmacotherapy for alcoholic patients with AH, and rely on psychotherapeutic approaches. Even with a support team of addiction specialists, relapse to drinking and recurrent AH remains a risk [6].

Because the patient with AH has usually been drinking up to the time of presentation, he or she is at risk of alcohol withdrawal syndrome once admitted to the hospital. The risk of seizures is greater if there is a history of previous alcohol induced seizure ('rum fits'). It is our practice to initiate a protocol of 'symptom-triggered management' using the Clinical Institute Withdrawal Assessment of Alcohol [11]. All patients admitted with acute alcohol toxicity are placed on nutritional supplements (see below), including thiamine.

Patients presenting with AH often have the clinical features of the systemic inflammatory response syndrome (SIRS). For example, leukocytosis is a defining feature of both entities. AH often occurs in patients with cirrhosis and portal hypertension. Cirrhosis leading to ascites is associated with splanchnic vasodilatation, peripheral vasodilatation, reduced systemic vascular resistance, and high cardiac output, all of which can result in systemic hypotension [12]. The challenge for the managing physician is to determine whether clinical phenomena such as tachycardia, hypotension, and leukocytosis are accounted for by the combined effects of alcohol, AH, and the hemodynamic consequences of portal hypertension or whether in addition, the patient has an infectious process contributing to the systemic inflammatory response. Patients with AH are immunocompromised by malnutrition and impaired liver function. They are at risk of pneumonia, particularly aspiration

pneumonia after vomiting or upper endoscopy, spontaneous bacterial peritonitis and urinary tract infection. Therefore, on admission, it is appropriate that all patients with AH undergo an extensive screening for infection, with chest radiographs, blood cultures, urine cultures, and where appropriate, diagnostic paracentesis. There are no data to show that administration of antibiotics in the absence of a confirmed infection will improve the outcome of severe AH, although this is a hypothesis that could be tested in a clinical trial. Once a positive culture or diagnostic chest radiograph is identified, the patient should be treated with appropriate antibiotics. As will be discussed later, patients who meet all criteria for corticosteroids, except for an identified infection, should have antibiotics started and quickly transitioned to corticosteroids [13].

The hemodynamic consequences of portal hypertension that lead to the overlap with SIRS are also the forces that make patients with AH at high risk for kidney failure due to hepatorenal syndrome [12]. In addition, AH patients are at risk from nephrotoxins, particularly nephrotoxic radiocontrast agents, aminoglycosides, and non-steroidal anti-inflammatories. The inclusion of serum creatinine or urea in the short-term prognostic instruments in Table 1 is testament to the grim prognostic significance of new-onset kidney failure in this patient population [14]. Avoidance of 'by rote' contrast enhanced CT scanning when patients with AH present to the emergency room, and removal of non-steroidal agents from protocol admission orders will limit the exposure of these patients to nephrotoxins. Treatment for hepatorenal syndrome with albumin and vasoconstrictors should be started early after careful daily measurement of urinary output, and serum creatinine to identify early acute kidney injury is key to management [15]. As will be discussed below, the salutary effect of pentoxifylline found in some studies appears to be confined to protecting patients with AH from developing hepatorenal syndrome.

Treatment of the inflamed liver

Background

AH occurs in approximately 20% of heavy drinkers. The treatment of severe AH remains controversial and is one of the main challenges in alcoholic liver disease [1].

Survival at 1 or 2 months has been the most common primary outcome adopted in prior studies evaluating pharmacological therapies in patients with severe AH. Most of the studies were underpowered because of the use of inappropriate criteria of disease severity. Indeed, reproducible criteria to identify patients at significant risk of early death are a prerequisite in order to calculate the number of patients needed for studies, assuming a one or two-sided type I error ≤0.05 and a power ≥80%. A significant proportion of studies evaluating corticosteroids were conducted before the era of DF, when short-term survival in the untreated control arms ranged from 0 to 81% [16]. DF has been validated by several groups as a reproducible criterion to identify patients at high risk of early mortality. In the absence of treatment, the spontaneous survival of patients with a DF ≥32 has fluctuated between 50% and 65% [17–19]. Conversely, because spontaneous survival at 28 days among patients with a DF <32 is close to 90% [18], it is impossible to observe any effect of short-term treatment on survival in this subgroup. Nowadays, experts require the use of DF for studies using 1- or 2-month survival as the primary endpoint. The MELD, the Glasgow, and the ABIC scores may be considered as alternative or additional tools to accurately define disease severity.

Corticosteroids

Randomized controlled trials evaluating corticosteroids in patients with AH have yielded inconsistent results, attributed to the wide differences of disease severity between studies [20]. Meta-analyses of the literature of the fifteen randomized controlled trials from three different groups concluded that the survival effect of corticosteroids was restricted to severe disease [20–22]. Conversely, Cochrane meta-analyses questioned the efficacy of corticosteroids in AH regardless of disease severity [23,24], although their most recent meta-analysis reported that corticosteroids significantly reduced mortality in the subgroup of trials that enrolled patients with a DF of at least 32 or hepatic encephalopathy [24].

The analysis of individual data from the five most recent randomized controlled trials [17,19,25–27], which included 418 randomized patients, confirmed the efficacy of corticosteroid in severe AH. The patients allocated to corticosteroids treatment (n = 221) had higher 28-day survival than patients allocated to non-corticosteroids treatment (n = 197): 80% *vs.* 66%. In multivariate analysis, leukocytes, DF, Lille Model, encephalopathy, and corticosteroid treatment were associated independently with short-term survival [28]. Corticosteroid treated patients had an early and greater improvement of liver function and a better response to the assigned therapy assessed by the Lille model. This analysis should end the controversy surrounding the short-term efficacy of corticosteroids in severe AH.

New management of patients according to the response to steroids

Early identification of responders with a substantial improvement in hepatic function following treatment with corticosteroids constitutes an advance in the management of severe AH [29]. After 7 days of treatment, physicians may indentify responders to medical therapy using a model, referred to as the Lille model [7]. The Lille model is highly predictive of death at 6 months and a score above 0.45 predicted 75% of the deaths. This approach highlights the benefits obtained from strategy integrating the impact of treatment upon the evaluated endpoint.

Using the Lille model, the recent meta-analysis of individual data observed that the survival impact of corticosteroids seemed to be restricted to patients classified as responders, either complete or partial [16]. This study confirms the need for adapting corticosteroid therapy to response to treatment. A subgroup analysis was performed according to the percentile distribution of the Lille score: ≤35th, 35–70th, and ≥70th percentile [16]. Patients were classified as: complete responders (Lille score ≤0.16, ≤35th percentile); partial responders (Lille score between 0.16–0.56, 35–70th percentile); and null-responders (Lille score >0.56, ≥70th percentile). This approach identified three patterns of responses, complete, partial, and null, with significant differences in survival benefit: 91% *vs.* 79% *vs.* 53%, p <0.0001. Corticosteroids showed a significant effect on 28-day survival in complete (hazard ratio 0.18) and in partial responders (hazard ratio 0.38), but not in null responders. In summary, using this classification, this study showed that the survival impact of corticosteroids was significant in complete and partial responders, whereas it appeared negligible in null responders [16]. This new classification raises questions concerning management of severe AH. It is speculated that corticosteroids may be sufficient in complete responders and that novel pharmacological therapies are relevant for intermediate responders.

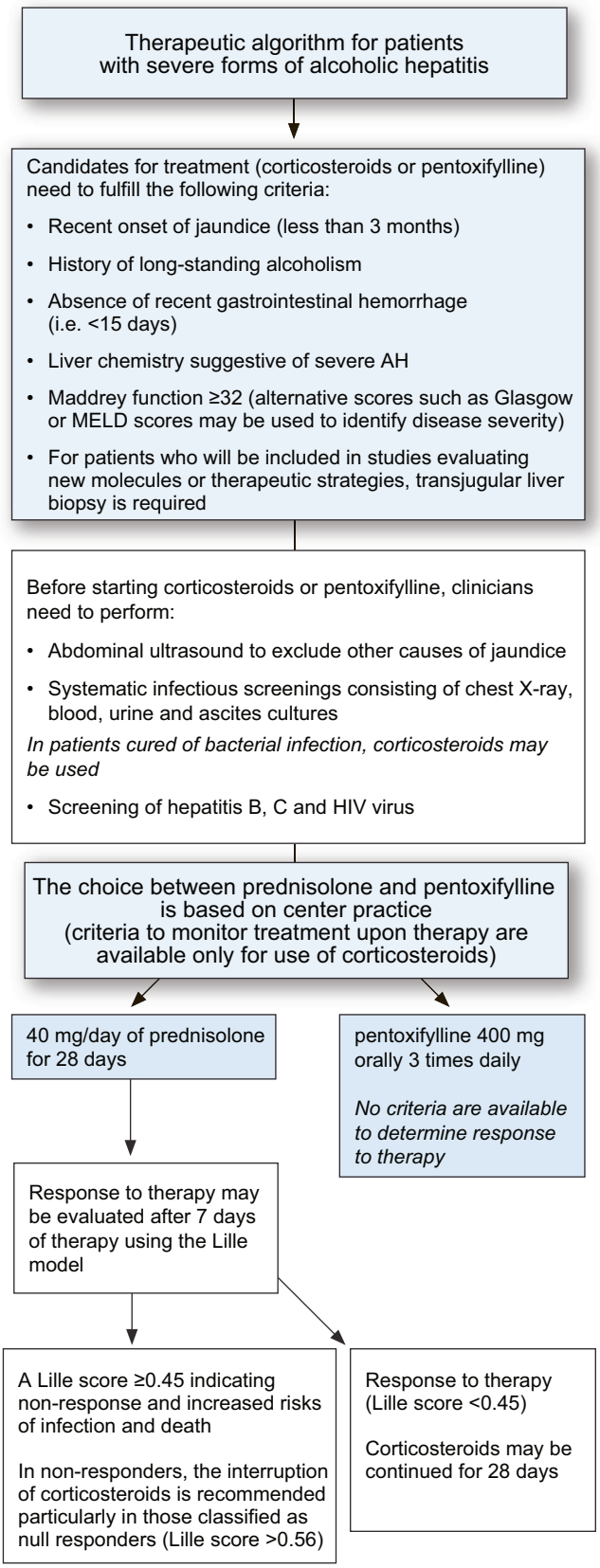

Fig. 1. Therapeutic algorithm for patients with severe forms of alcoholic hepatitis.

Infection has classically been viewed as a contraindication for corticosteroid treatment, no doubt on account of the close relationship between infection and the use of immunological agents such as corticosteroids. Conversely, in cirrhotic patients, the severity of liver dysfunction is an independent predictive factor of the development of infection. As corticosteroids induce early improvement in hepatic function, the Lille group investigated whether the corticosteroid-associated improved liver function, observed in responders with severe AH, outweighed the potential deleterious effects of corticosteroids on infections that often accompany severe AH [13]. Prior to initiation of corticosteroids, 25.6% of patients were already infected. In patients recovering from infection, prednisolone was started after a median time of 8 days. There were no significant differences in survival between patients who had been treated for infection prior to initiation of corticosteroids and the remainder. Furthermore, the probability of acquiring infection after corticosteroids had been started was drastically lower in responders (Lille score <0.45) compared with non-responders (11% *vs.* 43%). At first glance, infection might be considered a major factor contributing to death. However, it is not an independent prognostic factor, and early response to therapy seems to be more important for predicting both survival and the clinical significance of infection. The most likely hypothesis is that early improvement in liver function is the most important factor contributing to decreased risk of infection, and to patient survival. An algorithm for therapeutic strategy for patients with severe alcoholic hepatitis is provided in Fig. 1.

Non-responders do not derive any benefit from corticosteroids and require a new strategy. An early withdrawal of corticosteroids and a switch to either pentoxifylline [28] or molecular adsorbent recirculating system (MARS) are not efficacious. In summary, management of non-responders remains a challenge, and apart from advocating continued abstinence, we lack readily available therapies that work. It is for this reason that the debate on treatment of AH with liver transplantation has reopened (see below).

Pentoxifylline

In a randomized controlled trial of 101 patients with severe AH (DF ≥32), mortality rate was lower in pentoxifylline patients than in placebo patients (24% *vs.* 46.1%) [30]. The survival benefit of pentoxifylline appears to be related to a significant reduction in development of hepatorenal syndrome in pentoxifylline-treated patients (relative risk 0.29). Contrary to corticosteroids, the effect of pentoxifylline was related to prevention of hepatorenal function but not to improvement of liver function [30]. At the end of the treatment period, the two groups had similar values of DF, prothrombin time, and bilirubin levels. The preventive effect of pentoxifylline on hepatorenal syndrome was confirmed in two recent randomized controlled trials. In a randomized controlled trial of 335 cirrhotic Child C patients, 6-month survival of pentoxifylline patients (70%) was not significantly different from that of placebo patients (68.5%) whereas the probability of being free of renal failure at 6 months was significantly higher in the pentoxifylline group (90.9%) than in the placebo group (79.4%) [31]. In a sensitivity analysis restricted to the 55 patients enrolled with severe AH (DF ≥32) treated with corticosteroids, 6-month survival was not significantly different in pentoxifylline (76.9%) than in placebo patients (79.3%). The last randomized trial of 70 cirrhotic patients with ascites showed a lower occurrence of hepatorenal syndrome in pentoxifylline patients than in placebo

patients: 28.6% *vs.* 5.7% [32]. A randomized controlled trial of 68 patients with severe AH (DF ≥32) compared the efficacy of pentoxifylline and prednisolone. Pentoxifylline-treated patients had higher 3-month survival than corticosteroids patients: 85.3% *vs.* 64.7% [33]. Six patients who received corticosteroids developed hepatorenal syndrome as compared to none in the pentoxifylline group [33]. In summary, pentoxifylline seems to reduce the risk of hepatorenal syndrome in patients with severe AH, and perhaps to reduce short-term mortality in so far that it is related to acute kidney failure.

Enteral nutrition

Total enteral tube feeding was compared to corticosteroids in a randomized controlled trial [25]. The formula of the enteral diet was a low-fat diet in which medium-chain triglycerides and oleic acid accounted for most of its lipid content, after considering the deleterious effects of a high-fat diet on alcoholic liver injury in animal models. Mortality occurred earlier in the enteral group: 7 days *vs.* 23 days. During follow-up after the treatment period, deaths were observed more frequently in the corticosteroid group (10/27) than in the enteral group (2/24, $p = 0.04$). Those investigators recently suggested that combined treatment with enteral nutrition and corticosteroids could improve the outcome of patients with severe AH and merited investigation in a randomized controlled trial.

Anti-TNF-α

As mentioned above, TNF-α has been implicated in animal studies as an important cytokine mediator of AH, and therefore the anti-TNF-α strategy has been considered one of the most attractive approaches to developing future therapies for AH. This strategy was initially tested in a pilot randomized study of 20 patients with biopsy-proven severe AH treated by prednisolone 40 mg/day for 28 days who were randomized to receive infliximab 5 mg/kg IV (n = 10) or placebo (n = 10) [34]. At day 28, DF and IL-8 levels decreased significantly in the infliximab group. These data provided strong arguments in favor of future evaluation of infliximab, even though the study was not designed to evaluate the effects of infliximab on survival. A randomized controlled trial was stopped by the independent data safety monitoring board before the study accrued the planned enrollment of the 38 patients because of the unanticipated rate of deaths in the infliximab-treated group [35]. Indeed, after randomization of 36 patients, there were 7 deaths in the infliximab plus corticosteroid group and 3 deaths in the corticosteroid-only group. The probability of survival at 2 months was lower in the infliximab plus corticosteroid group (61%) than in the corticosteroids-only group (82%). The frequency of severe infections was significantly higher in the infliximab plus corticosteroid group. In a US multicenter study, 48 patients with moderate to severe AH were randomized in 2 groups treated by up to 6 subcutaneous injections of either etanercept or placebo for 3 weeks [36]. The 1-month mortality rates of placebo and etanercept patients were not significantly different, whereas the 6-month mortality rate was significantly higher in the etanercept group (58% *vs* 23%) [36]. Rates of infectious events were significantly higher in the etanercept group. In summary, anti-TNF-α agents are not effective for the treatment of patients with AH, and should not be considered outside the confines of an approved randomized clinical trial.

N-acetylcysteine

Fifty-two patients were randomized to receive N-acetylcysteine intravenously (n = 28) or a placebo perfusion (n = 24) along with adequate nutritional support for 14 days [37]. Survival rates at 1 and 6 months were not significantly different in N-acetylcysteine and control group. Early biological changes, documented infection rate at 1 month, and incidence of hepatorenal syndrome did not differ between the two groups. The investigators concluded that high doses of intravenous N-acetylcysteine therapy for 14 days conferred neither survival benefits nor early biological improvement in severe AH. Another randomized study of 101 patients compared corticosteroids to a novel antioxidant cocktail containing N-acetylcysteine. The odds of dying by 30 days were 2.4 greater for patients on antioxidants than patients on corticosteroids [19]. The investigators concluded that corticosteroids were superior to antioxidants. A recent randomized study observed that patients treated with corticosteroids and N-acetylcysteine had higher 1-month survival than patients treated with corticosteroids alone [38]. This benefit was not observed at 6 months. Nevertheless, this study is an important piece of work suggesting that corticosteroids and N-acetylcysteine may have synergistic effects.

Early liver transplantation and alcoholic hepatitis

Does the 6-month rule limit access to liver transplantation for the most severely ill patients?

At present, liver transplantation is not considered a therapeutic option for patients with AH. A panel of experts noted that the potential role of liver transplantation in managing patients with severe AH remains undecided [39]. In addition, members of UK liver transplant units listed AH as a contraindication for liver transplantation [40]. However, such recommendations have raised several concerns [41]. Indeed, optimal timing for liver transplantation in alcoholic patients varies drastically between transplant programs, and decisions on transplant eligibility should be made on an individual basis, with careful prediction of short-term survival. In the particular setting of non-responders to corticosteroids, strict application of a period of sobriety as a policy for transplant eligibility is unfair to such patients, as most of them will have died prior to the end of the 6-month sober period.

Clinicians fear that modifications in guidelines for liver transplantation of alcoholic patients, which are in conflict with public allocation preferences, may decrease public willingness to donate. It should be emphasized that such a concern was not raised in the setting of emergent liver transplantation proposed to patients with fulminant hepatic failure due to voluntary acetaminophen poisoning, or to active drug abusers with acute hepatitis B virus. It is important to make the public aware that most philosophers and ethicists feel that patients with self-inflicted diseases should have the same access to medical resources, and that personal responsibility should not influence the decision to transplant.

Early liver transplantation improves survival of patients

In severe AH, patients failing to respond to medical therapy can be identified early, and have a 6-month survival around 30%. As most deaths occur within 2 months, early liver transplantation (LT) in those patients is attractive but

highly controversial as it challenges the 6-month abstinence rule prior to LT [42]. Seven liver transplantation centers performed early liver transplantation in patients with severe AH failing to respond to medical therapy undergoing their first episode of liver disease and strictly selected using these criteria: absolute consensus of paramedical and medical staff, no co-morbidities, social integration, and supportive family members [43]. Non-responders were identified using Lille score ≥0.45 or worsening of liver function by day 7. This case-controlled study showed an unequivocal improvement of survival in patients who received early transplantation. The investigators concluded that despite the fact that early LT for severe AH patients who fail medical therapy contravenes the 6-month abstinence rule, these results support future evaluation of LT in a carefully-selected subgroup of patients with severe AH failing to medical therapy. However, early liver transplantation is relevant only for a minority of patients whereas new therapeutic strategies are urgently needed for the majority of non-responders.

Key Points

- Alcoholic hepatitis is associated with a histologic picture consisting of ballooned hepatocytes, Mallory bodies and lobular neutrophils
- In the severest clinical presentation of alcoholic hepatitis, in which serum bilirubin levels are markedly elevated, death is common despite stopping drinking
- Cessation of alcohol consumption is the *sine qua non* of therapy for alcohol hepatitis, and in the milder forms is sufficient for clinical recovery
- Severe AH may progress to multisystem organ failure. The advent of acute kidney injury and the emergence of infection are worrisome events
- Patients with a Maddrey Discriminant Function ≥32 (alternative scores such as Glasgow or MELD scores may be used to identify disease severity) should be considered as candidates for corticosteroids or pentoxifylline treatment
- We advocate the use of Lille score at day 7 to plan stopping corticosteroids or completing a 28-day course
- New treatments or strategies are required to improve the probability of being alive within the year following the onset of the disease

Conflict of interest

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Diseases and Conditions

Alcohol use disorder

By Mayo Clinic Staff

Alcohol use disorder (which includes a level that's sometimes called alcoholism) is a pattern of alcohol use that involves problems controlling your drinking, being preoccupied with alcohol, continuing to use alcohol even when it causes problems, having to drink more to get the same effect, or having withdrawal symptoms when you rapidly decrease or stop drinking.

Unhealthy alcohol use includes any alcohol use that puts your health or safety at risk or causes other alcohol-related problems. It also includes binge drinking — a pattern of drinking where a male consumes five or more drinks within two hours or a female downs at least four drinks within two hours. Binge drinking causes significant health and safety risks.

If your pattern of drinking results in repeated significant distress and problems functioning in your daily life, you likely have alcohol use disorder. It can range from mild to severe. However, even a mild disorder can escalate and lead to serious problems, so early treatment is important.

Alcohol use disorder can be mild, moderate or severe, based on the number of symptoms you experience. Signs and symptoms may include:

- Being unable to limit the amount of alcohol you drink
- Wanting to cut down on how much you drink or making unsuccessful attempts to do so
- Spending a lot of time drinking, getting alcohol or recovering from alcohol use
- Feeling a strong craving or urge to drink alcohol
- Failing to fulfill major obligations at work, school or home due to repeated alcohol use
- Continuing to drink alcohol even though you know it's causing physical, social or interpersonal problems
- Giving up or reducing social and work activities and hobbies
- Using alcohol in situations where it's not safe, such as when driving or swimming
- Developing a tolerance to alcohol so you need more to feel its effect or you have a reduced effect from the same amount
- Experiencing withdrawal symptoms — such as nausea, sweating and shaking — when you

don't drink, or drinking to avoid these symptoms

Alcohol use disorder can include periods of alcohol intoxication and symptoms of withdrawal.

- **Alcohol intoxication** results as the amount of alcohol in your blood stream increases. The higher the blood alcohol concentration is, the more impaired you become. Alcohol intoxication causes behavior problems and mental changes. These may include inappropriate behavior, unstable moods, impaired judgment, slurred speech, impaired attention or memory, and poor coordination. You can also have periods called "blackouts," where you don't remember events. Very high blood alcohol levels can lead to coma or even death.
- **Alcohol withdrawal** can occur when alcohol use has been heavy and prolonged and is then stopped or greatly reduced. It can occur within several hours to four or five days later. Symptoms include sweating, rapid heartbeat, hand tremors, problems sleeping, nausea and vomiting, hallucinations, restlessness and agitation, anxiety, and occasionally seizures. Symptoms can be severe enough to impair your ability to function at work or in social situations.

What is considered one drink?

The National Institute on Alcohol Abuse and Alcoholism defines one standard drink as any one of these:

- 12 ounces (355 milliliters) of regular beer (about 5 percent alcohol)
- 8 to 9 ounces (237 to 266 milliliters) of malt liquor (about 7 percent alcohol)
- 5 ounces (148 milliliters) of unfortified wine (about 12 percent alcohol)
- 1.5 ounces (44 milliliters) of 80-proof hard liquor (about 40 percent alcohol)

When to see a doctor

If you feel that you sometimes drink too much alcohol, or it's causing problems, or your family is concerned about your drinking, talk with your doctor. Other ways to get help include talking with a mental health provider or seeking help from a support group such as Alcoholics Anonymous or a similar type of self-help group.

Because denial is common, you may not feel like you have a problem with drinking. You might not recognize how much you drink or how many problems in your life are related to alcohol use. Listen to relatives, friends or co-workers when they ask you to examine your drinking habits or to seek help. Consider talking with someone who has had a problem drinking, but has stopped.

If your loved one needs help

Many people with alcohol use disorder hesitate to get treatment because they don't recognize they have a problem. An intervention from loved ones can help some people recognize and accept that they need professional help. If you're concerned about someone who drinks too much, ask a professional experienced in alcohol treatment for advice on how to approach that

person.

Genetic, psychological, social and environmental factors can impact how drinking alcohol affects your body and behavior. Theories suggest that for certain people drinking has a different and stronger impact that can lead to alcohol use disorder.

Over time, drinking too much alcohol may change the normal function of the areas of your brain associated with the experience of pleasure, judgment and the ability to exercise control over your behavior. This may result in craving alcohol to try to restore good feelings or reduce negative ones.

Risk factors for alcohol use disorder include:

- **Steady drinking over time.** Drinking too much on a regular basis for an extended period or binge drinking on a regular basis can lead to alcohol-related problems or alcohol use disorder.
- **Age.** People who begin drinking at an early age, and especially in a binge fashion, are at a higher risk of alcohol use disorder. Alcohol use may begin in the teens, but alcohol use disorder occurs more frequently in the 20s and 30s. However, it can begin at any age.
- **Family history.** The risk of alcohol use disorder is higher for people who have a parent or other close relative who has problems with alcohol. This may be influenced by genetic factors.
- **Depression and other mental health problems.** It's common for people with a mental health disorder such as anxiety, depression, schizophrenia or bipolar disorder to have problems with alcohol or other substances.
- **Social and cultural factors.** Having friends or a close partner who drinks regularly could increase your risk of alcohol use disorder. The glamorous way that drinking is sometimes portrayed in the media also may send the message that it's OK to drink too much. For young people, the influence of parents, peers and other role models can impact risk.

Alcohol depresses your central nervous system. In some people, the initial reaction may be stimulation. But as you continue to drink, you become sedated.

Too much alcohol affects your speech, muscle coordination and vital centers of your brain. A heavy drinking binge may even cause a life-threatening coma or death. This is of particular concern when you're taking certain medications that also depress the brain's function.

Impact on your safety

Excessive drinking can reduce your judgment skills and lower inhibitions, leading to poor choices and dangerous situations or behaviors, including:

- Motor vehicle accidents and other types of accidental injury, such as drowning
- Relationship problems
- Poor performance at work or school

- Increased likelihood of committing violent crimes or being the victim of a crime
- Legal problems or problems with employment or finances
- Problems with other substance use
- Engaging in risky, unprotected sex, or becoming the victim of sexual abuse or date rape
- Increased risk of attempted or completed suicide

Impact on your health

Drinking too much alcohol on a single occasion or over time can cause health problems, including:

- **Liver disease.** Heavy drinking can cause increased fat in the liver (hepatic steatosis), inflammation of the liver (alcoholic hepatitis), and over time, irreversible destruction and scarring of liver tissue (cirrhosis).
- **Digestive problems.** Heavy drinking can result in inflammation of the stomach lining (gastritis), as well as stomach and esophageal ulcers. It also can interfere with absorption of B vitamins and other nutrients. Heavy drinking can damage your pancreas or lead to inflammation of the pancreas (pancreatitis).
- **Heart problems.** Excessive drinking can lead to high blood pressure and increases your risk of an enlarged heart, heart failure or stroke. Even a single binge can cause a serious heart arrhythmia called atrial fibrillation.
- **Diabetes complications.** Alcohol interferes with the release of glucose from your liver and can increase the risk of low blood sugar (hypoglycemia). This is dangerous if you have diabetes and are already taking insulin to lower your blood sugar level.
- **Sexual function and menstruation issues.** Excessive drinking can cause erectile dysfunction in men. In women, it can interrupt menstruation.
- **Eye problems.** Over time, heavy drinking can cause involuntary rapid eye movement (nystagmus) as well as weakness and paralysis of your eye muscles due to a deficiency of vitamin B-1 (thiamine). A thiamine deficiency also can be associated with other brain changes, such as irreversible dementia, if not promptly treated.
- **Birth defects.** Alcohol use during pregnancy may cause miscarriage. It also may cause fetal alcohol syndrome, resulting in giving birth to a child who has physical and developmental problems that last a lifetime.
- **Bone damage.** Alcohol may interfere with the production of new bone. This bone loss can lead to thinning bones (osteoporosis) and an increased risk of fractures. Alcohol can also damage bone marrow, which makes blood cells. This can cause a low platelet count, which may result in bruising and bleeding.
- **Neurological complications.** Excessive drinking can affect your nervous system, causing numbness and pain in your hands and feet, disordered thinking, dementia, and short-term memory loss.
- **Weakened immune system.** Excessive alcohol use can make it harder for your body to

resist disease, increasing your risk of various illnesses, especially pneumonia.

- **Increased risk of cancer.** Long-term excessive alcohol use has been linked to a higher risk of many cancers, including mouth, throat, liver, colon and breast cancer. Even moderate drinking can increase the risk of breast cancer.
- **Medication and alcohol interactions.** Some medications interact with alcohol, increasing its toxic effects. Drinking while taking these medications can either increase or decrease their effectiveness, or make them dangerous.

Here's some information to help you get ready for your appointment, and what to expect from your doctor.

Consider your drinking habits, taking an honest look at how often and how much you drink. Be prepared to discuss any problems that alcohol may be causing. You may want to take a family member or friend along, if possible.

Before your appointment, make a list of:

- **Any symptoms you've had,** including any that may seem unrelated to your drinking
- **Key personal information,** including any major stresses or recent life changes
- **All medications,** vitamins or other supplements that you're taking, and their doses
- **Questions to ask** your doctor

Some questions to ask include:

- Do you think I drink too much or show signs of problem drinking?
- Do you think I need to cut back or quit drinking?
- Do you think alcohol could be causing or worsening my other health problems?
- What's the best course of action?
- What are the alternatives to the approach that you're suggesting?
- Do I need any medical tests for underlying physical problems?
- Are there any brochures or other printed material that I can have? What websites do you recommend?
- Would it be helpful for me to meet with a professional experienced in alcohol treatment?

Don't hesitate to ask any other questions.

What to expect from your doctor

Be ready to answer questions from your doctor, which may include:

- How often and how much do you drink?
- Do you have any family members with alcohol problems?
- Do you sometimes drink more than you intend to drink?

- Have relatives, friends or co-workers ever suggested you need to cut back or quit drinking?
- Do you feel like you need to drink more than you previously did to get the same effect?
- Have you tried to stop drinking? If so, was it difficult and did you have any withdrawal symptoms?
- Have you had problems at school, at work or in your relationships that may be related to alcohol use?
- Have there been times that you behaved in a dangerous, harmful or violent way when you were drinking?
- Do you have any physical health problems, such as liver disease or diabetes?
- Do you have any mental health issues, such as depression or anxiety?
- Do you use recreational drugs?

You're likely to start by seeing your primary care doctor. If your doctor suspects you have a problem with alcohol, he or she may refer you to a mental health provider.

To assess your problem with alcohol, your health care provider will likely:

- **Ask you several questions related to your drinking habits.** The health care provider may ask for permission to speak with family members or friends. However, confidentiality laws prevent your health care provider from giving out any information about you without your consent.
- **Perform a physical exam.** Your health care provider may do a physical exam and ask questions about your health. There are many physical signs that indicate complications of alcohol use.
- **Lab tests and imaging tests.** While there are no specific tests to diagnose alcohol use disorder, certain patterns of lab test abnormalities may strongly suggest it. And you may need tests to identify health problems that may be linked to your alcohol use. Damage to your organs may be seen on tests.
- **Complete a psychological evaluation.** This evaluation includes questions about your symptoms, thoughts, feelings and behavior patterns. You may need to fill out a questionnaire to help answer these questions.
- **Use the DSM-5 criteria.** The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), published by the American Psychiatric Association, is often used by mental health providers to diagnose mental health conditions and by insurance companies to reimburse for treatment.

Treatment for alcohol use disorder can vary, depending on your needs. Treatment may involve a brief intervention, individual or group counseling, an outpatient program, or a residential inpatient stay. Working to stop the use of alcohol to improve quality of life is the main treatment goal.

Treatment for alcohol use disorder may include:

- **Detox and withdrawal.** Treatment may begin with a program of detoxification or detox — withdrawal that's medically managed — which generally takes two to seven days. You may need to take sedating medications to prevent withdrawal symptoms. Detox is usually done at an inpatient treatment center or a hospital.
- **Learning skills and establishing a treatment plan.** This usually involves alcohol treatment specialists. It may include goal setting, behavior change techniques, use of self-help manuals, counseling and follow-up care at a treatment center.
- **Psychological counseling.** Counseling and therapy for groups and individuals help you better understand your problem with alcohol and support recovery from the psychological aspects of alcohol use. You may benefit from couples or family therapy — family support can be an important part of the recovery process.
- **Oral medications.** A drug called disulfiram (Antabuse) may help to prevent you from drinking, although it won't cure alcohol use disorder or remove the compulsion to drink. If you drink alcohol, the drug produces a physical reaction that may include flushing, nausea, vomiting and headaches. Naltrexone (Revia), a drug that blocks the good feelings alcohol causes, may prevent heavy drinking and reduce the urge to drink. Acamprosate (Campral) may help you combat alcohol cravings once you stop drinking. Unlike disulfiram, naltrexone and acamprosate don't make you feel sick after taking a drink.
- **Injected medication.** Vivitrol, a version of the drug naltrexone, is injected once a month by a health care professional. Although similar medication can be taken in pill form, the injectable version of the drug may be easier for people recovering from alcohol use disorder to use consistently.
- **Continuing support.** Aftercare programs and support groups help people recovering from alcohol use disorder to stop drinking, manage relapses and cope with necessary lifestyle changes. This may include medical or psychological care or attending a support group.
- **Treatment for psychological problems.** Alcohol use disorder commonly occurs along with other mental health disorders. If you have depression, anxiety or another mental health condition, you may need talk therapy (psychotherapy), medications or other treatment.
- **Medical treatment for health conditions.** Many alcohol-related health problems improve significantly once you stop drinking. But some health conditions may warrant continued treatment and follow-up.
- **Spiritual practice.** People who are involved with some type of regular spiritual practice may find it easier to maintain recovery from alcohol use disorder or other addictions. For many people, gaining greater insight into their spiritual side is a key element in recovery.

Residential treatment programs

For a serious alcohol problem, you may need a stay at a residential treatment facility. Most residential treatment programs include individual and group therapy, support groups, educational lectures, family involvement and activity therapy.

Residential treatment programs typically include licensed alcohol and drug counselors, social

workers, nurses, doctors and others with expertise and experience in treating alcohol use disorder.

You'll need to focus on changing your habits and making different lifestyle choices.

- **Consider your social situation.** Make it clear to your friends and family that you're not drinking alcohol. Develop a support system of friends and family who can support your recovery. You may need to distance yourself from friends and social situations that impair your recovery.
- **Develop healthy habits.** For example, good sleep, regular physical activity, managing stress more effectively and eating well all can make it easier for you to recover from alcohol use disorder.
- **Do things that don't involve alcohol.** You may find that many of your activities involve drinking. Replace them with hobbies or activities that are not centered around alcohol.

Avoid replacing conventional medical treatment or psychotherapy with alternative medicine. But if used in addition to your treatment plan when recovering from alcohol use disorder, these techniques may be helpful:

- **Yoga.** Yoga's series of postures and controlled breathing exercises may help you relax and manage stress.
- **Meditation.** During meditation, you focus your attention and eliminate the stream of jumbled thoughts that may be crowding your mind and causing stress.
- **Acupuncture.** With acupuncture, hair-thin needles are inserted under the skin. Acupuncture may help reduce anxiety and depression.

Many people with alcohol problems and their family members find that participating in support groups is an essential part of coping with the disease, preventing or dealing with relapses, and staying sober. Your doctor or counselor can suggest a support group. These groups are also often listed on the Web and sometimes in the phone book.

Here are a few examples:

- **Alcoholics Anonymous.** Alcoholics Anonymous (AA) is a self-help group of people recovering from alcoholism that offers a sober peer group built around 12 steps as an effective model for achieving total abstinence.
- **Women for Sobriety.** Women for Sobriety is a nonprofit organization offering a self-help group program for women who want to overcome alcoholism and other addictions. It focuses on developing coping skills related to emotional and spiritual growth, self-esteem and a healthy lifestyle.
- **Al-Anon and Alateen.** Al-Anon is designed for people who are affected by someone else's alcoholism. Alateen groups are available for teenage children of those with alcoholism. In sharing their stories, family members gain a greater understanding of how the disease affects the entire family.

Early intervention can prevent alcohol-related problems in teens. If you have a teenager, be alert to signs and symptoms that may indicate a problem with alcohol:

- Loss of interest in activities and hobbies and in personal appearance
- Bloodshot eyes, slurred speech, problems with coordination and memory lapses
- Difficulties or changes in relationships with friends, such as joining a new crowd
- Declining grades and problems in school
- Frequent mood changes and defensive behavior

You can help prevent teenage alcohol use:

- Set a good example with your own alcohol use.
- Talk openly with your child, spend quality time together and become actively involved in your child's life.
- Let your child know what behavior you expect — and what the consequences will be if he or she doesn't follow the rules.

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Mannitol metabolism during pathogenic fungal–host interactions under stressed conditions

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Numerous plants and fungi produce mannitol, which may serve as an osmolyte or metabolic store; furthermore, mannitol also acts as a powerful quencher of reactive oxygen species (ROS). Some phytopathogenic fungi use mannitol to stifle ROS-mediated plant resistance. Mannitol is essential in pathogenesis to balance cell reinforcements produced by both plants and animals. Mannitol likewise serves as a source of reducing power, managing coenzymes, and controlling cytoplasmic pH by going about as a sink or hotspot for protons. The metabolic pathways for mannitol biosynthesis and catabolism have been characterized in filamentous fungi by direct diminishment of fructose-6-phosphate into mannitol-1-phosphate including a mannitol-1-phosphate phosphatase catalyst. In plants mannitol is integrated from mannose-6-phosphate to mannitol-1-phosphate, which then dephosphorylates to mannitol. The enzyme mannitol dehydrogenase plays a key role in host–pathogen interactions and must be co-localized with pathogen-secreted mannitol to resist the infection.

Keywords: Mannitol, reactive oxygen species (ROS), mannitol dehydrogenase (MTD), mannitol-1-phosphate-5-dehydrogenase (MPD), polyols

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Introduction

Mannitol, a six carbon non-cyclic sugar liquor, is a polyol commonly found in plants and fungi. In plant species, mannitol appears to be, in every way, to be the most expansive, being found in more than 70 families (Lewis and Smith, 1967; Ruijter et al., 2003). In plants, mannitol is made despite sucrose and is a phloem-translocated photoassimilate. Mannitol has various capacities in the plants including serving as a carbon stockpiling compound (Lewis, 1984), as a store of reducing power (Stacey, 1974; Loescher, 1987; Stoop and Pharr, 1992), as a compatible osmolyte (Brown and Simpson, 1972; Yancey et al., 1982) and in osmoregulation (Hellebust, 1976). Mannitol has similarly been shown to be an oxygen radical quencher both *in vitro* (Smirnoff and Cumbes, 1989) and *in vivo* (Shen et al., 1997a,b). Mannitol is the essential translocated sugar when the sucrose pool is drained (Davis and Loescher, 1990). It may moreover be incorporated in the utilization of photochemical impulses (Jennings et al., 2002).

Mannitol is the most widely recognized polyol in fungi, where it is found in spores, fruiting bodies, and mycelia (Solomon et al., 2007), and is considered to be the abundant most of all dissolvable starches inside mycelia and fruiting bodies (Lewis and Smith, 1967; Horer et al., 2001; Dulermo et al., 2009). In fungi, mannitol is a storage or a translocated carbohydrate, and

is essential in spore germination under starvation conditions (Horikoshi et al., 1965; Lewis and Smith, 1967; Dijkema et al., 1985; Witteveen and Visser, 1995). Mannitol additionally extinguishes reactive oxygen species (ROS; Smirnoff and Cumbes, 1989; Chaturvedi et al., 1997; Voegele et al., 2005), prompting the speculation that it can assume a cell reinforcement part in host–pathogen interactions. As a case in point, mannitol-deficient mutants of *Cryptococcus neoformans* are less harmful than the wild type strain, probably because of the way that mannitol ensures protection against oxidative executing by phagocytic cells (Chaturvedi et al., 1996a,b).

Mannitol production and secretion are required for the pathogenicity of several fungal pathogens of both animals and plants (Chaturvedi et al., 1996a,b). In infecting tobacco, *Alternaria alternata* secretes mannitol, which is induced by host leaf extracts (Jennings et al., 1998). This observation revealed that fungal pathogens secrete mannitol to quench the ROS that mediate plant defenses. In response, pathogen-induced mannitol dehydrogenase (MTD) in the plant catabolize the pathogen's secreted mannitol, thus protecting the plants ROS-mediated defenses. Mannitol deficient mutants of *A. alternata* created by target gene disruption had reduced pathogenicity on tobacco (Vélëz et al., 2007, 2008), confirming that mannitol production and secretion was a significant factor for pathogenicity of this fungus.

Mannitol Biosynthesis in Fungi

The metabolic pathway for mannitol biosynthesis and catabolism is well described in filamentous fungi. Mannitol metabolism in fungus is cyclical process (Hult and Gatenbeck, 1978). **Figure 1** depicts the pathways of mannitol synthesis in organisms. In this cycle, mannitol-1-phosphate 5-dehydrogenase (MPD; EC 1.1.1.17) was proposed to decrease Fructose-6-phosphate into mannitol-1-phosphate utilizing the NADH cofactor, processed by dephosphorylation by mannitol-1-phosphate phosphatase (MPP; EC 3.1.3.22), into an inorganic phosphate mannitol. Mannitol would then be oxidized to fructose by MTD (EC 1.1.1.138) utilizing the $NADP^+$ cofactor. At last, fructose would be phosphorylated to fructose-6-phosphate by a hexokinase (HX; EC 2.7.1.1). Dephosphorylation of mannitol-1-phosphate into mannitol by means of MPP was portrayed as being irreversible. Thus, the proposed cycle would run in one direction with MPD as a catabolic enzyme system (Calmes et al., 2013).

The physiological functions of mannitol in fungi have been widely studied, and sometimes just as widely disputed. Mannitol serves as a store carbon source and as reservoir of reducing power (Hult et al., 1980), and other functions of mannitol have been proposed that incorporate stress resilience (Ruijter et al., 2003) and spore dispersal (Trail and Xu, 2002). The mannitol biosynthetic mutants served as a very useful model when in tobacco inoculation experiments, mutants with reduced mannitol production demonstrated an equally parallel reduction in disease symptoms (Vélëz et al., 2007).

A few fungal systems build stress resistance by collecting mannitol. The conidia of the cereal pathogen *Gibberella zeae* were promptly changed to chlamydospore-like structures (CLS) in cultures supplemented with high measures of mannitol (Reyneri, 2006; Son et al., 2012). These morphological changes in CLS, mannitol amassing may be specifically identified with expanded CLS stress resistance. Numerous parasitic species amass mannitol in their hyphae and spores, up to 10–15% of the dry weight (Witteveen and Visser, 1995). It can be hypothesized, based on information gained from model species, that this mannitol will be important for spore germination or hyphal protein during pathogenesis.

Mannitol Biosynthesis in Plants

In higher plants more than 13 polyols are confirmed (Bieleski, 1982; Lewis, 1984), in which polyol mannitol may be the most wide spread, being found in >100 types of plants in 70 families (Lewis and Smith, 1967; Jennings, 1984; Stoop et al., 1996). **Table 1** lists plants that contain mannitol. In celery, mannitol assumes a huge part as an osmoprotectant and in addition interchange carbon and energy source (Loescher et al., 1995; Pharr et al., 1995a,b; Stoop et al., 1996; Nuccio et al., 1999; Loescher and Everard, 2000; Williamson et al., 2013). Mannitol is produced in higher plants from mannose-6-phosphate through the activity of a NADPH-mannose-6-phosphate reductase (M6PR). Mannose-6-phosphate is converted to mannitol-1-phosphate by the assistance of the M6PR enzyme, and then mannitol-1-phosphate is dephoshorylated by a phosphatase to mannitol (Rumpho et al., 1983; Loescher et al., 1992).

Figure 2 illustrates the pathways of mannitol metabolism in plants. In celery, mannitol synthesis occurs in mature leaves where M6PR is localized in the cytosol of green palisade and spongy parenchyma tissues and bundle-sheath cells (Everard et al., 1993; Loescher et al., 1995). M6PR activity is regulated by light and the development stage of the plant tissue. In mature leaves of celery, the M6PR activity is high but in sink tissues such as roots and unstressed, immature leaves, no M6PR activity is detected (Everard et al., 1993, 1997; Stoop and Pharr, 1994; Jennings et al., 2002).

Role of Mannitol and MTD during Plant–Pathogen Interactions

In fungi, mannitol plays a role in metabolism and a role in pathogenesis. In response to pathogen invasion, plants produce ROS in the extracellular space or apoplast for defense. In plants ROS are generated by a plasmalemma-embedded NADPH oxidase and/or a pH-dependent peroxidase (Bolwell and Wojtaszek, 1997). The ROS created in this "oxidative blast" serve different parts in the plant response. They serve as signals for the start of downstream resistance components, including the hypersensitive response (HR) and systemic acquired resistance

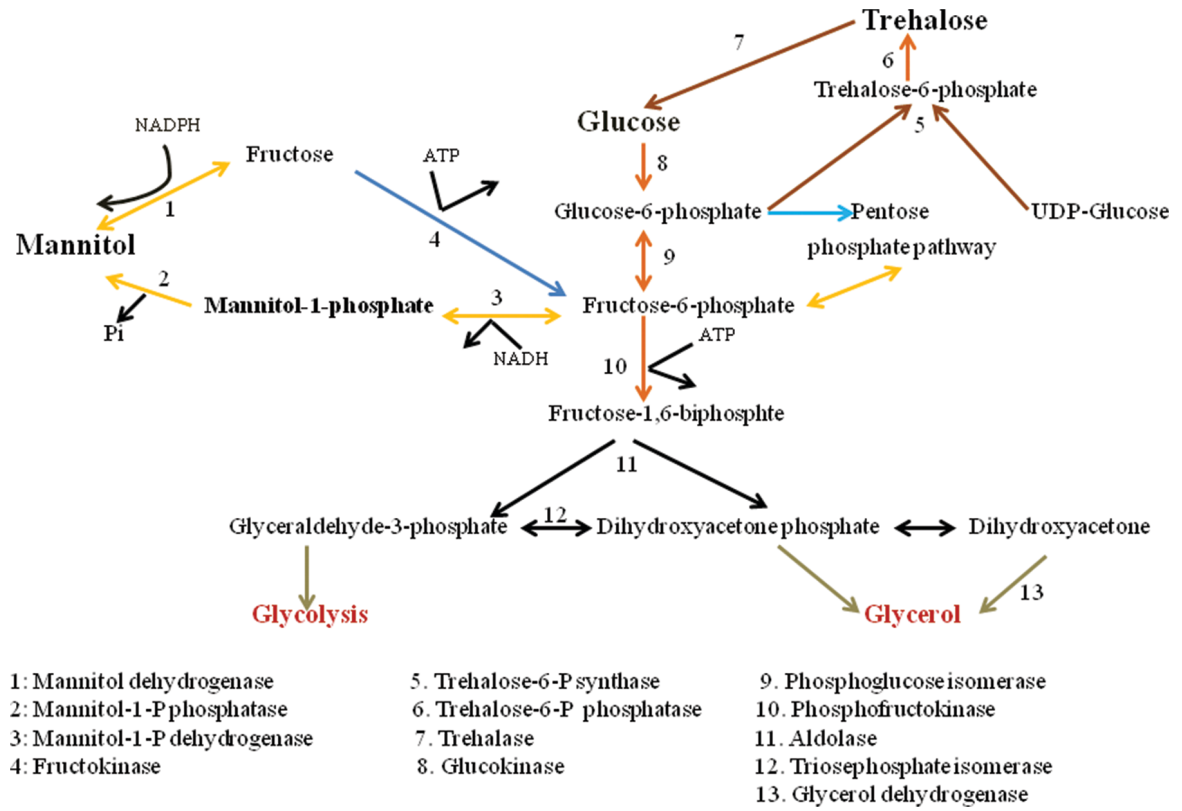

FIGURE 1 | Carbohydrate conversion and mannitol metabolic pathways in fungi.

(SAR). Some pathogens use strategies to circumvent these ROS-mediated defenses by the detoxification of ROS generated by host. It is reported that some plant and animal pathogenic fungi apparently use mannitol to detoxify ROS generated in the host environment.

The animal pathogen *C. neoformans* secretes large amounts of mannitol during the infection processes, and a mannitol low-producing mutant had reduced pathogenicity and oxidative stress tolerance (Chaturvedi et al., 1996a). The survival rate of *C. neoformans* was increased on addition of mannitol *in vitro* under the oxidative stress caused by ROS (Chaturvedi et al., 1996a,b). Virulent races of the tomato fungal pathogen *Cladosporium fulvum* produce and secrete mannitol during infection, while mutants unable to produce mannitol are non-pathogenic (Joosten et al., 1990). Mannitol secretion during the infection process is also reported in *Uromyces fabae* (a rust fungus), with mannitol accumulation in the apoplast paralleling high levels of fungal MTD activity (fungal mannitol biosynthesis) in the haustoria (Voegele et al., 2005). Fungal pathogen *A. alternata* also produces and secretes mannitol (Jennings et al., 1998) when treated with host plant (tobacco) extracts. Vélëz et al. (2008) reported that after treatment with the host extracts, the induction of genes for mannitol biosynthesis takes place and the pathogenicity was reduced in mannitol null mutants, which confirms that mannitol is a pathogenicity factor in fungi.

A key indication that mannitol may have a part in plant–pathogen communications came from the observation that the celery mannitol catabolic catalyst MTD was a pathogen-induced protein in celery (**Figure 3**; Williamson et al., 1995). Tobacco does not produce mannitol, MTD and corresponding protein and RNA accumulation is induced in fungus infected tobacco (Jennings et al., 1998).

Mannitol and MTD assume noteworthy parts in photosynthesis and in salt and oxidative stress resilience. Studies likewise implicate mannitol and MTD in plant–pathogen safeguards. To discover MTD in mannitol-containing plants (e.g., celery, parsley, and snapdragon), three non-mannitol plants (tobacco, tomato, and *Arabidopsis*) have been found to contain pathogen-incited MTD, where mannitol might act as a signal molecule (Jennings et al., 1999; Chan et al., 2011; Wyatt et al., 2014). Moreover, expression of a celery MTD in tobacco confers resistance to the mannitol secreting fungus *Alternaria*. Thus, MTD appears to represent a new class of resistance gene with potential for introducing increased fungal resistance in plants (Jennings et al., 1999).

Plants use ROS both as antimicrobial operators and as signal molecules to start differing safeguard reactions. Pathogens have developed numerous modes to sidestep these barriers. Pathogens incorporate mannitol, a ROS quencher, whose creation is fundamental for pathogenicity by such differing types as the tomato pathogen *Cladosporium* and the human pathogen

TABLE 1 | Representative plants that contain mannitol.

Family	Species	Tissue	References
Apiaceae	*Apium graveolens* (celeric and celery)	Leaf, Petiole,	Barker, 1955; Bourne, 1958
	Daucus carota (carrot)	Root	Pharr and Stoop, 1993
	Pastinaca sativa (parsnip)	Leaf, Petiole	Salmon et al., 1995
	Petroselinum crispum (parsley)		Obaton, 1929; Barker, 1955
	Oenanthe crocata (Hemlock Water dropwort)		Bourne, 1958
Arecaceae	*Cocos nucifera* (coconut)	Seedlings, Embryo	Bourne, 1958
Asteraceae	*Scorzonera hispanica* (black salsify or Spanish salsify)	Root, Seeds	Patkowska and Konopinski, 2008
Bromeliaceae	*Ananas comosus* (pineapple)		Pharr and Stoop, 1993
Brassicaceae	*Brassica oleracea* (cauliflower, cabbage)	Seed	Barker, 1955; Bourne, 1958
Bromeliaceae	*Ananas sativus* (pineapple)	Fruit	Bourne, 1958
Buxaceae	*Buxus sempervirens* (common boxwood)		Zimmerman and Zeigler, 1975
Cannaceae	*Canella winterana* (Canella or Barbasco)	Stem, Bark	Pharr and Stoop, 1993
Cactaceae	*Opuntia vulgaris*		Bourne, 1958
Combretaceae	*Laguncularia racemosa* (white mangrove)	Leaves	Zimmerman and Zeigler, 1975
	Terminalia arjuna (Arjuna)	Bark	Pharr and Stoop, 1993
	Terminalia chebula (Yellow Myrobalan)	Fruit	Pharr and Stoop, 1993
	Terminalia myriocarpa (Panisaj, Hollock)		
	Terminalia oliveri Brandis (Than)		
Compositae	*Lactuca sativa* (lettuce)		Bourne, 1958
Convolvulaceae	*Ipomoea purge* (Bindweed or Jalap)		
Cucurbitaceae	*Citrullus vulgaris* (watermelon)	Pericarp	Barker, 1955
	Cucurbita pepo (pumpkin, squash)	Fruit	Barker, 1955
Convolvulaceae	*Ipomea batatas* (sweet potato)	Root	
Euphorbiaceae	*Manihot utilissima* (cassava, manioc)		Zimmerman and Zeigler, 1975
Fabaceae	*Phaseolus vulgaris* (green been, French bean)		Barker, 1955; Bourne, 1958
	Pisum spp. (pea)		Zimmerman and Zeigler, 1975
	Spartium junceum (Spanish broom)		Barker, 1955; Bourne, 1958
	Cercis siliquastrum (Judas tree, Redbud)		
Gnetaceae	*Ephedra distachya*		Zimmerman and Zeigler, 1975
Gramineae	*Agropyron repens*		Zimmerman and Zeigler, 1975
	Andropogon annulatus		Zimmerman and Zeigler, 1975
Liliaceae	*Asparagus officinalis* (asparagus)		Barker, 1955
Oleaceae	*Forestiera acuminate* (swamp privet)	Bark, Leaf	Barker, 1955; Zimmerman and Zeigler, 1975
	Fraxinus americana (white ash)	Root, Wood	Bourne, 1958
	Fraxinus excelsior (European ash)	Leaf, Bark	Barker, 1955; Zimmerman and Zeigler, 1975
	Fraxinus ornus (flowering ash)	Leaf, Bark	Barker, 1955; Zimmerman and Zeigler, 1975
	Jasminum nudiflorum (winter jasmine)	Bark, Flower	Bourne, 1958; Zimmerman and Zeigler, 1975
	Jasminum officinale (poet's jessamine)	Leaf, Bark	Barker, 1955; Loescher et al., 1992
	Ligustrun vulgare (common privet)	Root, Stem	Barker, 1955; Zimmerman and Zeigler, 1975
	Olea europaea (olive)		Trip et al., 1965
	Olea glandulifera		
	Syringe vulgaris (common lilac)		
Plantanaceae	*Platanus orientalis* (oriental plane tree)	Stem, Bark, Leaves	Bourne, 1958
Rosaceae	*Prunus laurocerasus* (cherry, laurel)	Fruit	Bourne, 1958
Rubiaceae	*Coffea arabica* (coffee)	Seed	Bourne, 1958
	Gardenia (several species)		Barker, 1955; Bourne, 1958
Scrophulariaceae	*Veronica* (speedwell)	Leaf, Stem	Barker, 1955; Rumpho et al., 1983; Pedersen et al., 2007

Cryptococcus. The pathogen mannitol acts in extinguishing ROS and intervening with the formation of host barriers that ought to be more impervious to assault by the plants. MTD assumes a part in host–pathogen cooperations, must be colocalized with pathogen-emitted mannitol. In early stages of plant infection, the separation of infection structures and fungal protection against extracellular ROS created by oxidative burst were related with mannitol aggregation in hyphae and conidia, separately. Amid tissue colonization, albeit the fast change of plant sugars into mannitol in response to hyphae

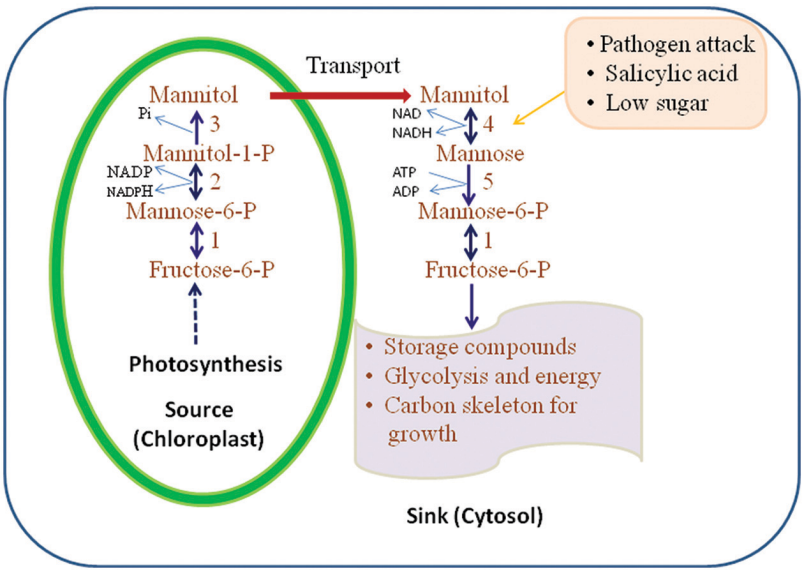

FIGURE 2 | Mannitol biosynthesis and catabolism as proposed in higher plants. 1, mannose; 6-P isomerise; 2, mannose-6-P reductase; 3, mannitol-1-phosphate dehydrogenase (M1PDA); 4, mannitol-1-dehydrogenase; 5, hexokinase (HX; adapted and modified from Stoop et al., 1996).

intrusion may not be specifically connected to necrosis, the polyol likely is involved in fungal protection against intracellular isothiocyanate-induced oxidative stress (Calmes et al., 2013).

In plants, polyols (mannitol or sorbitol) play a major role in sugar transport. Polyol/monosaccharide transporters (PMTs) are involved in phloem loading and unloading (Dusotoit-Coucaud et al., 2010). Polyols being a major sugar in the metabolism of some fungi (Solomon et al., 2007), PMTs have also been recommended to assume a part in plant–fungal interactions.

Mannitol Accumulation and Metabolism in Fungi

Fungi aggregate large amounts of polyols interacellulary, up to a several hundred millimoles every litre (Ruijter et al., 2003). *Aspergillus niger* creates various diverse polyols, including glycerol, erythriol, and D-mannitol (Witteveen and Visser, 1995). The production of the individual polyols in *A. niger* relies on the growth conditions and developmental stage. It recommends that polyols have important functions in fungal physiology. The hexitol D-mannitol is collected in numerous fungal species (Jennings, 1984). In *A. niger* conidiospores, D-mannitol is the prevalent carbon-containing compound and makes up 10–15% of the dry weight (Witteveen and Visser, 1995; Ruijter et al., 2003). The high amassings of mannitol in conidia of *A. niger* (Witteveen and Visser, 1995; Ruijter et al., 2003) and a few other organisms, for example, *Aspergillus oryzae* (Horikoshi et al., 1965), and *Aspergillus clavatus* (Corina and Munday, 1971; Ruijter et al., 2003), support a role in the survival of spores. During spore germination in *A. niger* mannitol is quickly metabolized, suggesting that it assumes a part in the capacity of carbon or reducing power (Witteveen and Visser, 1995; Ruijter et al., 2003). Correspondingly, in the basidiomycete *Agaricus bisporus* fruiting bodies, mannitol contributes up to half of the dry weight and is accepted to be a fundamental source of carbon that preserves the mushroom after harvest (Hammond and Nichols, 1975; Ruijter et al., 2003).

Mannitol is crucial for the security of spores against cell harm which happens under high temperature, drying, or increasing stress conditions. Mannitol creation is to avoid oxidative harm, for example, mannitol generation within the host by the human pathogen *C. neoformans* (Chaturvedi et al., 1996b) and secretion of mannitol by the phytopathogenic fungus *A. alternata* during plant disease (Jennings et al., 1998; Trontin et al., 2014). Interestingly, the plant increases MTD activity in light of parasitic contamination, probably for the evacuation of mannitol to balance the fungal protection mechanism (Jennings et al., 1998). In *A. niger,* mannitol is included in conidial stress resistance, especially oxidative and high-temperature stresses (Ruijter et al., 2003), and in *Stagonospora nodorum* the vicinity of mannitol is needed for asexual sporulation (Solomon et al., 2006). Since *A. niger* is a saprophyte and is pathogenic only in immuno-deficient people, it likely does not utilize mannitol generation for barrier amid development yet rather utilizes mannitol to guarantee maximal resilience of spores to survive unfavorable conditions. Numerous parasitic species aggregate trehalose and/or mannitol in their propagules, despite the fact that the levels differ (Dijksterhuis and Samson, 2002). Case in point, the trehalose level in *Aspergillus nidulans* spores is high compared to that in *A. niger* (Fillinger et al., 2001; Ruijter et al., 2003). Evidently, there is a species-particular inclination for mannitol or trehalose accumulation in conidia. In *A. nidulans* conidia, trehalose is imperative for long term spore survival and spore germination, proposing as a part of capacity carbon

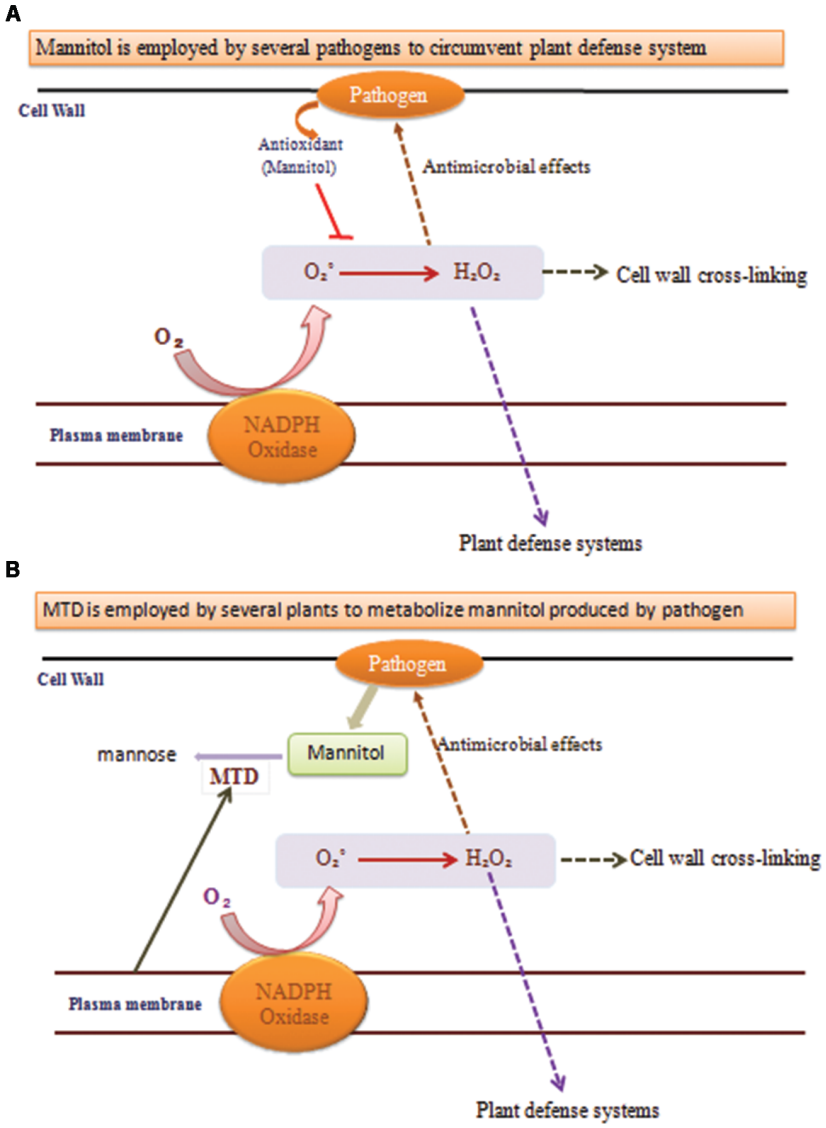

FIGURE 3 | Functions of mannitol and mannitol dehydrogenase (MTD) during plant–pathogen interactions. (A) When pathogens uses mannitol to circumvent plant defense system it quenches the ROS production and the processes marked with dotted arrows do not occur, leading to pathogen replication. **(B)** When plants use MTD to metabolize mannitol produced by pathogens then the processes marked with dotted arrows occur and provide resistance against the pathogen.

(Fillinger et al., 2001). The high mannitol level in *A. niger* conidia is not needed for the viability of conidiospores amid delayed stockpiling or spore germination, since inactivation of *mpdA* gene had no antagonistic impacts (Ruijter et al., 2003).

Accumulation of Mannitol in Plants

Abiotic Stress-imposed Mannitol Accumulation in Plants

In higher plants alditols and mannitol are osmolytes and solutes that provide resistance against various abiotic stresses (Hema et al., 2014). In a few plants mannitol and sorbitol collect in response to stress, working as osmolytes or compatible cytoplasmic solutes (Loescher, 1987). Changed tobacco plants encoding a quality mannitol-1-phosphate dehydrogenase (*mtlD*) brought about mannitol gathering, these changed tobacco plants survived, while non-changed plants were severely injured or killed when presented to 250 mol m^{-3} NaCl, indicating a function of mannitol in salt resilience (Tarczynski et al., 1993). Askari and Pepoyan (2012) reported that transgenic potato plants transformed with *mtlD* showed enhanced tolerance against salinity owing to the mannitol production due to *mtlD* activity. Similarly, a mannitol-synthesizing transgenic peanut plant has been shown to have high level of tolerance against salinity

and drought stresses attributing to the abiotic stress mitigating potentials of mannitol (Bhauso et al., 2014).

A positive connection between carbon apportioning into mannitol and salt anxiety was found in celery, which creates mannitol characteristically (Kann et al., 1993). Celery grown in hydroponic nutrient solution with salinity (equal to 30% sea water) showed dry weight gain equal to control plants grown at normal nutrient level. However, fresh weight gains under high salinity were reduced as compared to control. This indicates that water content of plants decreased as salinity increased but the total assimilatory ability was unaffected. Mannitol concentration is progressively increased when total salinity of the growth solution was increased. Mannitol enhances the development of transgenic wheat submerged and saltiness stress both at the callus and entire plant level (Tilahun et al., 2003). In tobacco, these finding are similar to use the *mtlD* gene (Tarczynski et al., 1992; Karakas et al., 1997) and same as *Arabidopsis* (Thomas et al., 1995). In transgenic wheat, the measure of mannitol collection was in the low end of the concentrations reported for tobacco and *Arabidopsis*.

Numerous plant species collect polyols and cyclitols in leaves because of water stress (Noiraud et al., 2000). Mannitol and sorbitol are the most ubiquitous polyols found in plants. Mannitol is synthesised in the cytoplasm from fructose-6-phosphate under drought conditions, and these polyols accumulate up to 80% of the total solutes involved in the osmotic adjustment process of some species, like peach and celery (Lo Bianco et al., 2000). Mannitol protects thiol-regulated enzymes (e.g., Phosphoribulokinase) against hydroxyl radicals, which are abundant during the oxidative stress process associated with water stress (Shen et al., 1997a). Under osmotic stress (salt- and water-stress driven) mannitol accumulation is attributable to a reduction in the catabolism of mannitol in green tissues (Stoop and Mooibroek, 1998). Mannitol production induced by water stress has been widely observed in plant species, e.g., tomatoes (Wang et al., 2000), sugarcane (Cha-um and Kirdmanee, 2008), rice, and sorghum (Cha-um et al., 2009).

In *Olea europaea*, mannitol works as an antioxidant osmoprotectants against oxidative stress coming about because of salt/dry spell push and even sun oriented irradiance (Melgar et al., 2009; Cimato et al., 2010). Mannitol aggregation has as of late been proposed to ensure salt-treated leaves in full daylight from heat stress incited oxidative stress to a more noteworthy degree than leaves developing under incomplete shading (Cimato et al., 2010; Artur et al., 2011). Production of mannitol is useful for *C. neoformans* to oppose other environmental stresses like as heat and osmotic stresses and the mutant of *C. neoformans* performed that at minimum levels of mannitol was more vulnerable to heat and osmotic stress (Al-Fakih, 2014).

Biotic Stress-imposed Mannitol Accumulation in Plants

The polyol mannitol extinguishes reactive oxygen species (ROS) both *in vitro* and *in vivo* (Smirnoff and Cumbes, 1989; Chaturvedi et al., 1997; Voegele et al., 2005). Studies have recommended that mannitol may be imperative in pathogenesis to cell reinforcement barriers by both plants and animals (Chaturvedi et al., 1996a,b). Jennings et al. (1998) estimated that pathogens secrete mannitol to extinguish ROS amid contamination of tobacco plants, in light of the fact that tobacco (a non-producer of mannitol) communicates a mannitol-debasing compound (MTD) when tested with parasitic elicitors and inducers of plant safeguard reactions. MTD changes over the pathogen-prompted mannitol to mannose, that is permitting the ROS intervened plant protection reaction compelling against the fungi. Transgenic tobacco plants that constitutively express MTD have increased resistance against *A. alternata* (Jennings et al., 2002). Mannitol combination happens with either sucrose blend, as in celery or with raffinose saccharide amalgamation, as in olive.

MTD as a Pathogen-response (PR) Protein

Mannitol dehydrogenase is one catalyst in the catabolism of mannitol. Mannitol is not found in all plants, but is a photosynthetic product in over 100 species in a number of diverse families (Loescher and Everard, 2000). The regulation of its primary catabolic enzyme MTD is quite complex, responding to factors including salts and simple sugars (Williamson et al., 2002). MTD has high amino acid sequence similarity to several pathogen-response (PR) proteins of unidentified function, and its expression is regulated by the endogenous PR-proteins inducers salicylic acid (SA) and hydrogen peroxide (H_2O_2; Williamson et al., 1995; Zamski et al., 2001; Jennings et al., 2002). Fungal pathogens secrete mannitol to quench the ROS that mediate plant defense responses. In response, pathogen-induced MTD in the plant might then catabolize the pathogen-secreted mannitol, thus protecting the plant's ROS-mediated defenses. MTD shows decreased activity in celery leaves exposed to high salinity, due to reduced amounts of MTD proteins (Stoop et al., 1995) and MTD transcripts (Williamson et al., 1995).

Mannitol is employed by the necrotrophic fungus *Botrytis cinerea* to overcome ROS toxicity induced during HR in plants thus making it to survive luxuriously on the necrotized dead tissue and lower plant growth and yield. Tomato plants overexpressing celery MTD owing to its PR-protein like activity exhibited enhanced resistance against *B. cinerea* (Patel et al., 2015). Also the secretion of MTD does not follow the established ER/Golgi pathway as for other PR proteins (Cheng et al., 2009; Cheng and Williamson, 2010).

Roles of Mannitol and Other Polyols

Osmoregulation

Polyol generation is a common feature during the growth of many organisms. In many filamentous fungi and particularly in yeasts, glycerol is the favored osmoprotectant. In the yeast *Saccharomyces cerevisiae,* glycerol contributes to the osmotic capability of the cell. Change of a *S. cerevisiae* mutant in glycerol combination with qualities for bacterial MPD and plant sorbitol-6-phosphate dehydrogenase brought about mannitol

and sorbitol creation. On the other hand, strains with mutations in mannitol/sorbitol synthesis were more sensitive to salt stress than mutants changed with the gpd1 quality as demonstrated by Shen et al. (1999). They inferred that polyol collection has two capacities, encouraging osmotic alteration and supporting redox control. In *A. nidulans,* glycerol and erythritol are the major polyols included in osmoregulation. The levels of glycerol, arabinitol, and mannitol were likewise followed in *C. fulvum*-infected tomato plants under ordinary and prohibitive watering regimens for a time of eight days (Clark et al., 2003). Mannitol and malic corrosive parts in the regulation of diurnal leaf water relations were compared in *"Biancolilla"* (high-mannitol) and *"Cerasuola"* (low-mannitol) olive trees. Mannitol was the most abundant polyol distinguished at low osmotic weight, while arabinitol levels amassed at higher osmotic weight, with glycerol having a transitory accumulation (Lo Bianco and Avellone, 2014).

Mannitol is likewise one of the essential osmolytes needed for producing turgor required for ascospore release, and MTD action was seen in both asexual and sexual formative stages (Trail et al., 2002; Min et al., 2010). *G. zeae* amasses sugar alcohols (glycerol, erythritol, arabitol, and mannitol), especially against matric water stresses (Ramirez et al., 2004). Since matric potential is the significant segment of the aggregate water potential in soil and oat crop buildups, sugar alcohols, for example, mannitol may accumulate in cells cultivated under submerged conditions. Results showed that mannitol supplement in medium instigated the transformation of conidia to CLS in *G. zeae,* and a few qualities are included in this conidial adjustment. Expanded CLS imperviousness to external stresses may be gained from metabolic changes, including the accumulation of mannitol, glycogen, lipids, and chitin.

Quencher of ROS

Mannitol and likely other sugar alcohols may be utilized to protect against ROS. Mannitol has been indicated to extinguish ROS both *in vivo* and *in vitro* (Smirnoff and Cumbes, 1989; Chaturvedi et al., 1997; Hema et al., 2014). ROS assume a significant part in pathogen resistance for both plants and animals. In animals, ROS are created by phagocytic leukocytes (macrophages/neutrophils; Rotrosen and Gallin, 1987), while in plants ROS are delivered by a NADPH oxidase confined in the plasmalamella layer (Grant and Loake, 2000). In plants, ROS have an administrative part in activating plant safeguards (e.g., lignin creation, phytoalexin production, lipid peroxidation, and the touch reaction) and additionally having antimicrobial effects (Baker and Orlandi, 1995). More confirmation that fungi use mannitol, to anticipate oxidative damage can be found in *A. alternata,* a parasitic pathogen of tobacco (*Nicotiana tabacum* L.). At the point when *A. alternata* was grown in culture medium and amended with tobacco extracts, an increment in mannitol levels and discharge was observed (Jennings et al., 1998).

Mannitol, which is made by the tomato pathogen *C. fulvum,* was found in intercellular liquids of tomato leaves infected with harmful races of *C. fulvum.* However, no mannitol was discovered if avirulent races were utilized (Joosten et al., 1990). Glucose and fructose from the plant were metabolized to mannitol by the parasite. The mannitol could then be utilized to provide energy during sporulation or could be translocated to the spores specifically (Joosten et al., 1990). Since mannitol is traded or filtered inactively into the apoplast, it could likewise have a part in ROS extinguishing.

Storage Carbohydrate

Mannitol is implied to have a part in fungal systems as a stockpiling sugar (Lewis and Smith, 1967). Ballio et al. (1964) separated glycerol, erythritol, arabitol, mannitol, and trehalose from conidia of *Penicillium chrysogenum.* Mannitol is likewise concentrated in sclerotia of *Sclerotinia sclerotiorum, Claviceps purpurea, Claviceps nigricans*, and *Sclerotinia curreyana* (Cooke, 1969). Mannitol is found in spores of *A. oryzae, Myrothecium verrucaria, Neurospora sitophila, Neurospora crassa, A. bisporus, Sterostratum corticoides, Puccinia coronata, Puccinia graminis* f. sp. *tritici, Erysiphe graminis* f. sp. *hordei*, and *A. clavatus* (Lewis and Smith, 1967). Mannitol in the conidia of *A. oryzae* is metabolized at an early stage amid germination (Horikoshi et al., 1965). Horikoshi et al. (1965) concluded that mannitol was being utilized as the carbon hotspot for endogenous breath during the first ventures of germination, which was later managed by glucose.

In mycorrhiza, mannitol serves as sink for the translocation and storage of carbohydrate, which probably is not accessible to the host plant (Lewis and Smith, 1967; Koide et al., 2000; Calmes et al., 2013). This concept has also been applied to interaction between the fungus *C. fulvum* and tomato (*Lycopersicon esculentum* L.).

Regulating Cofactors

Hult and Gatenbeck (1978) proposed that the mannitol cycle could be utilized to control the coenzymes NADH and $NADP^+$, that is, an approach to produce NADPH to the detriment of NADH and ATP with every turn of the cycle. However, studies with the parasite *A. nidulans,* gave no support to the operation of the mannitol cycle or for NADPH generation in this fungus (Singh et al., 1988). As indicated by Singh et al. (1988) *A. nidulans* cultivated on NO_3 as a nitrogen source would build the interest for NADPH, and accordingly create an increment in the maximal particular exercises of the enzyme in the mannitol cycle.

MTDs of the several bacterial and fungal pathogens are mannitol 2-oxidoreductases which produce fructose in the forward reaction of mannitol cycle by using either NAD^+ (EC 1.1.1.67) or $NADP^+$ (EC 1.1.1.138) as a cofactor (Voegele et al., 2005). In Basidiomycetes (Hult et al., 1980), mannitol-1-P dehydrogenase seems to be absent, so mannitol is possibly formed by direct reduction of fructose through a MTD (EC 1.1.1.67 or EC 1.1.1.138). NAD^+-dependent MTD activity is reported in *P. graminis* f. sp. *tritici* axenic cultures (Maclean, 1990), and Clancy and Coffey (1980) have also shown NAD^+- and $NADP^+$- dependent MTD activity in *Melampsora lini* axenic cultures and uredospores (Voegele et al., 2005).

Regulation of pH

Jennings (1984) suggested that polyols could be utilized by fungus to control their pH. Polyols could achieve this by serving as sinks or hotspots for protons, as the polyols are made (decreased) or oxidized into different starches. Ribitol accumulation occurred after mannitol consumption and during unsaturated fatty-acid degradation when a hydrogen-acceptor may be needed. At the point when the salt stress is discharged, the convergance of Cl^- *in vivo* diminishes (Mostaert et al., 1995), and that of fructose 6-P would be expanded by the mannitol-catabolizing pathway with MTD and HX (Karsten et al., 1997a,b; Iwamoto et al., 2003). Regulation of mannitol turn over may allow the quick reaction to a change in the natural salt situation. Since fructose 6-P is metabolically at a limb point along the mannitol pathway including glycolysis and reductive and oxidative pentose phosphate cycles, the biosynthesis of mannitol by means of M1P could likewise be controlled by the supply of F6P (Iwamoto et al., 2003). F6P could hence work as a key control component for mannitol metabolism, in addition to the regulation of M1PDH action by NaCl.

Morphogenesis and Conidiation

Mannitol is needed for vegetative sporulation in *S. nodorum,* both *in planta* and *in vitro* (Solomon et al., 2006, 2007). Utilizing mutant strains that are not able to metabolize mannitol, the study found that subculturing of these strains on media without the polyol brought about the suspension of asexual sporulation.

The requirement of mannitol for completion of the life cycle of ascomycetes and morphogenesis in basidiomycetes is also thought to be dependent on the metabolism of mannitol, as indicated by an increase in both MTD and fructose 6-phosphate dehydrogenase activity during fruiting body development in *A. bisporus* (Stoop and Mooibroek, 1998). Mannitol is also accumulated in the unsporulated oocysts in the avian parasite *Eimeria tenella*. Mannitol enables sporulation of the oocysts outside of the host by functioning as the endogenous carbon and energy source (Allocco et al., 1999). Mannitol has been shown to be a vital factor against heat and oxidative stress in *Neosartorya fischeri* for ascospore development as well as stress resistance of conidia (Wyatt et al., 2014).

Future Prospects

It is clear that mannitol can play an important role in plant growth and responses to diverse biotic and abiotic stresses. Possible future of mannitol for osmoprotection, efficient growth and resistance to pathogens, the engineering of plants with mannitol metabolism is a desirable goal. There is a discriminating issue, as mannitol use in sink tissues is spatially divided from its union in experienced photosynthetic tissues. There is little knowledge about the cellular and sub-cellular location of MTD or the control of MTD expression in response to environmental and metabolic factors. It is crucial to study the mannitol transport, enzyme localization and transcriptional and post-transcriptional regulation of MTD expression. Mannitol and the regulation of its production and degradation study in plants, animals and fungi is a vast topic for future research.

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复方甘草酸苷改善酒精性肝病患者肝功能的Meta分析

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摘 要 目的：系统评价复方甘草酸苷对酒精性肝病（ALD）患者肝功能的影响。方法：计算机检索Pubmed、中国期刊全文数据库和万方数据库，收集复方甘草酸苷治疗ALD的随机对照试验（RCT），同时辅以手工检索，评价纳入研究质量，合并结果进行Meta分析。结果：共纳入12项RCT，合计838例患者。Meta分析结果显示，试验组治疗后丙氨酸氨基转移酶[MD=-42.70，95%CI（-52.71，-32.69），$P<0.01$]、天冬氨酸氨基转移酶[MD=-47.93，95%CI（-62.77，-33.09），$P<0.01$]和γ-谷氨酰转肽酶[MD=-92.32，95%CI（-99.05，-85.60），$P<0.01$]水平均显著低于对照组。结论：复方甘草酸苷能够显著改善ALD患者肝功能。

关键词 复方甘草酸苷；酒精性肝病；肝功能；Meta分析

Effects of Compound Glycyrrhizin on Liver Function in Patients with Alcoholic Liver Disease: a Meta-analysis

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ABSTRACT OBJECTIVE: To evaluate the effects of Compound glycyrrhizin on liver function in patients with alcoholic liver disease. METHODS: Retrieved from Pubmed, CNKI and Wanfang database, randomized controlled trials (RCTs) about Compound glycyrrhizin in the treatment of alcoholic liver disease were collected, supplementing literature recourse retrieval. Quality evaluation and Meta-analysis of included literatures were conducted. RESULTS: A total of 12 RCTs were included, involving 838 cases. Meta-analysis results showed that the levels of alanine aminotransferase [MD=-42.70, 95%CI(-52.71, -32.69), $P<0.01$] and aspartate aminotransferase [MD=-47.93, 95%CI(-62.77, -33.09), $P<0.01$] and γ-glutamyl transpeptidase [MD=-92.32, 95%CI (-99.05, -85.60), $P<0.01$] in experimental group was lower than control group after treatment. CONCLUSION: Compound glycyrrhizin can significantly improve liver function in alcoholic liver disease.

KEY WORDS Compound glycyrrhizin; Alcoholic liver disease; Liver function; Meta-analysis

酒精性肝病（Alcoholic liver disease，ALD）在发达国家较为常见，在我国患病率也有上升趋势[1]。对ALD患者进行积极治疗，减少肝损伤，并加以健康教育，对该病的预后具有重要的意义。临床研究发现，复方甘草酸苷无论对ALD还是非ALD，均有显著的疗效，但单项临床研究样本量较低。本研究旨在通过Meta分析系统评价复方甘草酸苷对ALD患者肝功能改善的整体效果，以为临床用药提供参考。

1 资料与方法

1.1 文献纳入/排除标准

纳入标准：文献研究类型为随机对照试验（RCT）；语种限制为中文和英文；试验组患者接受常规护肝治疗联用复方甘草酸苷，对照组患者采用常规治疗；ALD的诊断符合中华医学会肝脏病学分会制定的《酒精性肝病诊断标准》[2]；文献中可提取两组治疗前后丙氨酸氨基转移酶（ALT）、天冬氨酸氨基转移酶（AST）和γ-谷氨酰转肽酶（γ-GT）的肝功能水平。排除标准：研究对象为动物实验；疑似重复发表的文献；无法提取所需数据的文献。

1.2 检索策略及文献质量评价[3]

以主题词“复方甘草酸苷”、“酒精性肝病”检索中国期刊全文数据库（CNKI，1999-2012.07）和万方数据库（1990-2012.07），以主题词“Ambroxol Hydrochloride”、“secretory otitis media”检索Pubmed数据库（由建库至2012年5月16日），同时辅以手工检索法。文献质量采用Jadad质量记分法进行[4]评分，1～2分为低质量文献，3～5分为高质量文献。

1.3 统计学方法

采用Rev Man 5.1软件进行Meta分析，异质性检验采用q

[22] Ridker PM, Rifai N, Pfeffer MA, *et al*. Long-term effects of pravastatin on plasma concentration of C-reactive protein. The Cholesterol and recurrent events (CARE) investigators[J]. *Circulation*, 1999, 100(3): 230.

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检验，同时使用I^2进行异质性定量分析，若存在异质性，则采用随机效应模型进行分析；反之，则采用固定效应模型进行分析。连续变量资料采用均数差（MD）及其95%可信区间（CI）表示。发表偏倚检验通过倒漏斗图进行。

2 结果

2.1 文献检索结果

共检索文献28篇，均为中文文献，通过阅读摘要及全文，并按照文献纳入标准，最终共纳入12项RCT，合计838例患者进行本次Meta分析。

2.2 纳入研究的一般特征及方法学评价

纳入的RCT样本量最大为94例，最小为40例，纳入研究的一般特征详见表1。文献的偏倚评估从随机分配方案的产生、分配方案是否隐藏、是否采用盲法、是否有退出和失访4个方面进行。

表1 纳入研究文献一般特征及文献质量评价

Tab 1 General characteristics and quality evaluation of included literature

第一作者	发表年份	例数		年龄，岁	干预措施		结局指标
		对照组	试验组		对照组	试验组	
黎红光[5]	2006	46	46	18～70	给予还原型谷胱甘肽、天冬氨酸钾镁、维生素及氨基酸类药物，疗程为4周	在对照组基础上加用40～80 ml复方甘草酸苷注射液，疗程共4周	①②③
彭于仑[6]	2007	20	20	55～68	给予还原型谷胱甘肽1.2 g，疗程为2周	在对照组基础上加用80 ml复方甘草酸苷注射液，疗程共2周	①②③
郭莲怡[7]	2007	30	30	42～75	戒烟，口服多种维生素，静脉滴注维生素C，疗程为1个月	戒烟，口服多种维生素，给予60 ml复方甘草酸苷注射液，疗程共1个月	①②③
张美稀[8]	2008	45	45		硫普罗宁注射液0.2 g，每日1次，连续4周后改用硫普罗宁片，1次200 mg，1日3次，连续8周	给予60 ml复方甘草酸苷注射液，静脉滴注，每日1次，连续4周后改为复方甘草酸苷片，每次2片，1日3次，连续8周	①②③
董晓艳[9]	2008	30	35	28～62	戒烟，运动，调节饮食结构，疗程为12周	给予75 mg复方甘草酸苷注射液静脉滴注，每日1次，连续12周	①②③
郑学峰[10]	2008	18	22	42～75	戒烟，口服多种维生素，疗程为3个月	戒烟，口服多种维生素，给予100 mg复方甘草酸苷注射液静脉滴注，每日1次，疗程为3个月	①②③
马颖光[11]	2009	46	48	21～78	给予天冬氨酸钾镁、维生素及氨基酸类药物，疗程为4周	在对照组基础上，加用60 ml复方甘草酸苷注射液+20 ml丹参注射液，疗程为4周	①②③
索日娜[12]	2010	30	30	27～70	常规给予维生素，葡醛内酯片0.2 g口服，水飞蓟素4粒口服，每日3次，疗程为4周	在对照组基础上，加用60 ml复方甘草酸苷注射液，每日1次，疗程为4周	①②③
康惠东[13]	2010	31	31	30～60	常规给予保肝治疗，同时给予葡醛酸钠注射液0.266 g每日1次，疗程为2周	在对照组基础上，加用40 ml复方甘草酸苷注射液，每日1次，疗程为2周	①②③
袁索红[14]	2010	46	46		戒烟，口服多种维生素及支持治疗，疗程为4周	戒烟，口服多种维生素，加用40 ml复方甘草酸苷注射液，每日1次，疗程为4周	①②③
刘卫军[15]	2011	29	36	22～67	常规给予天冬氨酸钾镁、维生素及氨基酸类药物，疗程为4周	在对照组基础上，加用60 ml复方甘草酸苷注射液+还原型谷胱甘肽1.8 g，疗程为4周	①②③
安良敏[16]	2012	36	45	12～71	常规给予天冬氨酸钾镁、维生素及氨基酸类药物，疗程为3个月	在对照组基础上，加用60 ml复方甘草酸苷注射液+还原型谷胱甘肽1.8 g，疗程为3个月	①②③

注：①丙氨酸氨基转移酶；②天冬氨酸氨基转移酶；③γ-谷氨酰转肽酶

note：①alanine transferase enzyme；②aspartate aminotransferase enzyme；③γ-glutamyl

2.3 Meta分析结果

2.3.1 ALT水平 12项研究报道了治疗后的ALT水平，各研究间有异质性（$P<0.01$），故采用随机效应模型进行分析，详见图1。Meta分析结果显示，两组治疗后ALT水平比较差异有统计学意义[MD=－42.70，95%CI（－52.71，－32.69），$P<0.01$]，提示复方甘草酸苷能够显著改善ALD患者ALT水平。

2.3.2 AST水平 12项研究报道了治疗后的AST水平，各研究间有异质性（$P<0.01$），故采用随机效应模型进行分析，详见图2。Meta分析结果显示，两组患者治疗后AST水平比较差异有统计学意义[MD=－47.93，95%CI（－62.77，－33.09），$P<0.01$]，提示复方甘草酸苷能够显著改善ALD患者AST水平。

2.3.3 γ-GT水平 12项研究报道了治疗后的γ-GT水平，各研究间有异质性（$P<0.01$），故采用随机效应模型进行分析，详见图3。Meta分析结果显示，两组患者治疗后γ-GT水平比较差异有统计学意义[MD=－92.32，95%CI（－99.05，－85.60），$P<0.01$]，提示复方甘草酸苷能够显著改善ALD患者γ-GT水平。

2.4 发表偏倚

对报道了ALT水平的12项研究进行倒漏斗图分析，详见图4。图4左右对称性较差，提示存在发表偏倚风险。

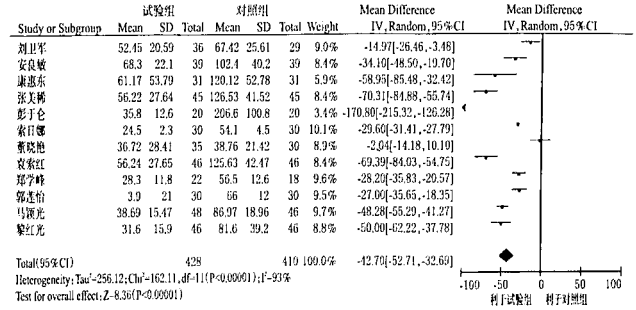

图1 两组患者治疗后ALT水平的Meta分析森林图

Fig 1 Forest plot of Meta-analysis of ALT levels in 2 groups after treatment

3 讨论

我国尚缺乏全国性大规模ALD研究的流行病学调查资料，但部分地区的流行病学调查显示，ALD发病率呈逐年上升趋势[17]。我国女性比男性易患ALD，中国人群ALD以酒精性肝炎为主，而西方国家的ALD则以酒精性脂肪肝为主，两者之间具有显著差异。ALD可能与饮食习惯和生活方式有关，随着人们生活水平的改变，社交和应酬的增加，以及酒文化的发

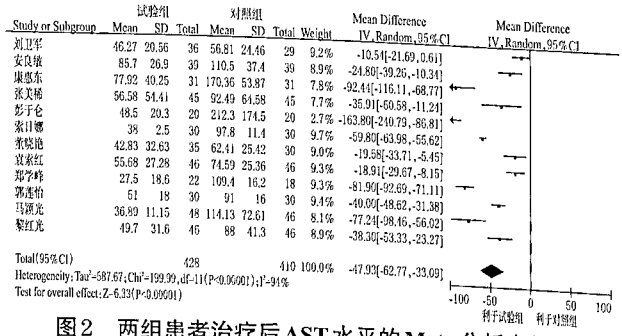

图2 两组患者治疗后AST水平的Meta分析森林图

Fig 2 Forest plot of Meta-analysis of AST levels in 2 groups after treatment

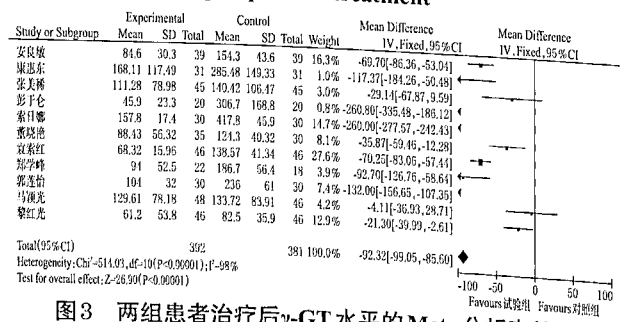

图3 两组患者治疗后γ-GT水平的Meta分析森林图

Fig 3 Forest plot of Meta-analysis for γ-GT levels in 2 groups after treatment

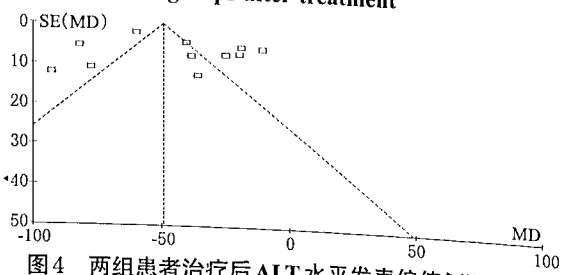

图4 两组患者治疗后ALT水平发表偏倚倒漏斗图

Fig 4 Funnel plot of publication bias for ALT levels in 2 groups after treatment

展，酒精滥用(Alcohol abuse)和酒精依赖(Alcohol dependence)已成为当今世界上日益严重的公共卫生问题[17-18]。对患者进行健康教育尤为重要，患者同时进行保肝治疗，对改变该病预后具有重要的意义。临床也不断探索新的用药组合，以探讨联合用药的效果。本次Meta分析提示，复方甘草酸苷对ALD患者肝功能具有显著的改善，ALT和AST水平经治疗后显著降低。

本次Meta分析进行发表偏倚分析时发现，“倒漏斗图”左右对称较差，提示存在阴性结果未发表的可能。本次研究纳入文献虽然表明采用随机方案，但随机方案的具体方法无从得知，因此无法评估选择性发表偏倚。同时，研究未纳入未发表的文献，根据文献质量评分标准，文献评分普遍较低。但本次研究将文献资料的一般特征进行详细描述，包括用药方式、年龄范围、样本量以及干预措施进行报道，使研究结果更加具有代表性，克服了样本量较小带来的偏倚。

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生脉注射液联合化疗治疗非小细胞肺癌的系统评价

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摘　要　目的：系统评价生脉注射液联合化疗治疗非小细胞肺癌的疗效。方法：计算机检索Pubmed、Cochrane library、EMbase、SCI、中国期刊全文数据库、中国生物医学文献数据库、万方数据库及维普数据库，并对纳入的文献进行严格的质量评价和Meta分析。结果：共纳入9项研究，合计585例患者。两组患者总有效率[OR=1.67，95%CI(1.20，2.33)，$P<0.01$]、生活质量量表(KPS)评分增加发生率[OR=3.33，95%CI(2.27，4.88)，$P<0.01$]、体质量增加发生率[OR=4.85，95%CI(2.85，8.24)，$P<0.01$]、骨髓抑制发生率[OR=0.39，95%CI(0.27，0.56)，$P<0.01$]、肝肾损害发生率[OR=0.31，95%CI(0.14，0.68)，$P<0.01$]、胃肠道反应发生率[OR=0.49，95%CI(0.33，0.73)，$P<0.01$]比较差异均有统计学意义，但总有效率分析纳入的研究可能存在发表偏倚。结论：生脉注射液联合化疗治疗非小细胞肺癌，在提高患者生存质量，降低化疗相关不良反应方面效果确切，且对化疗有协同增效作用，但尚需更多的研究加以证实。

关键词　生脉注射液；非小细胞肺癌；Meta分析

Systematic Review of Shengmai Injection Combined with Chemotherapy for Non-small Cell Lung Cancer

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ABSTRACT　OBJECTIVE：To evaluate the therapeutic efficacy of Shengmai injection combined with chemotherapy for non-small cell lung cancer (NSCLC). METHODS：Retrieved from Pubmed，Cochrane library，EMbase，SCI，CNKI，CBM，Wanfang database and VIP database，the quality of selected literatures was evaluated and Meta-analysis was conducted. RESULTS：A total of 9 studies were included，involving 585 patients. There were statistically significant differences in total effective rate[OR=1.67，95%CI (1.20，2.33)，$P<0.01$]，increase of KPS[OR=3.33，95% CI (2.27，4.88)，$P<0.01$]，increase of weight[OR=4.85，95% CI (2.85，8.24)，$P<0.01$]，rate of myelosuppression[OR=0.39，95% CI (0.27，0.56)，$P<0.01$]，liver and kidney damage[OR=0.31，95% CI (0.14，0.68)，$P<0.01$]，gastrointestinal reaction[OR=0.49，95% CI (0.33，0.73)，$P<0.01$]. However，publication bias may exist in the analysis of total effective rate. CONCLUSION：Shengmai injection plays good role in improving QOL and reducing chemotherapy related adverse reaction. Meanwhile，it may have synergistic effect with chemotherapy on NSCLC，which is well confirmed by more available evidence.

KEY WORDS　Shengmai injection；NSCLC；Meta-analysis

化疗是恶性肿瘤重要治疗手段之一[1]。非小细胞肺癌患者多数被发现时已属于中晚期，患者失去手术治疗的机会而需要接受化疗[2-4]。而化疗常导致患者胃肠道恶心、呕吐、骨髓抑制、体质量减轻，甚至由于感染、精神压力等不能坚持化疗，从而影响疗效和生存质量。中医中药在稳定癌灶、调节机体功能、提高免疫能力、改善临床症状、增加食欲、减轻放疗和化疗毒副作用及延长带瘤生存时间等方面有其独特的作用。生脉注射液是由人参、麦冬、五味子配伍而成的可供静脉注射的中药注射剂[5]。目前，生脉注射液越来越广泛地应用于临床。本研究旨在对生脉注射液联合化疗治疗非小细胞肺癌进行系统评价，以为临床合理用药提供依据。

1　资料与方法

1.1　纳入与排除标准

1.1.1　纳入标准　①研究对象：经病理学或细胞学诊断为非小细胞肺癌的住院患者，预计生存期≥3个月，化疗前血常规、肝肾功能正常。②干预措施：试验组为生脉注射液+常规化疗，对照组为常规化疗。③结局指标：主要结局指标为总有效率，次要结局指标为生存质量，包括生活质量量表(KPS)评分和体质量变化。④不良反应：骨髓抑制、肝肾损害和胃肠道反应。⑤研究类型：临床随机对照试验(RCT)。

1.1.2　排除标准　①非化疗方案治疗非小细胞肺癌。②生脉注射液联用其他阳性药物，但对照组没有用此阳性药物进行对照的用药方案。

1.2　文献检索

计算机检索Pubmed、Cochrane library、EMbase、SCI、中国期刊全文数据库(CNKI)、中国生物医学文献数据库(CBM)、万方数据库及维普数据库，中文检索词："生脉注射液"、"非小细胞肺癌"、"肿瘤"等；英文检索词："Shengmai Injection"、"non-small cell lung cancer"、"NSCLC"等。检索年限均为从建库时起至2012年6月。

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还原型谷胱甘肽联合异甘草酸镁治疗酒精性肝病的对照研究

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摘 要 目的:观察还原型谷胱甘肽片联合异甘草酸镁注射液静脉滴注治疗酒精性肝病的临床疗效。方法:60例酒精性肝病患者均戒酒,随机分为治疗组(30例)和对照组(30例)。治疗组给予还原型谷胱甘肽片联合异甘草酸镁注射液静脉滴注,对照组仅给予异甘草酸镁注射液静脉滴注,疗程均为4周。结果:2组患者治疗后临床症状、体征、实验室检查及彩超检查结果均有一定程度的改善,且治疗组疗效优于对照组,2组比较差异有统计学意义($P<0.05$)。结论:还原型谷胱甘肽片联合异甘草酸镁注射液治疗酒精性肝病疗效好,未见明显不良反应。

关键词 还原型谷胱甘肽片;异甘草酸镁;酒精性肝病

Therapeutic Efficacy of Reduced Glutathione Combined with Magnesium Isoglycyrrhizinate for Alcoholic Liver Disease

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ABSTRACT OBJECTIVE: To observe the clinical efficacy of oral dose of reduced glutathione combined with dripping injection of magnesium isoglycyrrhizinate for alcoholic liver disease. METHODS: A total of 60 patients with alcoholic liver disease who had been abstained from wine were randomly assigned to treatment group and control group (30 cases). Treatment group was given oral dose of reduced glutathione and intravenous dripping injection of magnesium isoglycyrrhizinate for 4 weeks. Control group was treated with intravenous dripping injection of magnesium isoglycyrrhizinate alone for 4 weeks. RESULTS: Clinical symptoms and signs, laboratory examination and B-ultrosound results were all improved in both groups, especially in the trial group. Significant differences were noted between the 2 groups ($P<0.05$). CONCLUSION: Oral dose of reduced glutathione combined with intravenous dripping injection of magnesium isoglycyrrhizinate is safe and effective for alcoholic liver disease without obvious adverse drug reaction.

KEY WORDS Reduced glutathione tablet; Magnesium isoglycyrrhizinate; Alcoholic liver disease

酒精性肝病(Alcoholic liver disease ,ALD)是因长期、大量饮用含乙醇的饮料所致的肝脏损害性病变。初期表现为酒精性脂肪肝,进而可发展成酒精性肝病,最后为酒精性肝硬化,严重危害人类健康。本病在欧美国家多见,在我国随着生活水平的提高,近年来的发病率也迅速升高[1]。我院采用还原型谷胱甘肽片联合静脉滴注异甘草酸镁注射液治疗酒精性肝病患者30例,取得较满意的疗效,现报道如下。

1 资料与方法

1.1 一般资料

60例ALD患者均为2007年7月—2009年7月在我院住院的患者,诊断均符合中华医学会肝脏病学分会脂肪肝和酒精性肝病学组制定的诊断标准[2]。排除病毒性肝炎、自身免疫性疾病、中毒性肝损伤等。全部病例随机分为2组,治疗组30例,其中男性27例,女性3例,平均年龄(40.3±10.6)岁。对照组30例,其中男性28例,女性2例,平均年龄(39.7±11.2)岁。全部患者均有程度不等的乏力、纳差及肝区不适,2组年龄、性别、病情、肝功能指标差异无统计学意义($P>0.05$),具有可比性。

1.2 治疗方法

2组均以戒酒为治疗前提,低脂饮食。2组均常规给予门冬氨酸钾镁、多种维生素。治疗组应用还原型谷胱甘肽片(阿拓莫兰,重庆药友制药有限责任公司),400 mg,tid,po;异甘草酸镁注射液(天晴甘美,江苏正大天晴药业股份有限公司)200 mg加入5%葡萄糖注射液250 mL中,静脉滴注,qd。对照组仅应用异甘草酸镁注射液200 mg加入5%葡萄糖注射液250 mL中,静脉滴注,qd。2组疗程均为4周。疗程结束后复查肝功能及肝脏彩超。治疗前、后检测患者的肝功能指标,包括丙氨酸氨基转移酶(ALT)、天冬氨酸氨基转移酶(AST)、直接胆红素(DBIL)、γ-谷氨酰转肽酶(GGT)、总胆红素(TBIL)。

1.3 疗效评价标准

治疗4周后评定疗效。临床疗效判断标准[3]:治愈,临床症状、体征消失,肝功能正常,彩超示无特异性表现;显效,临床症状、体征基本消失,肝功能基本正常,彩超示特异性表现明显改善;有效,临床症状、体征减轻,肝功能较治疗前好转,彩超示特异性表现有所改善;无效,临床症状、体征、肝功能及彩超检查等均无变化。各阶段ALD彩超表现为酒精性脂肪肝:肝回声细密增强,前场回声增强,后场回声衰减。酒精性肝病:肝实质增强、增粗,但回声衰减不明显。酒精性肝硬化:实质回声增强、增粗,并伴有门静脉增宽,脾脏增大、腹水及胆囊壁水肿等[4]。

1.4 统计学方法

采用SPSS 15.0软件进行统计学分析。试验数据用$\bar{x}\pm s$

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表示，等级资料采用非参数秩和检验，配对的计量资料采用t检验，多组间均数比较用方差分析。$P<0.05$表示差异有统计学意义。

2 结果

2.1 2组疗效比较

除对照组2例患者治疗后无明显变化外，治疗组及其余对照组患者治疗后乏力、食欲减退、腹胀、肝区疼痛等临床症状均有改善，体征、肝功能及彩超检查结果均有改善。2组疗效比较，治疗组优于对照组，差异有统计学意义（$Z=-2.243$，$P=0.025$），详见表1。

表1 2组疗效比较（n）

Tab 1 Comparison of therapeutic efficacies between 2 groups（n）

组别	例数	治愈	显效	有效	无效
治疗组	30	19	7	4	0
对照组	30	12	6	10	2

2.2 2组治疗前后肝功能指标比较

2组患者治疗后检测肝功能指标较治疗前均有较大程度的改善（$P<0.01$）。2组间比较，治疗组患者的ALT、AST、DBIL、GGT降低优于对照组，差异有统计学意义（$P<0.01$），但TBIL下降水平2组差异无显著性（$P=0.576$），详见表2。

表2 2组治疗前后肝功能指标比较（$\bar{x}\pm s$）

Tab 2 Comparison of liver function index between two groups（$\bar{x}\pm s$）

组别		ALT/U·L^{-1}	AST/U·L^{-1}	TBIL/μmol·L^{-1}	DBIL/μmol·L^{-1}	GGT/U·L^{-1}
治疗组	治疗前	116.01±2.41	126.80±2.55	34.60±1.71	14.67±1.51	194.67±2.01
	治疗后	37.73±2.53	44.23±3.02	20.83±1.57	7.37±1.35	72.07±3.55
对照组	治疗前	114.53±19.81	118.03±3.75	28.70±1.60	15.47±3.07	202.03±1.79
	治疗后	55.57±2.11	67.63±1.83	20.26±1.33	9.13±1.07	85.40±1.61

2.3 不良反应

治疗期间，2组患者生命体征平稳，未见明显的不良反应。

3 讨论

随着生活水平的提高，ALD在我国发病呈上升趋势，目前已经仅次于病毒性肝病，成为肝病的第二大病因。酒精主要在肝脏代谢降解，长期大量饮酒可加重肝脏的代谢负担，从而导致肝损伤，引起ALD。乙醇的代谢使正常的肝脏氧化还原反应失调，其代谢产生的乙醛对肝细胞有多种毒性，可抑制谷胱甘肽的合成，使清除自由基的功能减退，除本身可转化为甘油三酯，在肝细胞内沉积外，还影响甘油三酯的氧化转运，形成脂质沉积肝细胞的脂肪肝，进一步可引起肝细胞的变性坏死和炎症细胞浸润而形成酒精性肝炎。代谢过程中产生的大量乳酸可使脯氨酸增加，促进胶原的合成和纤维化，最终导致酒精性肝硬化[1,5]。

还原型谷胱甘肽是由谷氨酸、半胱氨酸和甘氨酸组成的三肽，其含有活性巯基，可补充内源谷胱甘肽的不足，与乙醇在肝脏内的毒性代谢产物乙醛、氧自由基等结合，从而抑制肝组织氧化物的产生及甘油三酯的堆积，纠正低氧血症，保护肝细胞膜，防止乙醇引起的肝细胞变性坏死及肝脏纤维化等损害的发生，并通过转甲基、丙氨基反应，保护肝脏的合成，发挥解毒、灭活、激素等功能，并促进胆酸代谢，有利于消化道吸收脂肪及脂溶性维生素[6,7]。因此，还原型谷胱甘肽能通过上述多种途径阻断酒精对肝脏的损伤，从而达到治疗ALD的目的。

异甘草酸镁是甘草酸的单一反式旋光异构体，是具有抗炎、保护肝细胞膜、抗氧化等多种功能的肝细胞保护药。研究显示，异甘草酸镁较以往的甘草酸制剂（甘草酸单胺及甘草酸二胺）有更好的疗效和安全性[8]。异甘草酸镁具有很高的肝脏靶向性，它在肝脏内与$\delta^4$25β-还原酶的竞争作用强，从而更好地抑制了机体皮质醇灭活；同时异甘草酸镁本身具有类固醇样作用，对靶细胞上的类固醇受体具有亲和力。它的药物消除半衰期可达24 h，可以每天一次用药。动物实验表明，对于四氯化碳引起的肝组织损伤及纤维化，异甘草酸镁可使肝组织损伤明显减轻，抑制胶原的合成，具有降低肝纤维化、改善肝组织学的作用[9]。异甘草酸镁还具有促进肝细胞增殖的作用，并呈现一定的量效关系[10]。

笔者应用还原型谷胱甘肽片联合静脉滴注异甘草酸镁注射液治疗ALD，患者的临床症状、体征、肝功能检查及肝脏彩超检查均明显改善，肝功能改善程度均高于仅应用异甘草酸镁组，提示两者联用有协同作用。文献报道多应用还原型谷胱甘肽注射剂治疗，但患者输液时间长，依从性相对较差。还原型谷胱甘肽口服剂型的应用可减少患者输液时间，患者易于接受。并且，联合用药未见明显不良反应。当然，ALD的治疗关键还是在戒酒。

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5

Acute Renal Failure

INTRODUCTION

Acute renal failure (ARF) is a clinical syndrome of diverse etiologies. It is characterized by a sudden decline in renal function as assessed by glomerular filtration rate (GFR), is clinically manifested by rising concentrations of blood urea nitrogen (BUN) and creatinine, and is frequently associated with decreased urinary output. The purpose of this chapter is to provide the reader with a stepwise approach to the differential diagnosis and management of clinical ARF. As do many others, we advocate an initial clinical evaluation aimed at distinguishing between prerenal, post-renal, and intrinsic renal causes of ARF. A list of the major causes of ARF based on such a mechanistic approach is given in Table 1.

It is apparent that the differential diagnosis of this syndrome involves consideration of a wide variety of possible etiologies. It is the clinician's task to orchestrate the findings of history and physical examination, in conjunction with laboratory and imaging data, so as to strive at a reasonable diagnosis. Surprisingly, in view of the breadth of the differential diagnosis and the acute nature of the problem, this can usually be accomplished. Accurate diagnosis is important, as accurate therapy is dependent upon it. Although the majority of hospital-acquired ARF may require only supportive care, most forms of pre-renal and post-renal failure require specific interventions which may result in dramatic resolution of the clinical syndrome. Moreover, some forms of intrinsic ARF may benefit from specific therapy directed at the underlying disease, as in rapidly progressive glomerulonephritis, or an alteration in current therapy, as in allergic interstitial nephritis. It is beyond the scope of this chapter to discuss all the entities in Table 1 in any detail, thus we will focus primarily upon the process of differential diagnosis and the general principles of the management of the patient with hospital-acquired ARF.

Scope of ARF

The incidence of ARF in the general population is not well described. In hospitalized patients the incidence ranges between 1–5%. This indicates ARF is a relatively common disorder in these patients, and highlights the fact that in many cases, ARF occurs as a complication of other serious medical disorders. As such, it is an ominous prognostic factor, and is associated with excessive morbidity and a high mortality. Mortality from ARF varies significantly depending upon etiology, thus accurate diagnosis has prognostic as well as therapeutic implications.

The mortality of ischemic ARF, often called acute tubular necrosis (ATN), in trauma patients or patients with medical or surgical illness—associated shock, is around 60%. There has been no substantial reduction in this mortality in re-

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cent years, even with the evolution of more effective forms of dialytic therapy. In post-partum ARF or ARF due to nephrotoxic injury, as with radiocontrast or antibiotics, the mortality is much lower, ranging from 2–15%. Those forms of ARF associated with substantial urine output (non-oliguria) are associated with a more favorable survival rate than oliguric forms. The explanation for this is uncertain, but may reflect less severe injury conferred by toxins compared to ischemia, or that non-oliguric patients' fluid balance and nutrition is easier to manage.

Pathogenesis

The pathogenesis of ATN, referring to ischemic or nephrotoxic forms of intrinsic ARF, is not well understood, but is the subject of considerable experimental study. In most all experimental models, both renal blood flow and GFR decline early after the initial insult. This period, lasting from hours to days, is termed the initiation phase. Gradually, renal blood flow returns towards normal, while GFR remains depressed. This is called the maintenance phase. The degree of renal failure which results is variable, and depends upon the nature and severity of the insult. Over days to weeks, renal function returns towards normal as the kidney repairs or regenerates. In clinical ARF, recovery occurs after two to three weeks unless the patient succumbs to uremia or other complications. The reason for the persistent high mortality of ARF, despite seemingly adequate dialytic support, is not clear. To some degree this reflects the seriousness of comorbid medical conditions, but the presence of ARF itself seems to be a poor prognostic factor.

Some experimentalists feel that the course of ATN may be modified during the initiation phase by using vasodilators to improve renal blood flow or diuretics to establish a solute diuresis. Results of such maneuvers have been variable and inconsistent, as have such maneuvers when attempted in the clinical setting, possibly because it is difficult to distinguish between the initiation and maintenance phases of ATN clinically, thus salutary efforts may have been made too late. It is generally agreed that there is no currently available method of reversing established ATN, although efforts directed towards improving renal cellular metabolism or minimizing oxidative damage to

Table 5–1 Causes of Acute Renal Failure

I. Pre-renal Failure
  A. Hypovolemia
  B. Cardiovascular failure
    1. myocardial failure
    2. vascular pooling
  C. Inadequate plasma oncotic pressure
    1. nephrotic syndrome
    2. cirrhosis and hepatorenal syndrome

II. Vascular Obstruction
  A. Arterial obstruction
    1. renal artery emboli
    2. atheroembolic renal disease
  B. Venous obstruction

III. Intrinsic Renal Disease
  A. Glomerulonephritis
  B. Tubulointerstitial nephritis
  C. Acute presentation of chronic renal failure
  D. Intrinsic acute renal failure
    1. Ischemic
      a. hemorrhagic hypotension
      b. severe volume depletion
      c. surgical aortic cross-clamping
      d. cardiac and biliary surgery
      e. defective cardiac output
      f. crush syndrome and other trauma
      g. septic shock
      i. pregnancy
      j. pancreatitis
    2. Nephrotoxic
      a. antibiotics
      b. heavy metals
      c. endogenous pigments: hemoglobin, myoglobin
      d. radiographic contrast agents
      e. drugs
      f. organic solvents
      h. fungicides and pesticides
      i. uric acid
      j. ethylene glycol and methanol

IV. Post-renal Failure
  A. Urinary bladder obstruction
  B. Extrinsic obstruction of the ureters
  C. Intraluminal obstruction of the ureters
  D. Retroperitoneal fibrosis

reperfused kidneys show some promise in the laboratory. In contrast, it is widely accepted that establishing a solute diuresis prior to renal insult may abort or attenuate the severity of resultant ATN. This knowledge is reflected by the use of mannitol in vascular surgery and volume repletion prior to radiocontrast studies.

Differential Diagnosis

The differential diagnosis of ARF is quite broad, as diverse conditions can result in the abrupt cessation of renal function. Structural disorders of blood vessels subserving the kidneys, inflammation of the glomeruli or tubulointerstitium, and obstruction of the kidneys may each present as ARF. Nearly all of these disorders can be rapidly excluded by a careful history, physical examination, simple renal imaging procedures, and a carefully examined urinary sediment.

The aorta or renal arteries can be occluded by dissection, thrombosis, or emboli. The two former conditions are seen often in patients with obvious evidence of atherosclerotic disease. The latter results either from dislodgement or a mural cardiac thrombus or disruption of atheromatous plaques in the aorta or renal arteries. A nuclear medicine renal blood flow study is the initial diagnostic imaging test, however, it may be non-diagnostic, and arteriography may be required. Whether to proceed to thrombolytic therapy or revascularization requires careful thought, and the patient's medical condition may dictate a conservative approach.

Acute glomerulonephritis (GN) and acute tubulointerstitial nephritis (TIN) are uncommon but important causes of ARF. If acute GN results in ARF, the presumption must be that the patient has rapidly progressive (crescentic) GN, and renal biopsy is indicated as soon as medically feasible. The urinary sediment examination is crucial to the accurate and rapid diagnosis of these disorders, as it will commonly demonstrate cellular casts, including red cell and/or white cell casts. Acute TIN often results from drug injury, in which case, eosinophiluria is an important clue.

ARF can occasionally be the mode of presentation of long-standing chronic renal disease, particularly when the patient has had little medical care. In this condition, the urinary sediment may reveal proteinuria and cylinduria, particularly broad, waxy casts. Renal ultrasound demonstrates small, shrunken kidneys, and other tests, including biopsy, are seldom helpful.

Notwithstanding the diagnostic considerations above, operationally, the major diagnostic decision is to determine whether the patient has intrinsic renal failure, usually from an ischemic or nephrotoxic insult, or whether renal function has been affected by inadequate delivery of blood to the kidney or obstruction of the urinary tract. When renal function can resume if blood flow were restored, the patient is said to have "pre-renal azotemia." Similarly, if renal function can resume if the urinary tract were unobstructed, the patient is said to have "post-renal" azotemia. If either of these processes is allowed to continue untreated, the kidneys may be subject to ischemic injury of sufficient severity that renal function remains depressed even when the insult is removed. These patients have established ARF.

Pre-renal azotemia results from an absolute or relative deficiency of blood volume, so that renal blood flow is substantially reduced. An absolute reduction in blood volume is often seen as a consequence of renal salt loss from excessive diuretic therapy, from vomiting, overt blood loss, or sequestration of extracellular volume as in hemorrhagic pancreatitis. A relative reduction in blood volume may occur when there is inadequate plasma oncotic pressure to maintain effective blood volume, as in nephrosis or cirrhosis, or when cardiac function is insufficient to provide for effective renal perfusion.

Post-renal azotemia results from obstruction, at any level, of the urinary tract. Bladder outlet obstruction from prostatic hypertrophy is the most common cause of this condition in men, while pelvic cancer accounts for most cases in females. The kidneys may be intrinsically obstructed by infilterative processes, or may be obstructed along the course of the ureters by tumor or retroperitoneal fibrosis from drugs or as a result of a leaking aortic aneurysm. Finally, renal calculi can be bilateral and cause obstruction or the patient may only have a single kidney. It is important to recognize that ARF rarely results from an insult to a single kidney if another kidney is present and normal. In the vast majority of cases, renal ultrasound is both highly sensitive and specific for obstruction. With retroperitoneal fibrosis and intrarenal obstruction from infiltrative disorders, the ultrasound may be non-diagnostic, thus computerized tomogra-

phy or retrograde ureterography may be required.

Diagnostic Assessment

The history, physical examination, basic laboratory information, and the microscopic and biochemical analysis of the urine are the mainstays of the evaluation of the patient with ARF. Review of the complete medical record, including flow sheets and nurses' notes is essential, particularly in hospital-acquired ARF.

The history may suggest recent hypotension, either profound or subtle, but sufficient to cause decreased renal profusion. Such hypotension may be associated with overt or covert volume losses, as with bleeding, or from excessive diuretic therapy or decompensated cardiac function, respectively. A history of drug therapy is also important, as many drugs, particularly antibiotics and non-steroidal anti-inflammatory agents, may cause nephrotoxic injury alone, or commonly in concert with decreased renal perfusion.

Patients with ARF have few symptoms and signs which contribute to establishing the diagnosis except those related to the causative disorder. Symptoms are related to the rapidity of onset and severity of dysfunction, and the patient's underlying condition. Azotemia, hyperkalemia, and acidemia are often asymptomatic unless the patient is very catabolic. Many patients quickly have symptoms of volume overload, which may reflect aggressive fluid therapy. Urine volume is an important sign, as anuria ($\leq$ 100 ml/d) is common in vascular catastrophes or complete obstruction, while polyuria ($\geq$ 1 liter/d) is characteristic of nephrotoxic injury. Since ischemic injury is operative in most patients with ARF, oliguria ($\leq$ 400 ml/d) is usual.

The physical examination should primarily focus on the questions of whether the patient is in a state of volume depletion or volume overload, or obstructed. Crucial elements of the examination include skin turgor, the cardiac and pulmonary exam, edema, and genitourinary examination as keys to these important questions. Accurate weights, blood pressure assessment for orthostatic hypotension, and proof of a properly draining urinary tract are essential.

The concentrations of blood urea nitrogen (BUN) and creatinine increase steadily during the course of ARF as production remains relatively constant while excretion is profoundly diminished. Daily increments of BUN and creatinine average 20–30 mg/dl and 1.3–1.5 mg/dl, respectively. More rapid incremental changes in the BUN may reflect excessive catabolism or gastrointestinal hemorrhage, while excessive increments in the serum creatinine often reflect muscle necrosis (rhabdomyolysis). Metabolic acidosis results from retention of endogenously produced acid, thus the serum bicarbonate concentration averages 17–18 meq/L in uncomplicated cases. Hyperkalemia (5.0–6.0 meq/L) may result from limited excretion and acidemia. Hyperphosphatemia occurs since phosphate excretion is negligible. This, in conjunction with inhibition of the gastrointestinal effects of Vitamin D, leads to hypocalcemia. The hematocrit falls over 5–7 days to a level of 23–27%, and may reflect volume overload, blood loss, and bone marrow supression.

Examination of the urine is extremely important in patients with ARF, since it may provide evidence of pre-renal or post-renal azotemia, or support the diagnosis of established ARF. The information gleaned from the urine is so critical that the physician should examine it personally.

The patient with pre-renal azotemia should have a concentrated urine with high urine to plasma ratios of urea, creatinine, and osmolality, and a low urinary sodium concentration. Similar values are found in patients with acute urinary obstruction. The patient with established ARF usually loses concentrating ability and the ability to conserve urinary sodium, with low urinary to plasma ratios of solute and high urinary sodium concentrations. Similar values are found in patients with ARF from chronic obstruction of the urinary tract. Indices of renal function such as the fractional excretion of sodium and the renal failure index are manipulations of these basic data, which should be interpreted cautiously if the patient has received diuretics in the 12–18 hours prior to their measurement. Nonetheless, biochemical analyses of urine accurately distinguish between pre- and post-renal failure in nearly 90% of cases.

The urine sediment is unremarkable in pre- and post-renal azotemia, and contains hyaline, and rarely, granular casts. Hematuria may be present if a catheter is in place. Conversely, the sediment in ARF demonstrates many granular casts, and most importantly, renal tubular epi-

thelial cells and other cellular debris, giving rise to a very "dirty" sediment. Experienced observers distinguish between pre- or post-renal failure and established ARF on the basis of the sediment in 85–90% of cases.

Imaging of the kidneys in patients with ARF should be limited to those studies which demonstrate whether there are two kidneys, whether there are calculi, and whether the urinary tract is obstructed. In our view the imaging test of choice is a technically well-done renal ultrasound. Plain films of the abdomen are often not helpful, and contrast procedures can be quite harmful. The nuclear scan should be reserved for anuric patients in whom arterial catastrophe is suspected.

Collectively, the history, physical examination, biochemical analysis of serum and urine, urinalysis, and renal ultrasound provide the necessary information needed by the clinician to determine whether the patient truly has established ARF or whether there is a readily reversible cause of acute renal dysfunction. At times the volume status of the patient is difficult to assess, and measurement of central venous pressure or pulmonary capillary wedge pressure is necessary. In our judgment these procedures are both over-utilized and time-consuming, and may often delay fluid resuscitation with perpetuation of renal ischemia.

Repetitive small challenges of isotonic saline, with close clinical assessment of effect, are often simultaneously diagnostic and therapeutic. In the pre-renal azotemia patient who is clinically volume depleted, fluid therapy with saline or blood may restore renal function. Similarly, improvement in cardiac function if heart failure is present may achieve the same result. Drainage of the urinary tract must be adequate in obstructed patients.

The use of diuretics and vasoactive hormones is widespread, but efficacy is lacking. The use of diuretics such as furosemide can be harmful if the patient is volume depleted. However, we believe that furosemide, if administered to euvolemic patients early in the course of ARF, may make oliguric patients non-oliguric and assist in their subsequent management. We administer graded doses of 1, 5, and 10 mg/kg of furosemide at hourly intervals over three-four hours to clearly euvolemic patients; restoration of urine output at any dose obviates further doses. Low-dose dopamine (1–3 mcg/kg/min) will promote urine flow in some patients, even without affecting systemic arterial pressure.

The use of mannitol prior to procedures associated with the development of ARF is widespread, and is probably efficacious in vascular surgical procedures. We advocate the use of isotonic bicarbonate and 100 ml of 20% mannitol in the fluid resuscitation of patients in whom ARF is due to rhabdomyolysis. There are no data of which we are aware which demonstrate that furosemide or dopamine have attenuated the mortality of established ARF.

TREATMENT OF ESTABLISHED ACUTE RENAL FAILURE

The fundamental assumption made in the treatment of ARF is that injured kidneys can recover. The major goal of therapy is to support the patient until this occurs. A secondary goal is to provide the proper metabolic milieu in which the patient can maintain adequate nutrition and resistance to infection, which is by far the major cause of death in ARF. To these ends, the assistance of experts in nutritional care and infectious diseases is critical in the management of these patients.

Some patients with ARF, particularly those who remain nonoliguric and in whom catabolism is not excessive, can be treated with fluid and protein restriction, in conjunction with adequate caloric support. Hyperkalemia can be controlled with exchange resins, glucose and insulin, and bicarbonate. The patient must be closely managed, however, and attention to routine details of patient care requires considerable effort. Moreover, the severity of renal dysfunction may limit the quantity and quality of nutritional support, with resultant catabolism of an unacceptable severity.

Dialytic support is required in many patients with ARF, and in our experience, is often required for a volume indication more so than for relief of symptoms of uremia. Generally accepted indications for dialysis include metabolic acidemia, hyperkalemia, hypercatabolic ARF, severe azotemia, volume overload, and uremia. Most nephrologists institute dialysis when the BUN and creatinine reach 110–130 mg/dl and 8–10 mg/dl, respectively.

Hemodialysis is widely available, and can be conducted with acceptable morbidity. An-

gioaccess is achieved with percutaneous catheters, and anticoagulation regimens are associated with minimal risk of bleeding. The procedure is usually performed on alternate days, although daily dialysis may be required if solute load is excessive or if required fluid volume administration results in volume overload.

Peritoneal dialysis, particularly if conducted with automatic cycling machines, offers similar efficacy without the risks of hypotension and bleeding which sometimes occur with hemodialysis. Additionally, peritoneal dialysis can be performed without the highly trained technical personnel needed for hemodialysis.

Recently artificial membranes have been made available which are sufficiently permeable to make fluid removal rates in excess of 1.5 L/hour possible. The membranes perform adequately at blood flow rates of 60 ml/min, and thus do not require flow augmentation with a blood pump. When connected to an artery and vein by means of special catheters, a functioning circuit is achieved with minimal cardiovascular stress. If the filter is used solely to remove fluid, the procedure is known as slow continuous ultrafiltration (SCUF). Pressure can be applied across the membrane to augment fluid removal, and the patient can receive sufficient quantities of electrolyte solution so that the concentrations of BUN and creatinine can be lowered. When conducted in this fashion, the procedure is known as hemofiltration. Operationally, the membrane is utilized continuously for several days. In our view its major use is in the patient who requires several liters of parenteral nutrition and antibiotic therapy each day. When used in this manner, volume overload is controlled. In many patients solute removal needs are sufficiently minimal, and the major personnel requirements of frequent hemodialysis are avoided.

SUMMARY

Properly managed, the mortality of ARF ranges from 2–65%, with higher mortalities being related to post-operative or traumatic ARF, underlying age and comorbidity, and infection. Patients rarely die of renal failure, but instead, die of their underlying disease or infection. The high mortality of ARF, persistent despite putatively adequate renal replacement therapy, suggests that future therapeutic success may depend upon advances in fighting infectious diseases, nutritional support, and in prophylactic measures effective in decreasing the incidence and/or severity of ARF.

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Interaction of Glycyrrhizin and Glycyrrhetinic Acid with DNA

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Glycyrrhizin (GL), a molecule of glycyrrhetinic acid (GA), is an aqueous extract from licorice root. These compounds are well known for their anti-inflammatory, hepatocarcinogenesis, antiviral, and interferon-inducing activities. This study is the first attempt to investigate the binding of GL and GA with DNA. The effect of ligand complexation on DNA aggregation and condensation was investigated in aqueous solution at physiological conditions, using constant DNA concentration (6.25 mM) and various ligands/polynucleotide (phosphate) ratios of 1/240, 1/120, 1/80, 1/40, 1/20, 1/10, 1/5, 1/2, and 1/1. Fourier transform infrared and ultraviolet (UV)-visible spectroscopic methods were used to determine the ligand binding modes, the binding constants, and the stability of ligand–DNA complexes in aqueous solution. Spectroscopic evidence showed that GL and GA bind DNA via major and minor grooves as well as the backbone phosphate group with overall binding constants of $K_{GL-DNA} = 5.7 \times 10^3\ M^{-1}$, $K_{GA-DNA} = 5.1 \times 10^3\ M^{-1}$. The affinity of ligand–DNA binding is in the order of GL > GA. DNA remained in the B-family structure, whereas biopolymer aggregation occurred at high triterpenoid concentrations.

Introduction

Licorice, the root of *Glycyrrhiza glabra*, an extremely important traditional Middle Eastern medicine, has been widely used during the past millennia for its taste and medical potential. Licorice extracts contain many components, including glycyrrhizin (GL), various sugars, flavonoids, and saponoids. GL (Fig. 1), accounting for 10%–25% of licorice root extract, is considered the primary ingredient (Eisenbrand, 2006). GL (3-*O*-[2-*O*-β-D-glucopyranuronosyl-α-D-glucopyranuronosyl]-18β-glycyrrhetinic acid) is hydrolyzed to 18β-glycyrrhetinic acid (GA; 3β-hydroxy-11-oxo-18β, 20β-olean-12-en-29-oic acid; Fig. 1) (Krausse *et al.*, 2004) *in vivo*, which is responsible for most of its pharmacological properties. It has been used in clinic as a remedy for the treatment of chronic hepatitis (Van Rossum *et al.*, 1998), tumor (Nishino *et al.*, 1984; Lee *et al.*, 2008), and human immunodeficiency virus (HIV) infection (Hattori *et al.*, 1989; Salvi *et al.*, 2003). GL suppresses reproduction of some DNA and RNA viruses, including the HIV, inactivating herpes simplex virus particles irreversibly (Pompei *et al.*, 1979; Harada *et al.*, 1999). GL inhibits liver cell injury caused by many chemicals and is used in the treatment of chronic hepatitis and cirrhosis in Japan. Presumably, GL inhibits protein kinase activity (Pompei *et al.*, 1979; Harada *et al.*, 1999). Several proteins have been reported to serve as GL targets. These are mostly casein kinase II (CK-II) substrates such as lactoferrin or lipoxigenase (Shimoyama *et al.*, 1996; Hatomi *et al.*, 2000). Recombinant HIV-1 reverse transcriptase has been shown to efficiently bind with recombinant human CK-II and to require phosphorylation for its normal enzymic activity (Harada *et al.*, 1998). Acting as a CK-II inhibitor, GL used at a high concentration (100 mM) completely inhibits phosphorylation of reverse transcriptase, which may account for its antiviral activity (Harada *et al.*, 1999).

GA is a pentacyclic triterpenoid derivative of β-amyrin. GA is effective against chronic hepatitis but also contributes to the side-effect aldosteronism. As only GA appears in the blood circulation after oral administration, it is considered to play an important role in the biological action (Oketani *et al.*, 1985; Ishida *et al.*, 1989).

Understanding the mechanism of GA action may expedite development of new drugs based on GA or its derivatives and reduce the risk of side effects. Thus, we examined the interaction of DNA adducts with two pentacyclic triterpenoid derivatives GL and GA in aqueous solution at pH 6–7 with ligands/DNA(P) molar ratios of 1/240 to 1/1 using Fourier transform infrared (FTIR) and 0.005–0.1 mM by ultraviolet (UV) measurements as well as molecular modeling. Structural analyses regarding the GL and GA binding sites, binding constants, and DNA secondary structure were performed. Our spectroscopic results provide a major structural analysis of triterpenoids–biopolymers interaction, which helps to elucidate the nature of this biologically important complexation *in vitro*.

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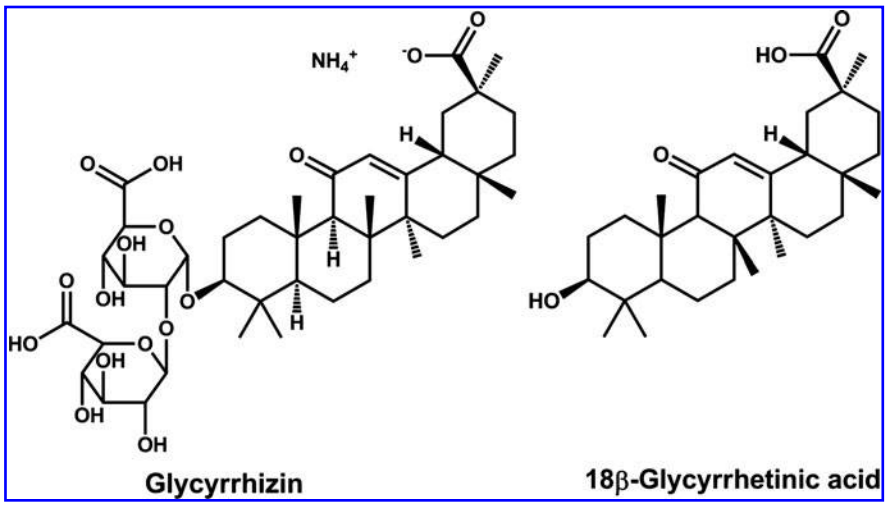

FIG. 1. Chemical structure of GL and 18β-GA. GL, glycyrrhizin; GA, glycyrrhetinic acid.

Materials and Methods

Materials

DNA sodium salt, GL, and GA were purchased from Sigma Chemical and used without further purification. To check the protein content of DNA solutions, the absorbance bands at 258 and 280 nm were used. The A_{258}/A_{280} ratio was 2.10 for DNA, showing that DNA samples were sufficiently free from protein (Marmur and Doty, 1961). Other chemicals were of reagent grade and used without further purification.

Preparation of stock solutions

DNA was dissolved to 0.5% (w/v; 0.0125 M) polynucleotide (phosphate) (pH 7) in NaCl solution for 24 h with occasional stirring to ensure the formation of a homogeneous solution. The final concentration of the DNA solution was spectrophotometrically determined at 258 nm using molar extinction coefficient $\varepsilon_{258} = 9250\,\mathrm{cm^{-1}\,M^{-1}}$ (DNA) (expressed as molarity of phosphate groups) (Vijalakshmi *et al.*, 2000).

GL and GA (0.05–12.5 mM) were dissolved in water and added dropwise to DNA solution (12.5 mM) to attain the desired ligand/DNA(P) molar ratios (r) of 1/240, 1/120, 1/80, 1/40, 1/20, 1/10, 1/5, 1/2, and 1/1 with a final DNA(P) concentration of 6.25 mM. The pH values of solutions were adjusted at 7.0 ± 0.2 using NaCl solution. The infrared spectra were recorded at 2 h after mixing GL and GA with DNA solution. For UV measurements, ligand concentrations of 0.005–0.1 mM were used with a constant DNA concentration of 0.5 mM.

FTIR spectroscopy measurements

Infrared spectra were recorded on a Nicolet FTIR spectrometer (Magna 550) equipped with a liquid nitrogen–cooled HgCdTe (MCT) detector and a KBr beam splitter. The spectra of ligands/DNA solutions were recorded using a cell assembled with ZnSe windows. Spectra were recorded and treated using the OMNIC software supplied by the manufacturer of the spectrophotometer. The spectra of the solutions were recorded after 2 h incubation of ligands with DNA solution, using ZnSe windows. The bands were measured in triplicates (three individual samples of the same DNA and ligand concentrations). For each spectrum, 100 scans were recorded with a resolution of $4\,\mathrm{cm^{-1}}$. The difference spectra ([polynucleotide solution + ligand solution] – [polynucleotide solution]) were obtained using a sharp DNA band at $968\,\mathrm{cm^{-1}}$ as internal reference (Kanakis *et al.*, 2005, 2007). This band, which is due to sugar C-C and C-O stretching vibrations, exhibited no spectral change (shifting or intensity variation) upon ligand–DNA complexation and was cancelled out upon spectral subtraction.

The intensity ratios of the bands due to several DNA in-plane vibrations related to A-T and G-C base pairs and the PO_2 stretching vibrations were measured with respect to the reference bands at $968\,\mathrm{cm^{-1}}$ (DNA) as a function of ligand concentrations with an error of ±3%. Similar intensity variations have been used to determine the ligand binding to DNA bases and backbone phosphate groups (Arakawa *et al.*, 2000).

The plots of the relative intensity (R) of several peaks of DNA in-plane vibrations related to A-T and G-C base pairs and the PO_2 stretching vibrations such as 1712 (guanine), 1662 (thymine), 1612 (adenine), 1490 (cytosine), and $1226\,\mathrm{cm^{-1}}$ (PO_2 groups) versus ligand concentrations were obtained after peak normalization using $R_i = I_i/I_{968}$, where I_i is the intensity of the absorption peak for pure DNA in the complex with i as the ligand concentration, and I_{968} is the intensity of the $968\,\mathrm{cm^{-1}}$ peak (DNA internal reference). The plot of intensity was drawn from $r = 1/240$ to 1/20 for GL-DNA and from $r = 1/240$ to 1/40 for GA-DNA. At higher ligand concentrations ($r = 1/40$ to 1/1), monitoring the intensity changes of DNA bands was not possible because of the overlapping of the GL and GA absorption bands with DNA vibrations. Therefore, the intensity changes shown are from $r = 1/240$ to $r = 1/20$ for GL-DNA and from $r = 1/240$ to $r = 1/40$ for GA-DNA complexes.

Absorption spectroscopy

The absorption spectra were recorded on a LKB model 4054 UV–visible spectrometer; quartz cuvettes of 1 cm were used and the absorption spectra were recorded with ligand concentrations of 0.005–0.1 mM and constant polynucleotide concentration of 0.5 mM. The binding constants of the ligand–DNA complexes were calculated as previously reported (Connors, 1987). It is assumed that the interaction

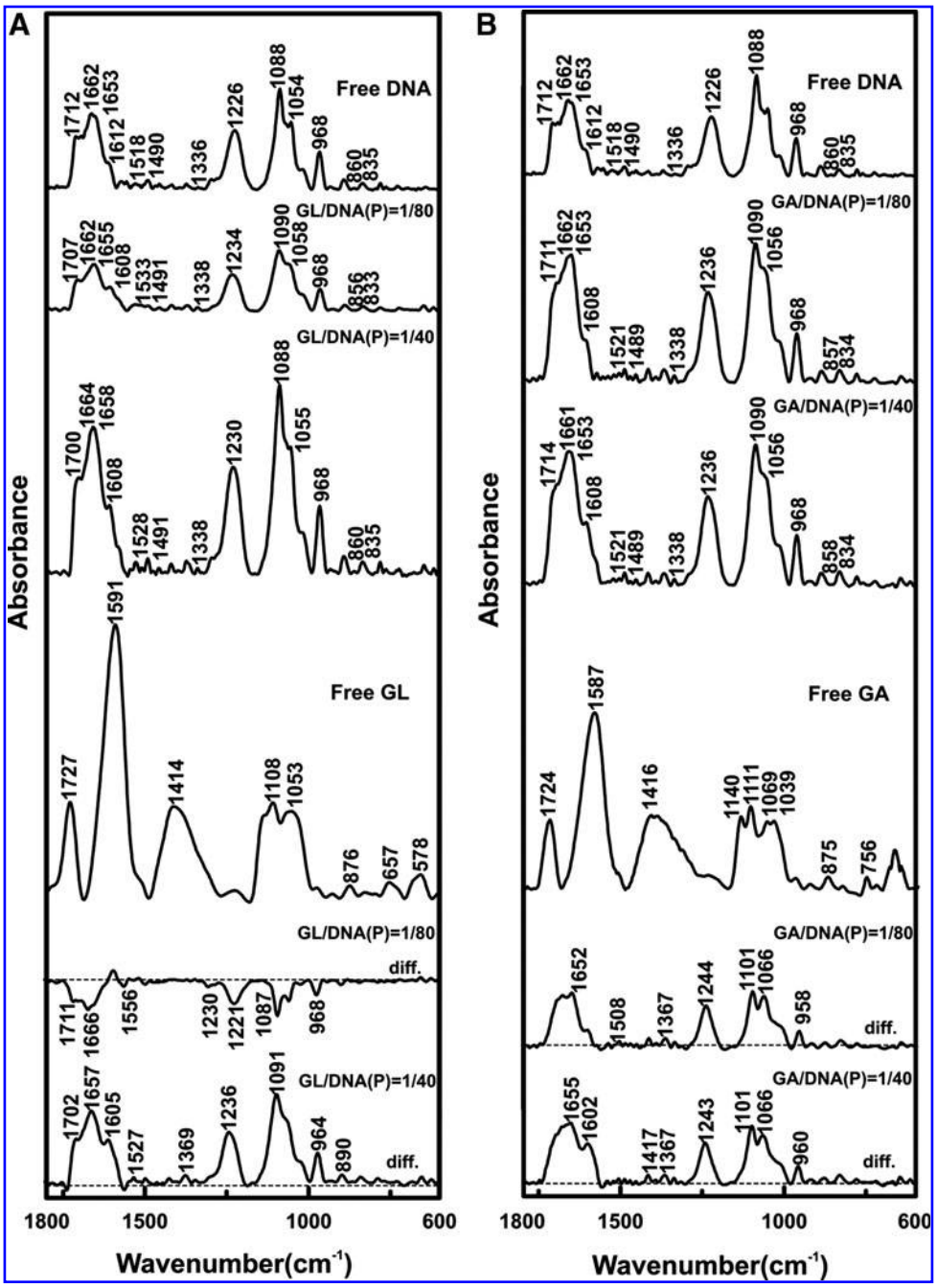

FIG. 2. Fourier transform infrared spectra in the region of 1800–600 cm^{-1} for calf thymus DNA, and **(A)** GL and **(B)** GA adducts in aqueous solution at pH = 7. DNA and two complexes spectra obtained at various GL and GA-DNA (phosphate) molar ratios (top three spectra); ligand and two difference spectra (bottom three spectra).

between the ligand [L] and the substrate [S] is 1:1; for this reason, a single complex SL (1:1) is formed. It was also assumed that the sites (and all the binding sites) are independent and the Beer's law is followed by all species. A wavelength is selected at which the molar absorptivities ε_S (molar absorptivity of the substrate) and ε_{11} (molar absorptivity of the complex) are different. Then, at total concentration S_t of the substrate, in the absence of ligand and with the light path length $b = 1$ cm, the solution absorbance is

$$A_o = \varepsilon_S b S_t \tag{1}$$

In the presence of ligand at total concentration L_t, the absorbance of a solution containing the same total substrate concentration is

$$A_L = \varepsilon_S b[S] + \varepsilon_L b[L] + \varepsilon_{11} b[SL] \tag{2}$$

where [S] is the concentration of the uncomplexed substrate, [L] is the concentration of the uncomplexed ligand, and [SL] is the concentration of the complex. This combined with the mass balance on S and L gives

$$A_L = \varepsilon_S b S_t + \varepsilon_L b L_t + \Delta\varepsilon_{11} b[SL] \tag{3}$$

where $\Delta\varepsilon_{11} = \varepsilon_{11} - \varepsilon_S - \varepsilon_L$ (ε_L is the molar absorptivity of the ligand). By measuring the solution absorbance against a reference containing ligand at the same total concentration L_t, the measured absorbance becomes

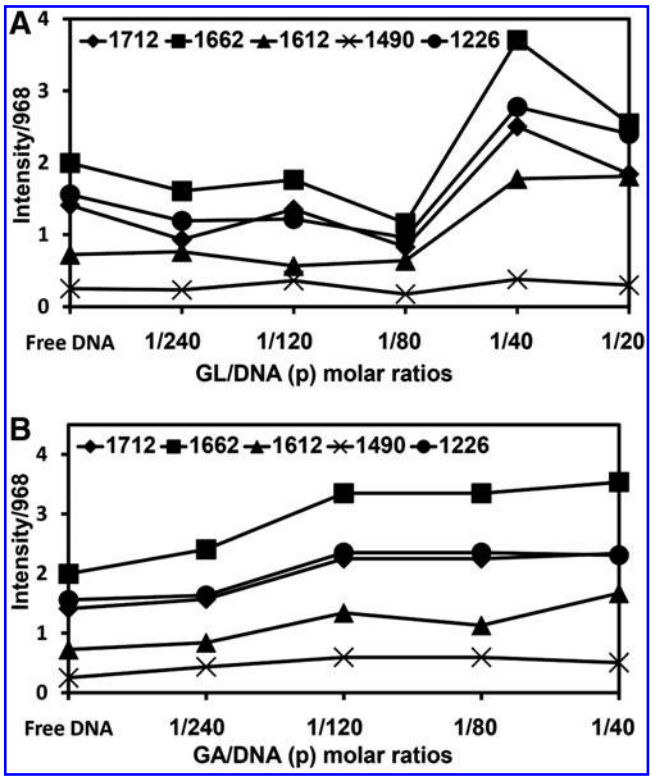

FIG. 3. Intensity ratio variations for several DNA in-plane vibrations as a function of **(A)** GL and **(B)** GA concentration. Intensity ratios for the DNA bands at 1712 (G, T), 1662 (T, G, A, C), 1612 (A), 1490 (C, G), and 1226 (PO_2 asymmetric) referenced to the DNA band at 968 cm^{-1}.

$$A = \varepsilon_S b S_t + \Delta\varepsilon_{11} b[\mathrm{SL}] \tag{4}$$

Combining equation (4) with the stability constant definition $K_{11} = [\mathrm{SL}]/[\mathrm{S}][\mathrm{L}]$ gives

$$\Delta A = K_{11}\Delta\varepsilon_{11} b[\mathrm{S}][\mathrm{L}] \tag{5}$$

where $\Delta A = A - A_o$. From the mass balance expression $S_t = [\mathrm{S}] + [\mathrm{SL}]$, we get $[\mathrm{S}] = S_t/(1 + K_{11}[\mathrm{L}])$, which is equation (5), giving equation (6) at the relationship between the observed absorbance change per centimeter and the system variables and parameters.

$$\frac{\Delta A}{b} = \frac{S_t K_{11}\Delta\varepsilon_{11}[L]}{1 + K_{11}[L]} \tag{6}$$

Equation (6) is the binding isotherm, which shows the hyperbolic dependence on free ligand concentration.

The double-reciprocal form of plotting the rectangular hyperbola $\frac{1}{y} = \frac{f}{d}\cdot\frac{1}{x} + \frac{e}{d}$ is based on the linearization of equation (6) according to the following equation:

$$\frac{b}{\Delta A} = \frac{1}{S_t K_{11}\Delta\varepsilon_{11}[L]} + \frac{1}{S_t \Delta\varepsilon_{11}} \tag{7}$$

Thus, the double-reciprocal plot of $1/\Delta A$ versus $1/[\mathrm{L}]$ is linear and the binding constant can be estimated from the following equation:

$$K_{11} = \frac{intercept}{slope} \tag{8}$$

Molecular modeling and docking

The crystal structures of DNA–ligand complex were selected from the protein databank (Web address: www.rcsb.org; PDB ID: 1D30) (Larsen *et al.*, 1989).

We docked GL and GA onto the oligonucleotide extracted from the crystal structures. For every individual model, the correlation between calculated binding and experimental values was analyzed to determine the most representative model.

To determine the preferred binding sites on DNA, docking studies were performed by AutoDock 4.2.3 software (Huey *et al.*, 2007) (Web address: http://autodock.scripps.edu).

The ligand structures were extracted from Pubchem (CID 16213697, 10114) files. To use the structures for docking, the universal force field (for ligands) (Rappe *et al.*, 1992) and Merck molecular force field 94 (for macromolecule) (Halgren, 1996) were minimized.

Docking to macromolecule was carried out using the Lamarckian genetics algorithm. For the local search, the so-called pseudo-Solis and Wets algorithm was applied using a maximum of 300 iterations per local search (Solis and Wets, 1981).

In AutoDock, the overall docking energy of a given ligand molecule in its active site is expressed as follows:

$$\Delta G = \Delta G_{vdW}\sum_{i,j}\left(\frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^{6}}\right) + \Delta G_{hbond}\sum_{i,j}\left(\frac{C_{ij}}{r_{ij}^{12}} - \frac{D_{ij}}{r_{ij}^{10}} + E_{hbond}\right) + \Delta G_{elec}\sum_{i,j}\frac{q_i - q_j}{\varepsilon(r_{ij})r_{ij}} + \Delta G_{tor}N_{tor} + \Delta G_{sol}\sum_{i_c,j} S_i V_j e^{(-r_{ij}^2/2\sigma^2)} \tag{9}$$

In equation (9), ΔG_{vdW}, ΔG_{hbond}, ΔG_{elec}, ΔG_{tor}, and ΔG_{sol} are free energy coefficients of van der Waals, hydrogen bond, electrostatic interactions, torsional term, and desolvation energy of oligonucleotide–ligand complex, respectively. r_{ij}, A_{ij}, B_{ij}, C_{ij}, and D_{ij} represent the interatomic distance, the depths of energy well, and the equilibrium separations between the two atoms, respectively. The first three terms are *in vacuo* force field energies for intermolecular interactions. The fourth term accounts for the internal steric energy of the ligand molecule.

The energies used and reported by AutoDock should be distinguished: there are docked energies, which include the intermolecular and intramolecular interaction energies and are used during dockings, and predicted free energies, which include the intermolecular energy and the torsional free energy and are only reported at the end of a docking (Morris *et al.*, 1998; Ali *et al.*, 2010).

We converted between the binding constant, $K_{binding}$, and the binding free energy change of binding, $\Delta G_{binding}$, using the following equation:

$$\Delta G_{binding} = -RT\ln K_{binding} \tag{10}$$

where R is the gas constant, 1.987 cal K^{-1} mol^{-1} and T is the absolute temperature, assumed to be room temperature, 298.15 K.

To analyze and display docking results, we used AutoDock Tools 1.5.4 (Sanner, 1999; Web address: http://mgltools.scripps.edu) and UCSF Chimera 1.5 (Couch *et al.*, 2006; Web address: www.cgl.ucsf.edu/chimera).

Results and Discussion

FTIR spectra of GL-DNA adducts

The IR spectral features of the GL-DNA interaction are presented in Fig. 2A. At $r=1/240$, no major GL-DNA interaction was observed as a result of minor spectral changes (intensity and shifting) of the guanine at 1712, thymine at 1660, adenine at 1612, cytosine at 1490, and the PO_2 band at 1226 cm^{-1} (asymmetric stretch) (Theophanides and Tajmir Riahi, 1985; Taillandier and Liquier, 1992; Loprete and Hartman, 1993; Andrushchenko *et al.*, 2001; Dovbeshko *et al.*, 2002; Ouameur and Tajmir-Riahi, 2004). At higher concentrations ($r=1/120$), the guanine band at 1712 shifted to 1706 cm^{-1}, the thymine band at 1660 shifted to 1664 cm^{-1}, the adenine band at 1612 shifted to 1608 cm^{-1}, and phosphate asymmetric band at 1226 shifted to 1234 cm^{-1}. The shifting was accompanied by intensity increase, mainly for guanine and thymine bands. The observed spectral changes can be related to GL interaction with guanine, adenine N7, thymine O2 bases and backbone phosphate group.

At $r=1/80$, decrease in the intensity of bases bands can be related to DNA stabilization upon GL interaction. At a higher GL concentration ($r=1/40$), the guanine band at 1712 shifted to 1700 cm^{-1}, the thymine band at 1660 shifted to 1664 cm^{-1}, the adenine band at 1612 shifted to 1608 cm^{-1}, and the phosphate asymmetric band at 1226 shifted to 1230 cm^{-1}.

Major intensity increase was observed for the guanine, thymine, adenine, and phosphate at this concentration (Figs. 2A and 3A). The observed spectral changes are due to major interaction of GL with bases and phosphate at this concentration. In the difference spectrum of GL-DNA ($r=1/40$), the positive features at 1702, 1657, 1605, 1236, and 1091 cm^{-1} are due to an increase in intensity of the DNA vibrations as a result of GL interaction with the G and A-T base pairs and phosphate backbone group (Fig. 2A, diff.; Fig. 3A).

At $r=1/20$, a major decrease in intensity was observed for the bases and phosphate vibrations, which is attributed to DNA aggregation in the presence of high GL concentrations (Fig. 3A).

It should be noted that GL-PO_2 binding occurred at all concentrations. Evidence for this comes from major shifting of the PO_2 asymmetric vibration from 1226 to 1230–1234 cm^{-1} ($r=1/240$ to $1/20$) (Fig. 3A). The observed shifting was accompanied by the variations in the intensity of the phosphate band at all concentrations ($r=1/240$ to $1/40$). The major intensity increase at $r=1/40$ is related to the maximum GL–phosphate interaction at this concentration.

In addition to a major spectral shifting of the PO_2 asymmetric band, the relative intensities of the asymmetric (ν_{as}) and symmetric (ν_s) vibrations were altered upon phosphate interaction (Alex and Dupius, 1989). The ν_s PO_2 (1088 cm^{-1}) and ν_{as} PO_2 (1226 cm^{-1}) were changed, with the ratio ν_s/ν_{as} going from 1.7 (free DNA) to 1.9 (GL-DNA complexes) at a high GL concentration (Fig. 2A). This showed that the maximum binding of GL to backbone phosphate group occurs at $r=1/40$.

No major intensity changes were observed for the cytosine band at 1490 cm^{-1}, indicating no major participation of cytosine in GL-DNA binding.

The absorption bands with medium intensity at 1653 cm^{-1} in the IR spectrum of free DNA and at 1651–1654 cm^{-1} in spectra of the GL–DNA adducts and in difference spectra are due to water deformation mode but not due to DNA vibrations (Tajmir-Riahi *et al.*, 2009).

Absorption spectroscopy revealed that addition of the aqueous GL to DNA solution resulted in a red shift of the DNA band at 258 to 260–263 nm in complexes, which is additional evidence for GL-DNA interaction (Fig. 4A).

FTIR spectra of GA-DNA adducts

At low GA concentration ($r=1/240$, $1/120$), major interaction was observed as a result of spectral changes in the GA-DNA interaction at this concentration. No major shifting was observed for the thymine band at 1662 ($r=1/240$, $1/120$), whereas the adenine band at 1612 shifted to 1606 cm^{-1} ($r=1/240$) and 1608 ($r=1/120$) and the phosphate asymmetric band at 1226 shifted to 1236 cm^{-1} ($r=1/240$, $1/120$). The observed shifting was accompanied by intensity increase of the guanine, adenine, and mainly thymine and phosphate bands upon GA complexation. A gradual increase of intensity of DNA vibrations with a maximum at $r=1/120$

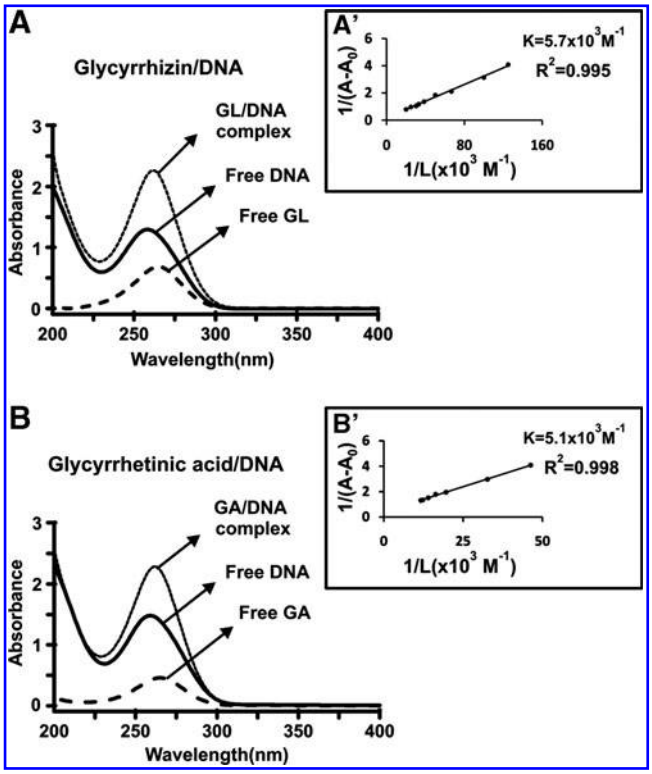

FIG. 4. Ultraviolet–visible results of calf-thymus DNA and **(A)** GL and **(B)** GA complexes: spectra of free DNA (0.5 mM); free GL, GL-DNA complex (0.03 mM); free GA, GA-DNA complex (0.08 mM). Plot of $1/(A - A_0)$ versus (1/ligand concentration) for GL, GA, and calf-thymus DNA complexes, where A_0 is the initial absorbance of DNA (258 nm) and A is the recorded absorbance at different GL and GA concentrations (0.005–0.1 mM) with a constant DNA concentration of 0.5 mM at pH 7.

indicates a major interaction of GA with DNA bases at this concentration. No major intensity change was observed at $r = 1/80$, $1/40$, indicating no more interaction at higher concentrations (Figs. 2B and 3B).

Additional evidence regarding GA interaction with the phosphate backbone group comes from the relative intensities of the asymmetric (v_{as}) and symmetric (v_s) vibrations of the phosphate group (Alex and Dupius, 1989). The v_s PO_2 (1088 cm^{-1}) and v_{as} PO_2 (1226 cm^{-1}) were changed, with the ratio v_s/v_{as} going from 1.7 (free DNA) to 1.5 (glycyrrhetinic–DNA complexes) upon GA complexation (Fig. 2B).

Absorption spectroscopy revealed that addition of aqueous GA to DNA solution resulted in a red shift of the DNA band at 258 to 260–263 nm in complexes, which is additional evidence for GA-DNA interaction (Fig. 4B).

DNA conformation

No alterations of B-DNA structure were observed upon GL and GA-DNA complexation as a result of no major spectral changes for B-DNA marker bands at 1226 cm^{-1} (PO_2 stretch), 1712 cm^{-1} (mainly guanine), and 836 cm^{-1} (phosphodiester mode) upon GL and GA complexation (Taillandier and Liquier, 1992; Loprete and Hartman, 1993; Tajmir-Riahi *et al.*, 1995) (Fig. 2).

In a B to A transition, the marker band at 836 cm^{-1} shifts toward a lower frequency at about 825–800 cm^{-1}, the guanine band at 1712 cm^{-1} appears at 1700–1695 cm^{-1}, and the phosphate band at 1226 cm^{-1} shifts toward a higher frequency at 1240–1235 cm^{-1} (Tajmir-Riahi *et al.*, 1995, 2009). In a B to Z conformational change, the sugar–phosphate band at 836 cm^{-1} appears at 800–780 cm^{-1}, the guanine band displaces to 1690 cm^{-1}, and the phosphate band shifts to 1216 cm^{-1} (Tajmir-Riahi *et al.*, 1995, 2009).

In the GL and GA-DNA complexes, shifting of the B-DNA marker bands at 1226 to 1230–1238 cm^{-1} is indicative of GL and GA interaction with phosphate and not due to B to A-DNA conformational change (Andrushchenko *et al.*, 2001; Polyanichko *et al.*, 2004; Marty *et al.*, 2009) (Fig. 2A, B).

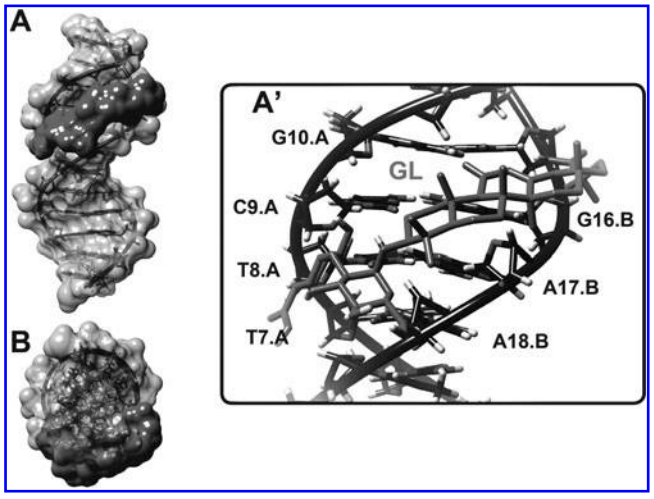

FIG. 5. Docking structure between $d(CGCGAATTCGCG)_2$ (PDB ID: 1D30) and GL. **(A)** Surface representation of $d(CGCGAATTCGCG)_2$ complexes with GL (displayed on side). **(A')** Close-up view of $d(CGCGAATTCGCG)_2$ complexes with GL. **(B)** Surface representation of d(CGC GAATTCGCG)$_2$ complexes with GL (displayed on top).

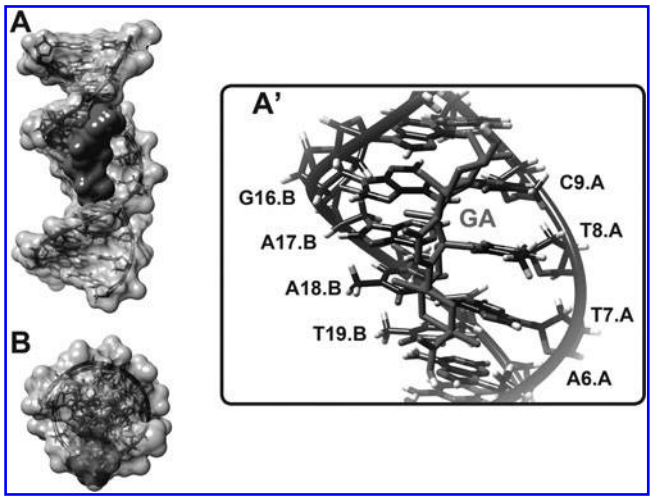

FIG. 6. Docking structure between $d(CGCGAATTCGCG)_2$ (PDB ID: 1D30) and GA. **(A)** Surface representation of $d(CGCGAATTCGCG)_2$ complexes with GA (displayed on side). **(A')** Close-up view of $d(CGCGAATTCGCG)_2$ complexes with GA. **(B)** Surface representation of d(CGC GAATTCGCG)$_2$ complexes with GA (displayed on top).

Stability of GL-DNA and GA-DNA complexes

The ligand binding constants were determined as described in the Materials and Methods section (UV–visible spectroscopy). The calculations of the overall binding constants were carried out using UV spectroscopy as previously reported (Connors, 1987). Concentrations of the complexed ligand were determined by subtracting absorbance of the free DNA at 258 nm from those of the complexed. Concentration of the free ligand was determined by subtraction of the complex ligand from total ligand used in the experiment. Our data of 1/[complex ligand] almost proportionally increased as a function of 1/[free ligand] (Fig. 4). The double reciprocal plot of $1/(A - A_0)$ versus 1/[ligand] is linear, and

Table 1. Comparison Between the Binding Free Energies of Glycyrrhizin and Glycyrrhetinic Acid Interactions

$\Delta G_{binding}$[a] (kcal/mol)	*Torsional energy* (kcal/mol)	*Internal energy* (kcal/mol)	*Intermol energy* (kcal/mol)	*Complex*
				Experimental data
−5.12				GL-ctDNA[b]
−5.06				GA-ctDNA
				Calculated data
−5.43	4.47	−2.24	−9.91	GL-1D30[c]
−3.86	0.89	0.16	−4.75	GA-1D30

[a]$\Delta G_{binding}$ is the sum of intermolecular energy and torsional free energy.
[b]Calf thymus DNA.
[c]$d(CGCGAATTCGCG)_2$.
GL, glycyrrhizin; GA, glycyrrhetinic acid.

the binding constant (K) can be estimated from the ratio of the intercept to the slope (Fig. 4), where A_0 is the initial absorbance of the free DNA at 258 nm and A is the recorded absorbance of DNA in the presence of different GL and GA concentrations. The overall binding constants are estimated to be $K_{GL\text{-}DNA} = 5.7 \times 10^3\ M^{-1}$ and $K_{GA\text{-}DNA} = 5.1 \times 10^3\ M^{-1}$.

Docking study

To determine the preferred binding sites on DNA, the GL and GA were docked to DNA. The dockings results are shown in Figures 5 and 6. The comparisons between experimental and calculated data are shown in Table 1.

The models show that GL is surrounded by G16.A, A17.B, A18.B, T7.A, T8.A, C9.A (Fig. 5), and phosphate groups with a binding energy of −5.43 kcal/mol (Table 1) and GA is surrounded by A17.B, A18.B, T19.B, T8.A, T7.A (Fig. 6), and phosphate groups with a binding energy of −3.86 kcal/mol (Table 1).

Data derived from GL, GA, and 1D30 docking shows different mood energy interactions. The structure that had the most compatibility with the FTIR results was selected. The FTIR and UV results showed only external binding of GL and GA to DNA (for GL mainly with G, A, and T; for GA with A and T). The selected docking data show minor groove binding into oligonucleotide (1D30) with a greater tendency to A (Figs. 5 and 6). Most known minor groove–binding drugs preferably recognize A-T sequences. However, minor groove binders are designed to have interaction with G-C pairs (Goodsell *et al.*, 1995; Kielkopf *et al.*, 1998a, b; Dervan and Edelson, 2003).

Conclusions

Based on our spectroscopic results and docking studies, the following points are important: GL and GA interact with DNA via external binding, with overall binding constants of $K_{GL\text{-}DNA} = 5.7 \times 10^3\ M^{-1}$, $K_{GA\text{–}DNA} = 5.1 \times 10^3\ M^{-1}$. GL binding is via G, A, T, and PO_2, whereas GA interacts with A, T, and PO_2 backbone group. The affinity of ligand–DNA binding is in the order of GL > GA. DNA remains in the B-family structure, whereas biopolymer aggregation occurs at high GL and GA concentrations.

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Disclosure Statement

No competing financial interests exist.

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Biochemical and histological effects of 26 weeks of glycyrrhizin treatment in chronic hepatitis C: A randomized phase II trial☆

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See Editorial, pages 473–475

***Background/Aims*: Phase I/II studies of 4 weeks duration have confirmed the ALT lowering effect of glycyrrhizin in Western chronic hepatitis C patients. Our aim was to determine the dose frequency of glycyrrhizin required to maintain the ALT response beyond 4 weeks and evaluate its effect on liver histology and quality of life.**

***Methods*: HCV-RNA-positive patients with elevated ALT and marked fibrosis or necro-inflammation who were not eligible for interferon therapy were treated for 4 weeks with six infusions weekly of glycyrrhizin. Patients with an ALT response at week 4 were randomized to continue treatment for 22 weeks in three dose frequency groups: 6×, 3× or once weekly.**

***Results*: 72/121 (60%) patients were randomized. At the end of treatment the ALT response was maintained in 60%, 24% and 9% of patients in the 6×, 3×, and once weekly groups, respectively ($p < 0.001$). In ALT responders the necro-inflammation score improved non-significantly compared to ALT non-responders. Quality of life assessed by SF-36 increased in patients treated with the study drug, albeit unrelated to the occurrence of ALT response.**

***Conclusions*: ALT responses induced by 4 weeks glycyrrhizin therapy can be maintained in a subset of chronic hepatitis C patients receiving at least three injections weekly. The observed ALT response did not translate in a significant histological improvement after 6 months treatment.**

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☆ S.W. Schalm who has taken part in this study has declared a relationship with the manufacturers of the drugs involved and he received funding from the drug companies involved to carry out their research. He received funding from the Foundation of Liver Research Rotterdam which enabled him to carry out the study.

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Keywords: **Stronger Neo-Minophagen; Alternative medicine; Clinical trial; Multicenter; Liver histology; Quality of life; Alanine aminotransferase**

1. Introduction

Chronic hepatitis C infection can be associated with progressive liver disease that may evolve insidiously to cirrhosis with an increased risk of hepatocellular carcinoma (HCC) and liver failure [1–3]. Antiviral treatment with (peg)interferon–ribavirin combination therapy has become successful in 50–85% of patients over the past decade [4,5]. For those not responding or those patients with absolute contraindications to (peg)interferon–ribavirin combination therapy, different treatment strategies have to be sought. These approaches might include viral suppressive or antifibrotic therapy. Studies on chronic hepatitis B and C have shown that persistent normalization of ALT is important in reducing the complications of chronic hepatitis, regardless of ongoing viral replication [6,7].

Glycyrrhizin, a natural compound extracted from the roots of *Glycyrrhiza glabra*, has in vitro antiviral effect against multiple viruses [8–12]. The underlying mechanism of its antiviral effect is not fully elucidated. Both inhibition and augmentation of T-cell cytotoxicity have been reported as well as stimulation of the endogenous production of interferon. It has been shown that glycyrrhizin alters cellular viral penetration and diminishes cell lysis through cell membrane stabilization [13–17]. In Japan glycyrrhizin has been used as a treatment for chronic hepatitis for more than 20 years. In a double-blind randomized placebo-controlled trial, Suzuki showed that in Japanese patients with chronic hepatitis the serum transaminases decreased during the treatment with glycyrrhizin given intravenously as Stronger Neo-Minophagen C (SNMC) [18]. After discontinuation of the medication the serum transaminases rebounded, but this could be prevented by maintenance therapy. In a retrospective study, Arase concluded that long-term usage of glycyrrhizin is effective in preventing HCC development when ALT normalizes during therapy in Japanese patients with chronic hepatitis C [19]. Besides pseudo-hyperaldosteronism, glycyrrhizin treatment has hardly ever been associated with side effects.

In contrast to Japan, the European experience with glycyrrhizin treatment has been limited. In two pilot studies of 4 weeks duration we confirmed the ALT lowering effect of glycyrrhizin in European patients even though there was no decrease in viremia [20,21]. We have now designed a study of 26-weeks treatment with glycyrrhizin to evaluate the dose and frequency required to maintain the initial ALT response beyond week 4. Furthermore, the effect of the 26-weeks treatment with glycyrrhizin on liver histology and quality of life was studied.

2. Patients and methods

2.1. Patients

Patients between 18 and 70 years of age were eligible if they met all inclusion criteria: serum antibodies against HCV; HCV-RNA-positive; serum alanine aminotransferase (ALT) levels at least twofold the upper limit of normal on two occasions in the 12 weeks before initiation of treatment; liver biopsy consistent with fibrosis stage 3–6 or necro-inflammation score 6–12 according to Ishak's score [22] and non-eligibility for interferon therapy (previous non-response or contraindications to interferon such as psychiatric co-morbidity or unwillingness to undergo interferon based treatment albeit fully informed about benefits and risks).

Patients were excluded if they had decompensated liver disease, hepatocellular carcinoma, other causes of liver disease, malignancy other than skin basocellular carcinoma in the previous 5 years; human immunodeficiency virus infection; immunosuppressive therapy; antiviral treatment in the preceding 3 months; pregnancy, breast-feeding; hypokalemia, hyperaldosteronism, myopathy, use of thiazide diuretics and liquorice addiction or if they were unwilling to use contraception for the whole study period including 3 months after treatment.

2.2. Study design

This randomized, open phase II clinical trial was conducted at tertiary care European centers. All patients provided written informed consent and the protocol was approved by each center's Institutional Ethics Committee. The Clinical Research Bureau of the University Medical Center Rotterdam coordinated the study and was responsible for verification of the inclusion criteria, randomization and data acquisition. An independent Contract Research Organization ensured that the study was conducted according to Good Clinical Practice.

All patients were treated with glycyrrhizin six times a week for the first 4 weeks. Glycyrrhizin was given as Stronger Neo-Minophagen C (SNMC, supplied by Minophagen Pharmaceutical Co. Ltd., Tokyo, Japan), consisting of 40 mg glycyrrhizin, 20 mg cysteine and 400 mg glycine in 20 ml physiological saline. Medication per visit consisted of 5 ampoules of 20 ml administered directly into a peripheral vein in a 3–5 min period through a 21 G butterfly needle or an indwelling 22 G plastic canula. All patients were treated as outpatients.

At week 4, patients with an ALT response, defined as a decrease of 50% or more of the baseline value or a serum ALT level $\leqslant 1.5 \times$ upper limit of normal, were randomized to one of the three study groups: glycyrrhizin administration six times per week, three times per week or once per week for an additional 22 weeks. Patients not tolerating the six times per week administration of the study drug were allowed to have the dose frequency reduced to a tolerable regimen.

Sample size was calculated using a simulation with ALT response at the end of treatment as primary outcome. Assuming that 70% of the included patients would continue beyond week 4 and ALT normalization of, respectively, 50%, 30% and 10% for the 6× weekly, 3× weekly and once weekly dose frequency group, at least 120 patients needed to be included to reach a power of 90% (trend test) at $\alpha = 0.05$ (two-sided).

2.3. Randomization

Randomization was done centrally at the coordination center directly after receipt of the week 4 patient data from the participating center. After verification of the week 4 ALT response, one of the 84 sealed opaque envelopes, prepared by the biostatistician according to a computer generated randomization list, containing the group alloca-

tion was drawn by a senior data manager. The group allocation was subsequently faxed to the participating center.

2.4. Patient assessment

Patients were assessed for safety, tolerance and efficacy at the end of weeks 2, 4 and then every 4 weeks. Follow-up visits to monitor potential late side effects were scheduled at 12 and 26 weeks after completion of therapy. Routine hematological and biochemical testing (including serum ALT) was done by the local laboratories of the participating centers. Quantitative serum HCV-RNA was assessed centrally at Rotterdam at the start of treatment, at week 4 and at the end of therapy week 26 by a standardized quantitative reverse transcription polymerase chain reaction (RT-PCR) assay with a sensitivity of 1000 copies/mL (Roche Cobas Amplicor v 2.0).

2.5. Liver histology

All patients had a liver biopsy performed in the 6 months preceding the start of therapy. A second liver biopsy was taken at the end of therapy. All liver biopsies were assessed both centrally at Rotterdam and externally at Goteborg by a team of two observers who were unaware of the patient's identity, timing of the biopsy and the treatment received. Biopsies were assessed for inflammation and fibrosis by the Ishak score [22].

2.6. Health Related Quality of Life (HRQoL)

The Short-Form36 (SF-36) questionnaire was filled in by the study participants at week 0, 4 and 26 to evaluate the quality of life under treatment. It includes eight multi-item scales on Physical Functioning (PF), Role limitations due to Physical Problems (RP), Bodily Pain (BP), General Health (GH), Vitality (VT), Social Functioning (SF), Role limitations due to Emotional problems (RE) and Mental Health (MH) [23,24].

Patients were not informed about their ALT values prior to filling in the SF-36 questionnaire.

2.7. Study endpoints

The primary measure of efficacy was ALT response, defined as serum ALT $\leqslant$1.5 time upper limit of normal at week 26. The secondary measure of efficacy was histological activity index at end of treatment (week 26) compared to histological activity index before start of treatment. The third outcome measure was the Health Related Quality of Life (HRQoL) at week 4 and week 26 compared to the HRQoL at week 0.

2.8. Statistical analysis

For the primary statistical analysis the Modified Intention To Treat population (M-ITT: all patients included in the study who fulfilled the inclusion criteria and who received at least one dose of glycyrrhizin after week 4) was selected in view of the aims of the study. The statistical analyses were performed with the use of the SPSS/PC software package (version 11.0 SPSS). The percentage ALT response between groups was compared using a χ^2 trend test. Kruskal–Wallis test was used to compare the median ALT between the three treatment arms. p-values below 0.05 were considered significant. Logistic regression analysis was performed to study possible correlations between baseline variables and ALT response.

Histological changes at the end of treatment were assessed using Kruskal–Wallis test for comparison between pre-treatment and post-treatment groups, and the Mann–Whitney Exact test for comparison between ALT responders versus non-responders. The difference in Quality of life between week 0 and 4, and between week 4 and 26 (or week 0 and 26) was assessed with one-way analysis of variance for each parameter of the SF-36 questionnaire. In a generalized linear model, the outcomes of SF-36 were compared between treatment arms and ALT responses at week 26.

3. Results

3.1. Characteristics of patients

One hundred and twenty one patients were enrolled between October 1999 and October 2002. The trial profile is shown in Fig. 1. After 4 weeks of six times weekly treatment, 72 of 115 patients were randomized. Two patients declined further study participation and were excluded from the M-ITT group. Another two patients were excluded from the M-ITT population: in these two patients the study drug was discontinued at 6 and 8 weeks because of the diagnosis of a colorectal cancer and a multiple myeloma, respectively. Retrospective determination of carcinoembryonic antigen (CEA) and paraproteinemia in a stored screening sample demonstrated that these two diagnoses were already subclinically present at inclusion and hence not related to the therapy. Thus a total of 68 out of 72 randomized patients fulfilled all inclusion criteria and received at least one dose of the study drug after week 4, constituting the M-ITT population. The baseline characteristics of the Total Study Population, M-ITT and three treatment arms are shown in Table 1. All groups showed similar demographic, biochemical and virological baseline characteristics.

3.2. Biochemical response

The evolution of serum ALT for the three different dose groups after randomization is shown in Fig. 2. The median ALTs at the end of treatment were $1.2 \times$ ULN, $1.9 \times$ ULN and $2.5 \times$ ULN for the 6×, 3×, 1× weekly dose groups, respectively ($p \leqslant 0.001$ Kruskal–Wallis test) (Fig. 2a). The pre-defined ALT endpoint $\leqslant$1.5 ULN was met in 60% (12/20), 24% (6/25) and 9% (2/23) of the patients in the 6×, 3× and 1× weekly dose groups, respectively ($p \leqslant 0.001$ χ^2 trend test). The initial ALT response, set as ALT $\leqslant$1.5 ULN or a 50% reduction from baseline value, was maintained in 75% (15/20), 48% (12/25) and 26% (6/23), respectively ($p \leqslant 0.001$ χ^2 trend test) (Fig. 2b). Complete ALT normalization (ALT $\leqslant$1.0 ULN) was observed in 30% (6/20), 12% (3/25) and 0% (0/23) for the three treatment groups, respectively ($p \leqslant 0.015$ χ^2 trend test). In a logistic regression analysis, none of the baseline characteristics (sex, age, genotype, viral load, presence of cirrhosis, height, weight, body mass index, baseline ALT, inflammation or fibrosis score) were predictive for ALT response. Between 50% and 95% of those with an ALT response after 4 weeks maintained the response when fully compliant with the assigned therapy of 3–6 times per week. Seventy-five percent of those assigned to 6 times per week were fully compliant; 95% compliance was observed in the 3 times per week group.

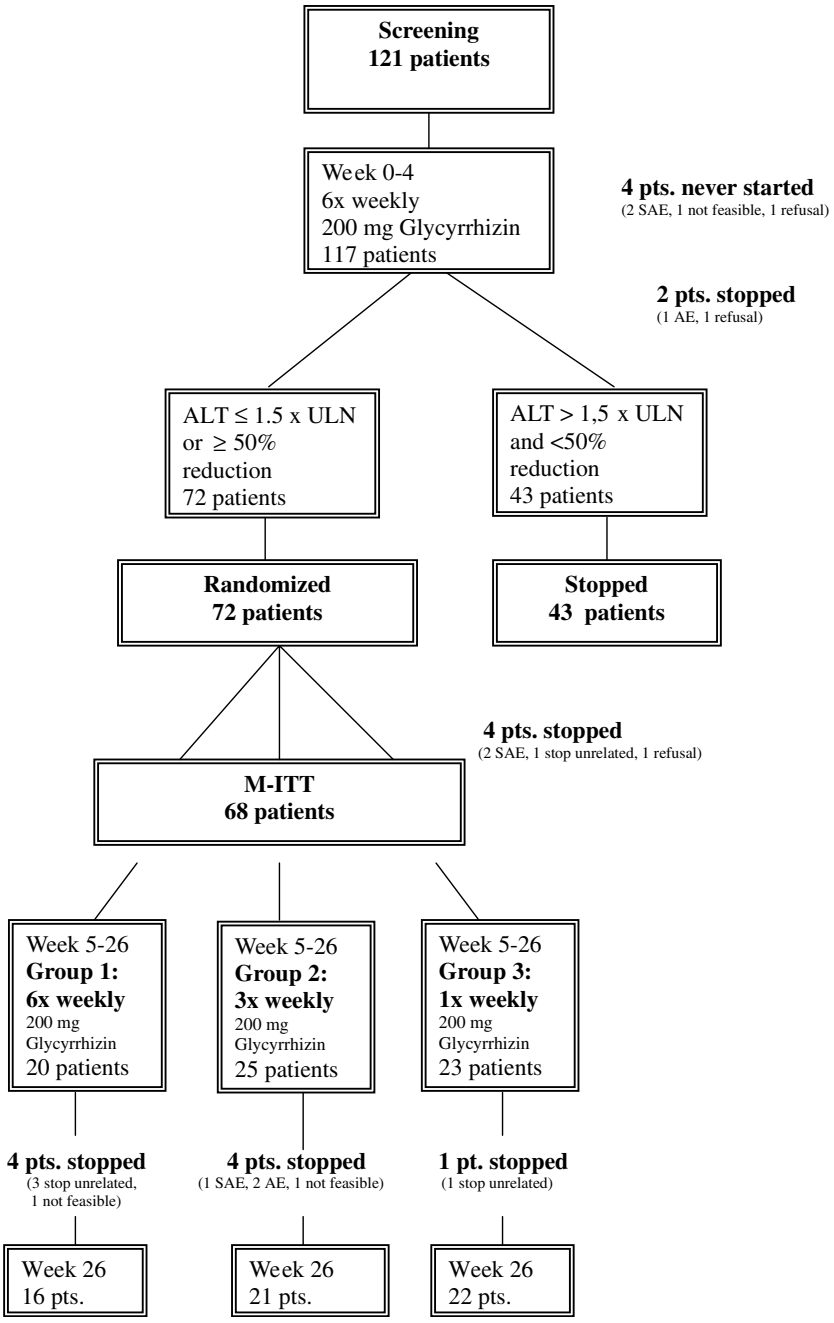

Fig. 1. Trial profile.

3.3. Histological response

Pre-treatment biopsies were available for central evaluation in 117 of the 121 patients. Post-treatment biopsies were available in 55 of the 56 M-ITT patients who completed the study. Due to inadequate sample size prohibiting reliable staging, pair-wise analysis of pre- and post-treatment biopsies was possible in 42 pairs. Twelve pairs were available in patients with an ALT response and 30 pairs in biochemical non-responders.

The median difference in Ishak scores between post and pre-treatment biopsies is shown in Fig. 3. Between randomization groups no significant differences were observed either in inflammation or fibrosis score. In ALT responders there was a tendency towards improvement in the inflammation score: median improvement −0.50, interquartile range [−1.75, 0.03] at the end of the treatment ($p < 0.07$; Mann–Whitney Exact test) while the ALT non-responders developed a worse inflammation score: median 1.00, interquartile range [−1.0, 2.0]. These findings were independently confirmed by a second reading of the biopsies by the external pathological team.

No changes in fibrosis score were detected in both responders and non-responders in this six months study.

Table 1
Patient characteristics

	Total study population	Week 4 non-randomized	Week 4 randomized	M-ITT	6× weekly	3× weekly	1× weekly
Number of patients	121	49	72	68	20	25	23
Sex, Male (%)	62	65	60	60	65	68	65
Age, year, mean (SD)	51 (10)	50 (12)	52 (10)	52 (10)	52 (9)	53 (9)	51 (11)
Caucasian (%)	87	92	83	84	80	84	87
Genotype 1 (%)	66	65	67	66	55	76	65
Cirrhosis (%) (Ishak 5 + 6)	50	43	55	55	56	61	50
Viral load, copies/ml (median)	4.2×10^6	3.6×10^6	4.3×10^6	4.2×10^6	5.5×10^6	4.0×10^6	2.1×10^6
Previous IFN therapy (%)	73	73	74	73	65	75	77
Median ALT, x ULN (range)	3.3 (1.5–11.2)	3.3 (1.6–11.2)	3.5 (1.5–10.1)	3.7 (1.5–10.1)	2.8 (1.6–10.1)	3.1 (2.1–9.0)	4.2 (1.5–9.4)
BMI, mean (SD)	26.9 (4.3)	27.1 (4.1)	26.8 (4.4)	26.6 (4.3)	26.8 (3.9)	25.8 (2.6)	27.4 (5.9)

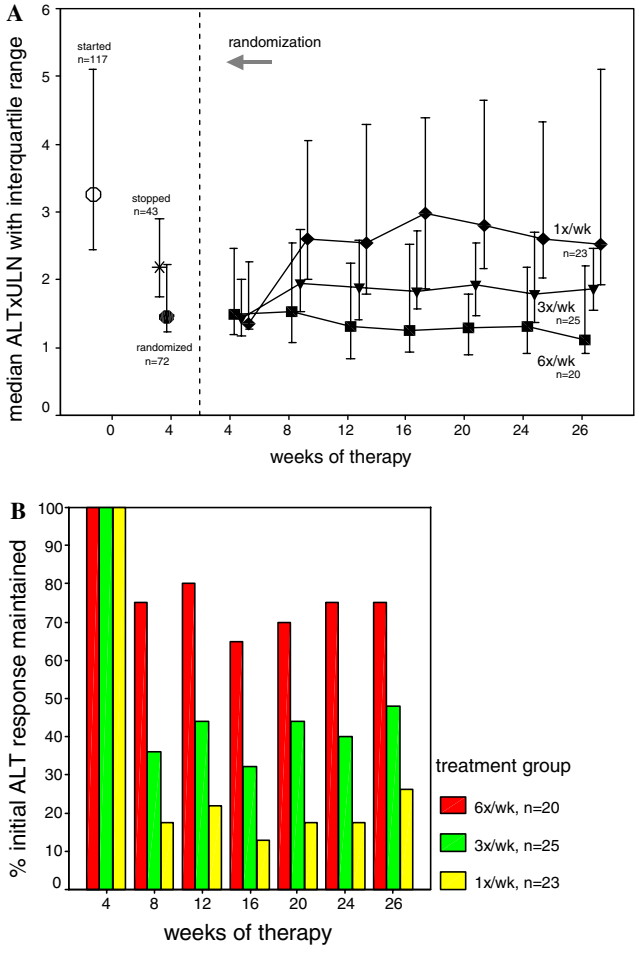

Fig. 2. On treatment ALT evolution. (A) The median ALT evolution from week 0 to week 4 (time of randomization) and from randomization to week 26 is shown for each treatment group together with the interquartile range (the 25th and 75th percentile). (B) Percentage of patients with an initial ALT response (ALT level $\leqslant 1.5 \times$ ULN or a decrease of at least 50% from baseline value) maintained during therapy. [This figure appears in colour on the web].

3.4. Quality of life

The SF-36 questionnaire was available in 111, 99 and 84 patients at week 0, 4 and 26, respectively. Patients completing the first 4 weeks of treatment had a significant improvement in five out of eight scales (all $p \leqslant 0.026$). The patients who had ALT response at week 4 had a comparable improvement in the same five parameters to those without an ALT response, suggesting that the administration of intravenous glycyrrhizin six times weekly in the study protocol rather than the ALT response induced the improvement in quality of life.

For the M-ITT population of 68 patients, the SF-36 was available in 64, 58 and 57 patients at week 0, 4 and 26, respectively. Only one scale (Physical Functioning) was statistically better at end of treatment compared to start of treatment (Fig. 4a). On the two assessments made at week 4 and 26 on treatment, no deterioration of any of the SF-36 parameters compared to the baseline values was seen, reflecting the good tolerability of intravenous glycyrrhizin during the 26 week treatment period.

Patients who continued treatment had overall higher SF-36 scores compared to patients who stopped treatment at week 4 according to protocol because of non-response (these differences were significant for Physical Functioning, Role limitations due to Physical problems and General Health, Fig. 4b).

3.5. Serum HCV-RNA

No changes in serum HCV-RNA were observed during the study period regardless of study arm or ALT response. The overall mean log difference between week 0 and week 4 was 0.04; the mean log decline of

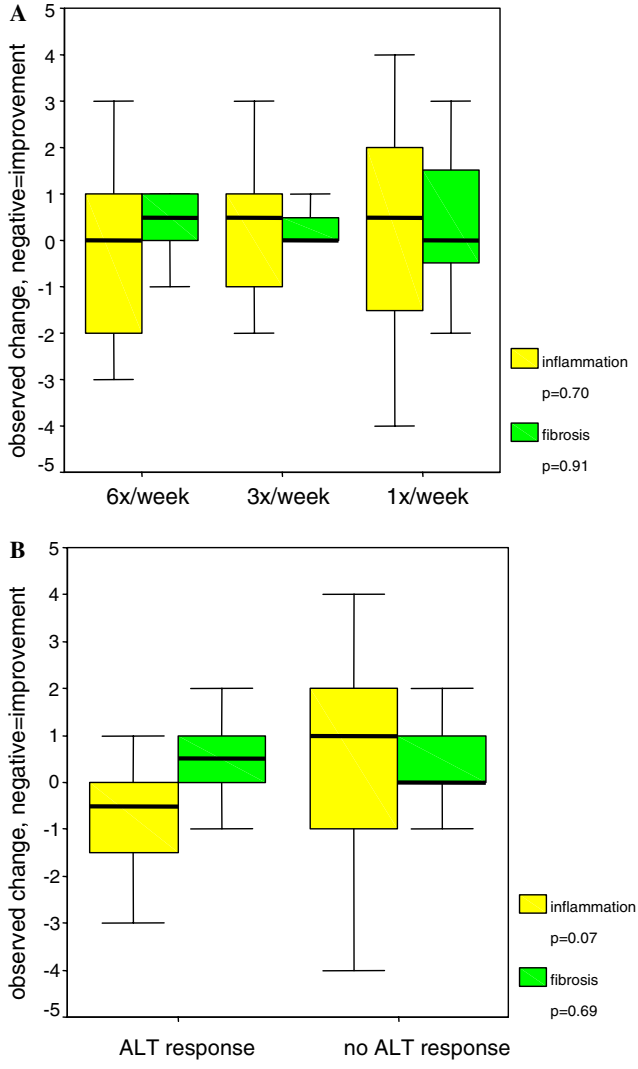

Fig. 3. Histology changes by randomization group (A) and by ALT response (B) between $t = 0$ and after 26 weeks of glycyrrhizin therapy (medians, 25–75% percentiles). Between randomization groups no significant differences were observed (A). Patients with an ALT response had a median improvement of 0.50 points in the HAI, whereas patients without ALT response had a deterioration of 1.0 point ($p < 0.07$; Mann–Whitney Exact test). Changes in fibrosis score between patients with ALT response and ALT non-response were not significant (B). [This figure appears in colour on the web].

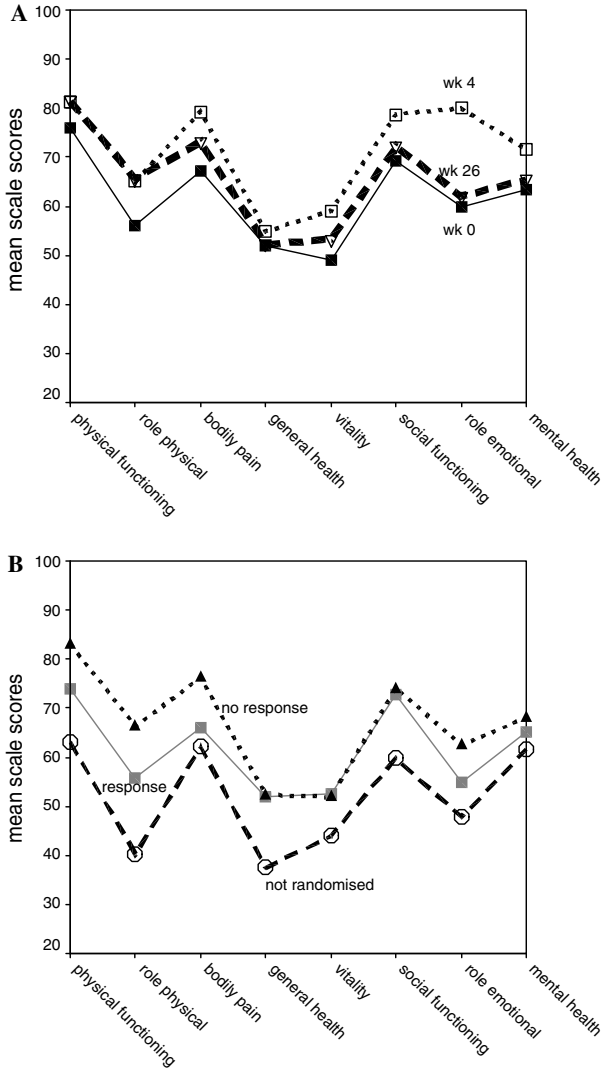

Fig. 4. Quality of life. (A) Mean HRQoL scale scores of the M-ITT population with a completed SF-36 questionnaire at week 0, week 4 and week 26 ($n = 53$ pairs). Only the Physical Functioning was significantly improved comparing week 0 with week 26. (B) Mean HRQoL scale scores at week 26 of the M-ITT population according to ALT response (SF-36 available in 18 patients with an ALT response and 39 patients without an ALT response) and of patients not randomized ($n = 23$). At week 26 the Physical Functioning, Role limitations due to Physical Problems and General Health were significant better for patients still on treatment compared to patients not randomized indicating the excellent tolerability of glycyrrhizin therapy.

HCV-RNA values between week 4 and 26 was 0.19, 0.07, 0.05 for 6×, 3× and 1× weekly dose groups, respectively.

3.6. Safety and side effects and tolerability (Table 2)

Treatment was well tolerated in the large majority of patients. In total, 15 serious adverse events (SAE) were reported in 14 patients from the total study population. All of these serious adverse events, except "hypokalemia and tachyarrhythmia", were unrelated to the study drug. In nine cases with SAE unrelated to the study drug the study medication was continued without change; in five patients the study drug was discontinued.

Patients who stopped or had dose reduction due to abnormalities (Adverse Events) considered to be related to glycyrrhizin had often symptoms or signs related to glycyrrhizin induced pseudo-aldosteronism (hypokalemia, ascites, hypertension and tachyarrhythmia). Thrombophlebitis related to the daily intravenous administration occurred only in one patient of the total study population.

Table 2
Adverse events, incidence and effect on study drug

	Number of patients	Effect on study drug
Related adverse events		
Hypokalemia (SAE)	1	Dose reduction
Tachyarrhythmia (1× SAE)	2 (1 with hypokalemia)	Dose reduction
Hypertension	7 (1 worsening of hypertension)	Treatment discontinuation in two patients (one with concomitant thrombophlebitis), dose reduction in five patients
Ascites	1	Discontinued
Thrombophlebitis	1	Discontinued
Other: combination of: Fatigue, perspiration, pain in the back, weight loss, sweating, loss of appetite, circulatory problems	2	Discontinued
Unrelated serious adverse events		
Colon carcinoma	1	Discontinued
Morbus Kahler	1	Discontinued
Bilateral pneumonia	1	Discontinued
Transient ischemic attack	1	Discontinued
Vasovagal reaction before injection	1	Discontinued
Vasovagal reaction after liver biopsy	1	None
Spinocellular Epithelioma of Larynx	1	None
Abdominal pain and suspected. Hemobilia after liver biopsy	1	None
Hepatocellular carcinoma	1	None
Admission for evaluation liver Transplantation	1	None
Rectal bleeding	1	None
Pain after liver biopsy	1	None
Attempt to suicide	1	None
Myocardial infarction	1	None

4. Discussion

Our study population consisted of patients who were not eligible for interferon therapy because of previous non-response or the presence of contraindications. The majority of the patients included had genotype 1, cirrhosis and a high viral load. The current treatment options for this difficult to treat population are limited. The 2002 NIH consensus on hepatitis C stressed the importance of organizing trials with antifibrotic, anti-inflammatory and immunomodulatory drugs in this patient group, including trials evaluating the efficacy of alternative and non-traditional therapies [11]. Two recent meta-analyses of complementary and alternative therapy for hepatitis C concluded that at the time of the analysis, scientifically insufficient data on glycyrrhizin therapy were present to evaluate its usefulness [25,26]. Our study is a randomized controlled trial evaluating the efficacy of 26 weeks of glycyrrhizin monotherapy in Western patients.

The majority of patients (60%) had a significant ALT decrease after 4 weeks treatment with 6 times weekly administration of glycyrrhizin, confirming our previous controlled study [11]. More than 80% of patients who continued six times weekly maintained the induction response observed at 4 weeks. The pre-defined study endpoint at 26 weeks with ALT levels $\leqslant$1.5 upper limit of normal was reached in 18 of the 45 (40%) patients who continued to receive at least three times weekly the study drug beyond week 4. So, a potentially relevant biochemical response can be maintained with 3–6 injections of glycyrrhizin per week in part of the patients who at present have no other proven treatment alternatives. As in our previous study, no effect on viremia was seen.

Our study was developed to have a statistical power of 90% for the primary endpoint. Fewer patients than pre-conceived were randomized at week 4; on the other hand, a higher fraction than estimated in the pre-study power analysis maintained ALT response in the 6× weekly group. A post hoc analysis would give a power of 86%, but the post hoc analysis approach is now considered fundamentally flawed. If one would calculate the sample size for a similar future study on the basis of our findings, the same number of 120 patients would be needed to obtain a power of 90%.

A Japanese controlled trial performed prior to the discovery of hepatitis C demonstrated histological improvement in glycyrrhizin treated patients [27]. The histological changes observed in the paired biopsies in our study show a non-significant decrease in inflammatory score with an unchanged fibrosis score in patients with an ALT response of $\leqslant$1.5 ULN at the end of 6 month study period. Supplementary studies with a larger sample size will be needed to demonstrate or negate a significant histological benefit.

The general concern that the frequent i.v. dosing of glycyrrhizin would be an important impediment in a Western population for this therapy was not substantiated. In fact, an improvement in quality of life parameters was observed during glycyrrhizin treatment. The observation that improvement in quality of life did not

correlate with ALT response makes interpretation of the findings hazardous. However, it points to the absence of important side effects of glycyrrhizin therapy.

In conclusion, this study shows that glycyrrhizin therapy is able to induce and maintain ALT response in a small proportion of chronic hepatitis C patients who, at present, have no proven therapeutic alternative. Although the observed effect requires frequent dosing, this treatment is usually well tolerated. The ALT response that is obtained by glycyrrhizin therapy may be capable of halting the necro-inflammation in the liver. This and its eventual effect on fibrosis progression mandates further study including histological evaluation after a longer treatment period in a bigger sample size before this treatment can be regarded as unequivocally beneficial to chronic hepatitis C patients.

Acknowledgement

All contributing physicians and research nurses were invaluable in achieving a high compliance to the intravenous study medication. We gratefully acknowledge the support of Professor H.E. Blum (University Hospital Freiburg, Germany). Mrs. U. Hahn (HPC), Mrs. A. Duquet (DRC), Mrs. K. Marinissen (IMRO Tramarko) as study monitors succeeded in high quality documentation of study findings. Prof. Tango, Prof. Suzuki and Prof. Iino were helpful with advice prior to and during the study as well as by reading the manuscript. This study was supported by Minophagen Pharmaceutical Co., Ltd., Japan.

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CME

Alcoholic Liver Disease

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These recommendations provide a data-supported approach. They are based on the following: (i) a formal review and analysis of the recently published world literature on the topic (Medline search); (ii) American College of Physicians Manual for Assessing Health Practices and Designing Practice Guidelines (1); (iii) guideline policies, including the American Association for the Study of Liver Diseases (AASLD) Policy on the development and use of practice guidelines and the AGA Policy Statement on Guidelines (2); and (iv) the experience of the authors in the specified topic. Intended for use by physicians, these recommendations suggest preferred approaches to the diagnostic, therapeutic, and preventive aspects of care. They are intended to be flexible, in contrast to the standards of care, which are inflexible policies to be followed in every case. Specific recommendations are based on relevant published information. To more fully characterize the quality of evidence supporting the recommendations, the Practice Guideline Committee of the AASLD requires a Class (reflecting the benefit vs. risk) and Level (assessing the strength or certainty) of Evidence to be assigned and reported with each recommendation (Table 1, adapted from the American College of Cardiology and the American Heart Association Practice Guidelines) (3,4).

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PREVALENCE AND NATURAL HISTORY

Alcoholic liver disease (ALD) encompasses a spectrum of injury, ranging from simple steatosis to frank cirrhosis. It may well represent the oldest form of liver injury known to mankind. Evidence suggests that fermented beverages existed at least as early as the Neolithic period (cir. 10,000 BC) (5). Alcohol remains a major cause of liver disease worldwide. It is common for patients with ALD to share the risk factors for simultaneous injury from other liver insults (e.g., co-existing non-alcoholic fatty liver disease, or chronic viral hepatitis). Many of the natural history studies of ALD and even treatment trials were performed before these other liver diseases were recognized, or specific testing was possible. Thus, the individual effect of alcohol in some of these studies may have been confounded by the presence of these additional injuries. Despite this limitation, the data regarding ALD are robust enough to draw conclusions about the pathophysiology of this disease. The possible factors that can affect the development of liver injury include the dose, duration, and type of alcohol consumption, drinking patterns, gender, ethnicity, and associated risk factors, including obesity, iron overload, concomitant infection with viral hepatitis, and genetic factors.

Geographic variability exists in the patterns of alcohol intake throughout the world (6). Approximately two-thirds of the adult Americans drink alcohol (7). The majority drink small or moderate amounts and do so without evidence of clinical disease (8–10). A subgroup of drinkers, however, drink excessively, develop physical tolerance and withdrawal, and are diagnosed with alcohol dependence (11). A second subset, alcohol abusers and problem drinkers, are those who engage in harmful use of alcohol, which is defined by the development of negative social and health consequences of drinking (e.g., unemployment, loss of family, organ damage, accidental injury, or death) (12). Failure to recognize alcoholism remains a significant problem and impairs efforts at both the prevention and the management of patients with ALD (13,14). Although the exact prevalence is unknown, approximately 7.4% of adult Americans were estimated to meet the Diagnostic and Statistical Manual of Mental Disorders, 4th edition, criteria for the diagnosis of alcohol abuse and/or alcohol dependence in 1994 (15); more recent data suggest 4.65% meet the criteria for alcohol abuse and 3.81% for alcohol dependence (16). In 2003, 44% of all deaths from liver disease were attributed to alcohol (17).

The population-level mortality from ALD is related to the per capita alcohol consumption obtained from national alcoholic beverage sales data. There are conflicting data regarding a possible lower risk of liver injury in wine drinkers (18,19). One epidemiological study has estimated that for every 1 l increase in per capita alcohol consumption (independent of the type of beverage), there was a 14% increase in cirrhosis in men and 8% increase in women (20). These data must be considered in the context of the limitations of measuring alcohol

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Table 1. Grading system for recommendations

Classification	Description
Class I	Conditions for which there is evidence and/or general agreement that a given diagnostic evaluation, procedure or treatment is beneficial, useful, and effective
Class II	Conditions for which there is conflicting evidence and/or a divergence of opinion about the usefulness/efficacy of a diagnostic evaluation, procedure, or treatment
Class IIa	Weight of evidence/opinion is in favor of usefulness/efficacy
Class IIb	Usefulness/efficacy is less well established by evidence/opinion
Class III	Conditions for which there is evidence and/or general agreement that a diagnostic evaluation/procedure/treatment is not useful/effective and in some cases may be harmful
Level of evidence	
Level A	Data derived from multiple randomized clinical trials or meta-analyses
Level B	Data derived from a single randomized trial or nonrandomized studies
Level C	Only consensus opinion of experts, case studies, or standard of care

Table 2. Quantity of alcohol in a standard drink

	Amount (g)	Range (g)
USA	12	9.3–13.2
Canada	13.6	13.6
UK	9.5	8–10
Europe	9.8	8.7–10.0
Australia and New Zealand	9.2	6.0–11.0
Japan	23.5	21.2–28.0

Adapted from Turner (262).
To standardize, many authorities recommend conversion to grams of alcohol consumed. To convert concentrations of alcohol, usually listed in volume percent (equivalent to the volume of solute/volume of solution × 100), the percentage of alcohol by volume (% v/v) is multiplied by the specific gravity of alcohol, 0.79 g/ml (263).

use and defining ALD. The scientific literature has also used a variety of definitions of what constitutes a standard drink (**Table 2**). Most studies depend on interviews with patients or their families to quantify drinking patterns, a method that is subject to a number of biases, which may lead to invalid estimates of alcohol consumption (21).

Although there are limitations of the available data, the World Health Organization's Global Alcohol database, which has been in existence since 1996, has been used to estimate the worldwide patterns of alcohol consumption and allow comparisons of alcohol-related morbidity and mortality (22). The burden of alcohol-related disease is the highest in the developed world, where it may account for as much as 9.2% of all disability-adjusted life years. However, even in the developing regions of the world, alcohol accounts for a major portion of the global disease burden, and is projected to take on increasing importance in those regions over time (22,23).

DISEASE SPECTRUM

The spectrum of alcohol-related liver injury varies from simple steatosis to cirrhosis. These are not necessarily distinct stages of evolution of the disease, but rather, multiple stages that may be present simultaneously in a given individual (24,25). These are often grouped into three histological stages of ALD, including fatty liver or simple steatosis, alcoholic hepatitis (AH), and chronic hepatitis with hepatic fibrosis or cirrhosis (26). The latter stages may also be associated with a number of histological changes (which have varying degrees of specificity for ALD), including the presence of Mallory's hyaline, megamitochondria, or perivenular and perisinusoidal fibrosis (24).

Fatty liver develops in about 90% of individuals who drink more than 60 g/day of alcohol (27), but may also occur in individuals who drink less (28). Simple, uncomplicated fatty liver is usually asymptomatic and self-limited, and may be completely reversible with abstinence after about 4–6 weeks (29). However, several studies have suggested that progression to fibrosis and cirrhosis occurs in 5–15% of the patients despite abstinence (30,31). In one study, continued alcohol use (>40 g/day) increased the risk of progression to cirrhosis to 30%, and fibrosis or cirrhosis to 37% (32).

Fibrosis is believed to start in the perivenular area and is influenced by the amount of alcohol ingested (33,34). Perivenular fibrosis and deposition of fibronectin occur in 40–60% of the patients who ingest more than 40–80 g/day for an average of 25 years. Perivenular sclerosis has been identified as a significant and independent risk factor for the progression of alcoholic liver injury to fibrosis or cirrhosis (33,35). Progression of ALD culminates in the development of cirrhosis, which is usually micronodular, but may occasionally be mixed micro- and macronodular (36).

A subset of patients with ALD will develop severe AH, which has a substantially worse short-term prognosis (37). AH also represents a spectrum of disease, ranging from mild injury to severe, life-threatening injury, and often presents acutely against a background of chronic liver disease (38,39). The true prevalence is unknown, but histological studies of patients with ALD suggest that AH may be present in as many as 10–35% of hospitalized alcoholic patients (40–42). Typically, symptomatic patients present with advanced liver disease, with concomitant cirrhosis in more than 50% of the patients, and superimposed

acute decompensation. However, even patients with a relatively mild presentation are at high risk of progressive liver injury, with cirrhosis developing in up to 50% of the patients (43,44). The likelihood that AH will progress to permanent damage is increased among those who continue to abuse alcohol. Abstinence from alcohol in one small series did not guarantee complete recovery. Only 27% of the abstaining patients had histological normalization, whereas 18% progressed to cirrhosis, and the remaining patients had persistent AH when followed for up to 18 months (45).

RISK FACTORS

Unlike many other hepatotoxins, the likelihood of developing progressive alcohol-induced liver disease or cirrhosis is not completely dose-dependent, as it occurs in only a subset of patients. A number of risk factors that influence the risk of development and progression of liver disease have been identified.

The amount of alcohol ingested (independent of the form in which it is ingested) is the most important risk factor for the development of ALD (46). The relationship between the quantity of alcohol ingested and the development of liver disease is not clearly linear (47,48). However, a significant correlation exists between per capita consumption and the prevalence of cirrhosis (49). The risk of developing cirrhosis increases with the ingestion of >60–80 g/day of alcohol for ≥10 years in men, and >20 g/day in women (6,50). Yet, despite drinking at these levels, only 6–41% of the individuals develop cirrhosis (6,51). In a population-based cohort study of almost 7,000 subjects in two northern Italian communities, even among patients with very high daily alcohol intake (>120 g/day), only 13.5% developed ALD (50). The risk of cirrhosis or non-cirrhotic chronic liver disease increased with a total lifetime alcohol intake of >100 kg, or a daily intake of >30 g/day (50). The odds of developing cirrhosis or lesser degrees of liver disease with a daily alcohol intake of >30 g/day were 13.7 and 23.6, respectively, when compared with non-drinkers (50).

The type of alcohol consumed may influence the risk of developing liver disease. In a survey of over 30,000 persons in Denmark, drinking beer or spirits was more likely to be associated with liver disease than drinking wine (18).

Another factor that has been identified is the pattern of drinking. Drinking outside of meal times has been reported to increase the risk of ALD by 2.7-fold compared with those who consumed alcohol only at mealtimes (52). Binge drinking, defined by some researchers as five drinks for men and four drinks for women in one sitting, has also been shown to increase the risk of ALD and all-cause mortality (53,54).

Women have been found to be twice as sensitive to alcohol-mediated hepatotoxicity and may develop more severe ALD at lower doses and with shorter duration of alcohol consumption than men (55). Several studies have shown differing blood alcohol levels in women vs. men after consumption of equal amounts of alcohol (56). This might be explained by differences in the relative amounts of gastric alcohol dehydrogenase, a higher proportion of body fat in women, or changes in alcohol absorption with the menstrual cycle (57). Based on epidemiological evidence of a threshold effect of alcohol, a suggested 'safe' limit of alcohol intake had been 21 units per week in men and 14 units per week in women who have no other chronic liver disease (58,59) (wherein a unit is defined as the equivalent of 8 g of ethanol). However, other data suggest that a lower quantity may be toxic in women, implying a lower threshold of perhaps no more than 7 units per week (47). A higher risk of liver injury may be associated with an individual's racial and ethnic heritage (60). The rates of alcoholic cirrhosis are higher in African-American and Hispanic males compared with Caucasian males and the mortality rates are the highest in Hispanic males (61). These differences do not seem to be related to differences in the amounts of alcohol consumed (62).

The presence and extent of protein calorie malnutrition have an important role in determining the outcome of patients with ALD. Mortality increases in direct proportion to the extent of malnutrition, approaching 80% in patients with severe malnutrition (i.e., <50% of the normal) (63). Micronutrient abnormalities, such as hepatic vitamin A depletion or depressed vitamin E levels, may also potentially aggravate the liver disease (64). Diets rich in polyunsaturated fats promote alcohol-induced liver disease in animals (65), whereas diets high in saturated fats may be protective. Obesity and excess body weight have been associated with an increased risk of ALD (66,67).

In addition to environmental factors, genetic factors predispose to both alcoholism and ALD (68–70). Children of alcoholics raised in adopted families had a significantly higher rate of alcohol dependence than adopted children of non-alcoholics, who served as controls (18% vs. 5%) (71). In population-based studies, monozygotic twins were approximately twice as likely to drink as dizygotic twins; among those who drank, monozygotic twins were more likely to have a similar frequency and quantity of alcohol consumption (72). Moreover, monozyotic twins had a significantly higher prevalence of alcoholic cirrhosis than dizygotic twins (73).

Finally, polymorphisms of genes involved in the metabolism of alcohol (including alcohol dehydrogenase, acetaldehyde dehydrogenase, and the cytochrome P450 system) and in those that regulate endotoxin-mediated release of cytokines have been associated with ALD (74,75). However, specific genetic abnormalities for susceptibility to alcohol abuse and the development of ALD have not yet been firmly established.

There is a clear synergistic relationship between chronic viral hepatitis and alcohol, resulting in more advanced liver disease jointly than separately. The combination of HCV and alcohol predisposes to more advanced liver injury than alcohol alone (76,77), with disease at a younger age, more severe histological features, and a decreased survival (78). In a large-cohort study of the effect of heavy alcohol abuse in patients with post-transfusion hepatitis C, the risk of cirrhosis was elevated 30-fold (79). Although the precise toxic threshold for alcohol is not known, and may be lower and non-uniform among patients at

risk, it seems prudent in light of these data to advise patients with hepatitis C to abstain from consuming even moderate quantities of alcohol.

DIAGNOSIS

The diagnosis of ALD is based on a combination of features, including a history of significant alcohol intake, clinical evidence of liver disease, and supporting laboratory abnormalities (80). Unfortunately, the ability to detect these is constrained by patient and physician factors, as well as diagnostic laboratory shortcomings. Denial of alcohol abuse and underreporting of alcohol intake are common in these patients (81,82). Physicians underestimate alcohol-related problems and make specific recommendations even less frequently (83,84). Both the physical findings and laboratory evidence for ALD may be non-diagnostic, especially in patients with mild ALD or early cirrhosis (85). Therefore, the clinician must have a low threshold to raise the issue of possible ALD, and has to rely on indirect evidence of alcohol abuse, such as questionnaires, information from family members, or laboratory tests to strengthen or confirm a clinical suspicion (86).

Screening for alcohol abuse

Clinicians commonly fail to screen patients, and thus fail to recognize or treat alcoholism appropriately (87). Clinical history that may suggest alcohol abuse or alcohol dependence includes the pattern, type, and amount of alcohol ingested, as well as evidence of social or psychological consequences of alcohol abuse. These may be suggested by other injuries or past trauma, such as frequent falls, lacerations, burns, fractures, or emergency department visits (88). Biochemical tests have been considered to be less sensitive than questionnaires in screening for alcohol abuse (89,90), but may be useful in identifying relapse (91,92). Various questionnaires have been used to detect alcohol dependence or abuse, and include the CAGE, the Michigan Alcoholism Screening Test, and the Alcohol Use Disorders Identification Test (89,93). A structured interview, using instruments such as the Lifetime Drinking History, is often used as a gold standard for quantifying lifetime alchohol consumption (94).

The CAGE questionnaire was originally developed to identify hospitalized inpatients with alcohol problems, and remains among the most widely used screening instruments. It has been faulted, however, on several measures—it focuses on the consequences of alcohol consumption rather than on the amount of actual drinking, and it refers to lifetime patterns of behavior, rather than short-term or recent changes. Its virtues, however, include its ease of implementation—it is short (four questions), simple (yes/no answers), and can be incorporated into the clinical history or self-administered as a written document. As a result of its longevity, it has been tested in a wide range of populations.

One meta-analysis of its characteristics, using a cutoff of more than two positive responses, found an overall pooled sensitivity and specificity of 0.71 and 0.90, respectively (95). The CAGE questionnaire is familiar to most physicians, and has been suggested for use in general screening (96) (**Table 3**). The Alcohol Use Disorders Identification Test is a 10-item questionnaire developed by the World Health Organization to avoid ethnic and cultural bias (97) and focus on the identification of heavy drinkers. It has a higher sensitivity and specificity than shorter screening instruments (with sensitivity ranging from 51 to 97%, and specificity from 78 to 96% in primary care) (98). It has been suggested that it has three advantages over other screening tests: it may identify drinkers at risk who are not yet alcohol-dependent; it includes a measure of consumption; and lastly, it includes both current and lifetime drinking time spans. It is more likely to detect problem drinking before overt alcohol dependence or abuse might be diagnosed, and thus may be more robust and effective across a variety of populations (99–101). One possible algorithm for clinicians suggests asking about the quantity of alcohol consumed, and the number of heavy drinking days in the preceding year (i.e., ≥ 5 drinks/day for men or ≥ 4 drinks/day for women), as well as administering a version of the Alcohol Use Disorders Identification Test questionnaire (102) (**Table 4**). An Alcohol Use Disorders Identification Test score of ≥ 8, or having had ≥ 1 heavy drinking days constitutes a positive screening test, and should prompt further evaluation to rule out an alcohol use disorder (102).

Table 3. The CAGE questionnaire (264)

1. Have you ever felt you should cut down on your drinking?
2. Have people annoyed you by criticizing your drinking?
3. Have you ever felt bad or guilty about your drinking?
4. Have you ever had a drink first thing in the morning to steady your nerves or to get rid of a hangover (eye-opener)?

Scoring: Each response is scored as 0 or 1, with a higher score indicative of alcohol-related problems, and a total of ≥ 2 being clinically significant.

Regardless of which screening instrument is selected, however, it is important for clinicians to incorporate screening into their general practice (98,103). This may be especially important, as some data suggest that these screening instruments may improve the ability of physicians to predict long-term clinical outcomes, including hospitalization for alcohol-related diagnoses (104).

One particular biomarker in longstanding use, gamma glutamyl transpeptidase (GGT), has been evaluated in a number of settings, including large population surveys (105,106). Unfortunately, its low sensitivity and specificity limit the usefulness of elevated GGT to diagnose alcohol abuse (107–109), the levels of which may fluctuate with extensive liver injury (110). Lower levels of GGT (<100) or a total bilirubin/GGT ratio >1 has been described as a predictor of 1-year mortality in patients with alcoholic cirrhosis (110), although this has not consistently added the prognostic ability to other lab tests (111). However, in combination with other biomarkers, GGT may help add independent information in diagnosing alcohol abuse

Table 4. AUDIT questionnaire (102)

Question	0	1	2	3	4
1. How often do you have a drink containing alcohol?	Never	Monthly or less	2–4 Times a month	2–3 Times a week	4 Or more times a week
2. How many drinks containing alcohol do you have on a typical day when you are drinking?	1 or 2	3 or 4	5 or 6	7–9	10 or more
3. How often do you have 5 or more drinks on one occasion?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
4. How often during the last year have you found that you were not able to stop drinking once you had started?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
5. How often during the last year have you failed to do what was normally expected of you because of drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
6. How often during the last year have you needed a first drink in the morning to get yourself going after a heavy drinking session?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
7. How often during the last year have you had a feeling of guilt or remorse after drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
8. How often during the last year have you been unable to remember what happened the night before because of your drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
9. Have you or someone else been injured because of your drinking?	No		Yes, but not in the last year		Yes, during the last year
10. Has a relative, friend, doctor, or other health-care worker been concerned about your drinking or suggested you cut down?	No		Yes, but not in the last year		Yes, during the last year

AUDIT, Alcohol Use Disorders Identification Test.
To score the AUDIT questionnaire, sum the scores for each of the 10 questions. A total ≥8 for men up to age 60, or ≥4 for women, adolescents, or men over the age of 60 is considered to be a positive screening test.

or problem drinking (112). Macrocytosis is seen in individuals abusing alcohol but lacks sensitivity. A combination of raised GGT and mean corpuscular volume or changes in these values over time in hospitalized patients may improve the sensitivity for diagnosing alcohol abuse. Multiple other candidate biomarkers that may detect alcohol use or abuse objectively have been studied (113,114). Carbohydrate-deficient transferrin has been the biomarker best studied, but has limited sensitivity and specificity (115). Its test characteristics are also influenced by a number of other factors, including age, gender, BMI, and other chronic liver diseases (116–118). Despite the enthusiasm about a possible quantitative, reliable assay of alcohol consumption or abuse, the lack of sensitivity and specificity prevent reliance on any single biomarker (119).

Diagnosis of ALD

The diagnosis of ALD is made by documentation of alcohol excess and evidence of liver disease (120). No single laboratory marker definitively establishes alcohol to be the etiology of liver disease. Furthermore, alcohol may be one of a number of factors causing liver injury, and the specific contributory role of alcohol alone may be difficult to assess in a patient with multifactorial liver disease. A number of laboratory abnormalities, including elevated serum aminotransferases, have been reported in patients with alcoholic liver injury, and used to diagnose ALD (121). Serum AST is typically elevated to a level of 2–6 times the upper limits of the normal in severe AH. Levels of AST >500 IU/l or ALT >200 IU/l are rarely seen with AH (other than alcoholic foamy degeneration or concomitant

acetaminophen overdose) (122), and should suggest another etiology. In about 70% of patients the AST/ALT ratio is >2, but this may be of greater value in patients without cirrhosis (123–125). Ratios >3 are highly suggestive of ALD (126).

Physical examination

Physical examination findings in patients with ALD may range from normal to those suggestive of advanced cirrhosis. As in other forms of chronic liver disease, physical examination features generally have low sensitivity, even for the detection of advanced disease or cirrhosis, although they may have higher specificity (127). Therefore, it has been suggested that the presence of these features may have some benefit in "ruling in" the presence of advanced disease (127). Features specific for ALD are perhaps even more difficult to identify. Palpation of the liver may be normal in the presence of ALD, and does not provide accurate information regarding liver volume (128). Certain physical examination findings have been associated with a higher likelihood of cirrhosis among alcoholics (129). Although some of the physical findings are more commonly observed in ALD (parotid enlargement, Dupuytren's contracture, and especially those signs associated with feminization) than in non-ALD, no single physical finding or constellation of findings is 100% specific or sensitive for ALD (130). Some of the physical examination features may also carry some independent prognostic information, with the presence of specific features associated with an increased risk of mortality over 1 year. These include (with their associated relative risks) hepatic encephalopathy (4.0), presence of visible veins across the anterior abdominal wall (2.2), edema (2.9), ascites (4.0), spider nevi (3.3), and weakness (2.1) (131). Although this is somewhat helpful clinically, findings from the physical examination must be interpreted with caution, as there is considerable heterogeneity in the assessment of each of these features when different examiners are involved (132). Several authors have reported the detection of a hepatic bruit in the setting of AH (133). This has been used in some centers as a diagnostic criterion for AH (134). However, the sensitivity, as well as the specificity of this finding is uncertain (135). In one series of 280 consecutive hospitalized patients, only 4 of 240 (or 1.7%) with AH and cirrhosis had an audible bruit (136). Caution about adopting this as a diagnostic criterion has therefore been advised (137).

It is important for physicians caring for these patients to recognize that ALD does not exist in isolation, and that other organ dysfunctions related to alcohol abuse may coexist with ALD, including cardiomyopathy (138,139), skeletal muscle wasting (140), pancreatic dysfunction, and alcoholic neurotoxicity (141). Evidence of these must be sought during the clinical examination, so that appropriate treatment may be provided (142).

Hepatic imaging

Imaging studies have been used to diagnose the presence of liver disease but do not have a role in establishing alcohol as the specific etiology of liver disease. However, the diagnosis of fatty change, established cirrhosis, and hepatocellular carcinoma may be suggested by ultrasound, CT scan, or magnetic resonance imaging and confirmed by other laboratory investigations (143,144). The major aim of imaging studies is to exclude other causes of abnormal liver tests in a patient who abuses alcohol, such as obstructive biliary pathology, or infiltrative and neoplastic diseases of the liver (145). Magnetic resonance imaging has been used as an adjunct to diagnose cirrhosis, and to distinguish end-stage liver disease related to viral hepatitis infection from ALD. Specific features that may be suggestive of alcoholic cirrhosis include a higher volume index of the caudate lobe, more frequent visualization of the right posterior hepatic notch, and smaller size of regenerative nodules of the liver in patients with cirrhosis on the basis of a comparison of ALD with chronic viral hepatitis (146). Although changes were identified on ultrasound and magnetic resonance imaging, it is unclear whether these results are generalizable (146,147).

Liver biopsy in ALD

Although not essential in the management of ALD, a liver biopsy is useful in establishing the diagnosis (144). As many as 20% of the patients with a history of alcohol abuse have a secondary or coexisting etiology for liver disease (148). In the absence of decompensated disease, clinical and biochemical indicators are poor markers of the severity of the liver disease and a biopsy is useful in establishing the stage and severity of the liver disease (144,149).

The histological features of alcohol-induced hepatic injury vary, depending on the extent and stage of injury. These may include steatosis (fatty change), lobular inflammation, periportal fibrosis, Mallory bodies, nuclear vacuolation, bile ductal proliferation, and fibrosis or cirrhosis (24). However, these may co-exist in the same biopsy, and are not individually pathognomonic of ALD. The clinical diagnosis of AH is made based on a typical presentation, with severe liver dysfunction in the context of excessive alcohol consumption, and the exclusion of other causes of acute and chronic liver disease. In a subset of patients with AH, a liver biopsy may show specific histological features, including confluent parenchymal necrosis, steatosis, deposition of intrasinusoidal and pericentral collagen, ballooning degeneration, and lobular inflammation affecting the perivenular regions in the earliest stages (34). The liver may be infiltrated with polymorphonuclear cells, typically clustered around cytoplasmic structures known as Mallory bodies (150), which represent aggregated cytokeratin intermediate filaments and other proteins. In addition to confirming the diagnosis and staging the extent of the disease, specific features on liver biopsy also convey prognostic importance. The severity of inflammation (i.e., degree of polymorphonuclear leukocyte infiltration) and cholestatic changes correlate with increasingly poor prognosis, and may also predict response to corticosteroid treatment in severe AH (151,152). Megamitochondria in AH may be associated with a milder form of AH, a lower incidence of cirrhosis, and fewer complications, with a good long-term survival (153). AH is associated with perivenular and pericellular fibrosis, which may be a harbinger of future

cirrhosis, especially in patients who continue to abuse alcohol or those who are co-infected with hepatitis C virus (33,154). Mallory bodies, giant mitochondria, neutrophilic infiltration, and fibrosis may be seen in conditions other than ALD (155).

Although a liver biopsy may not be practical in the management of all patients, it has been shown that physicians' clinical impression may correlate only moderately well with the histological findings on liver biopsy. Studies that have included a liver biopsy in all patients with presumed AH have shown histological confirmation in only 70–80% of the patients (156). However, the incentive to make a definitive histological diagnosis is partly dependent on the possible risks of a biopsy, as well as on the risks involved with particular treatments. If no treatment for ALD or AH is contemplated, based on noninvasive estimates of an individual patient's prognosis, it is usually not necessary to make a histological diagnosis. Alternatively, if an investigational treatment or a therapy with associated risk is contemplated, the risk–benefit ratio involved in pursuing a liver biopsy may change.

Recommendations:

1. Clinicians should discuss alcohol use with patients, and any suspicion of possible abuse or excess should prompt use of a structured questionnaire and further evaluation (Class I, level C).
2. For patients with a history of alcohol abuse or excess and evidence of liver disease, further laboratory tests should be done to exclude other etiologies and to confirm the diagnosis (Class I, level C).
3. Patients with ALD and suggestive symptoms should be screened for evidence of other end-organ damage, as appropriate (Class I, level C).
4. For patients with a clinical diagnosis of severe AH for whom medical treatment is contemplated, or for those in whom reasonable uncertainty exists regarding the underlying diagnosis, a liver biopsy should be considered. This decision will depend on local expertise and ability in performing a liver biopsy in patients with coagulopathy, the patient's severity of illness, and the type of therapy under consideration (Class I, level C).

PROGNOSTIC FACTORS

Prognosis in AH

Decisions regarding treatment are critically dependent on the ability to estimate a given patient's prognosis. Many individual clinical and laboratory features, along with specific histological features have also been tested as measures of disease prognosis. In AH, the Maddrey discriminant function, a disease-specific prognostic score, has been used to stratify a patient's severity of illness (157). The initial formula was derived in the context of clinical trials of AH, and later modified to Maddrey discriminant function (MDF) = 4.6 (patient's PT – control PT) + total bilirubin (mg/dl) (158). Patients with a score of ≥ 32 were at the highest risk of dying, with a 1-month mortality as high as 30–50% (151). In particular, those with evidence of both hepatic encephalopathy and an elevated discriminant function were at highest risk. Although relatively easy to use, and based on standard laboratory tests, several drawbacks to the use of the MDF have been noted. Although it is a continuous measure, its interpretation (using a threshold of 32) has converted it into an essentially categorical method of classification. Once patients have exceeded that threshold, their risk for dying is higher, but not specified. Dynamic models, which incorporate the changes in laboratory studies over time, have also been used to estimate the outcome in patients, including the change in bilirubin in the first week of hospitalization, which is significantly associated with the outcome of patients with AH treated with prednisolone (159).

Table 5 outlines some of the prognostic scoring systems used for patients with AH.

Table 5. Prognostic scoring systems used for patients with alcoholic hepatitis

Name	Derivation set	Elements				Test characteristics
1. Maddrey (modified) discriminant function (1989) (158)	n=66	MDF=4.6 (patient's PT – control PT) + total bilirubin (mg/dl)				Poor prognosis if score ≥32
2. MELD score (2001)[a] (160)	n=1,179	MELD score = $3.8 \times \log_e$(bilirubin in mg/dl) + $11.2 \times \log_e$(INR) + $9.6 \times \log_e$(creatinine mg/dl) + 6.4				Poor prognosis if >18
3. Glasgow alcoholic hepatitis score (2005) (161)	n=241	Score[b]:	1	2	3	
		Age	<50	≥50	—	Poor prognosis if score >8 (for score calculated on hospital day 1 or day 7)
		WCC	<15	≥15	—	
		Urea (mmol/l)	<5	≥5	—	
		PT ratio	<1.5	1.5–2.0	≥2	
		Bilirubin (mg/dl)	<7.3	7.3–14.6	>14.6	

MDF, Maddrey discriminant function; MELD, model for end-stage liver disease.
[a]The MELD score has also been used to estimate the 90-day mortality (166); an online calculator is available at http://www.mayoclinic.org/meld/mayomodel7.html.
[b]The GAH score is calculated by summing the points assigned for each of the five variables: age, white blood cell count, blood urea nitrogen, PT as a ratio of the patient's value to that of the control, and the bilirubin. This is done on hospital day 1 or on day 7.

Other scoring systems have also been proposed to stratify patients, including the combined clinical and laboratory index of the University of Toronto (131), the Beclere model (151), the model for end-stage liver disease (MELD) score (160), and the Glasgow AH Score (161). The diagnostic abilities of the latter two models have been tested against the MDF and other scoring systems for cirrhosis (such as the Child–Turcotte–Pugh score) in terms of specific test characteristics, including sensitivity and specificity, at least in some populations (162,163). Owing to the inherent trade-offs involved in setting test thresholds, optimal cut points are not clearly established for each of these indices. Some investigators have suggested specific cutoffs for these indices, including an MDF ≥ 32 or a MELD score >11, that seem to be roughly equivalent in their ability to detect patients with a poor prognosis, with similar sensitivity and specificity (162). Others have suggested higher MELD cutoffs of 18 (164), 19 (165), or 21 (166) (**Table 6**).

Several studies have also shown the utility of repeat testing and calculation of these indices during the course of hospitalization, including MELD or MDF score at 1 week, and degree of change. A change of ≥ 2 points in the MELD score in the first week has been shown to independently predict in-hospital mortality (164). The Glasgow AH Score was recently derived, and its test characteristics compared with the MDF and the MELD scores. Although it had an overall higher accuracy, it was substantially less sensitive for predicting the 1-month and 3-month mortality compared with either the MDF or the MELD (161). The degree of portal hypertension may be a sensitive marker for the severity of liver injury (167). A recently proposed scoring system combines measurements of a marker of portal hypertension, asymmetric dimethylarginine, and of its stereoisomer to predict the outcomes (168). This combined score has been compared with the Child–Turcotte–Pugh score, MELD, and MDF, and shown to have an overall sensitivity of 73% and a specificity of 83%, which were at least as good as those of other scoring systems (168). These results, however, require further validation.

As the aim of early detection of patients at highest risk of poor outcome requires maximization of the sensitivity of the test score, it would seem reasonable to use the MDF (with a cutoff of 32, and/or the presence of encephalopathy) to select patients for therapy.

Recommendation:

5. Patients presenting with a high clinical suspicion of AH should have their risk for poor outcome stratified using the Maddrey discriminant function, as well as other available clinical data. Evaluating a patient's condition over time with serial calculation of the MELD score is also justified (Class I, level B).

THERAPY

Therapy of ALD is based on the stage of the disease and the specific aims of treatment (169,170). Complications of cirrhosis, including evidence of hepatic failure (encephalopathy) as well as portal hypertension (ascites, variceal bleeding), are treated as in patients with non-ALD, with additional attention given to other organ dysfunctions associated specifically with alcohol (170).

Table 6. Comparisons of diagnostic indices

Author	Patient population	Outcome	AUROC
Sheth (162)	*N*=34 patients with alcoholic hepatitis hospitalized during 1997–2000. 21% 30-day mortality	MELD >11: Sensitivity 86% Specificity: 81% MDF ≥32: Sensitivity 86% Specificity 48%	MELD: 0.82 MDF: 0.86
Srikureja (164)	*N*=202 AH patients admitted during 1997–2002. 29 inpatient deaths	Admission MELD ≥18: Sensitivity 85% Specificity 84% Admission MDF ≥32: Sensitivity 83% Specificity 60% Admission CTP ≥12 Sensitivity 76% Specificity 80%	Admission MELD: 0.89 Admission CTP: 0.87 Admission DF: 0.81
Dunn (166)	*N*=73 AH patients admitted during 1995–2001. 16 deaths in 90 days. Outcome: 30-day mortality	Admission MELD >21: Sensitivity 75% Specificity 75% MDF >41: Sensitivity 75% Specificity 69	Admission MELD: 0.83 Admission MDF: 0.74
Soultati (165)	*N*=34 patients admitted during 2000–2005; 2 deaths/30 days, 5 deaths/90 days. Outcome: 30-day mortality	MELD ≥30.5: Sensitivity 1 Specificity 0.937 MDF ≥108.68 Sensitivity 1 Specificity 0.969	MELD: 0.969 MDF: 0.984

AH, alcoholic hepatitis; AUROC: area under the receiver operating characteristic curve, with optimal test results closest to 1; CTP, Child–Turcotte–Pugh score; DF, discriminant function; MDF, Maddrey discriminant function; MELD, model for end-stage liver disease.

Abstinence

Abstinence is the most important therapeutic intervention for patients with ALD (171). Abstinence has been shown to improve the outcome and histological features of hepatic injury, to reduce portal pressure and decrease progression to cirrhosis, and to improve survival at all stages in patients with ALD (171–174). However, this may be less likely to occur in female patients (172,175,176). This improvement can be relatively rapid, and in 66% of the patients abstaining from alcohol, significant improvement was observed in 3 months (177). Continued alcohol ingestion results in an increased risk of portal hypertensive bleeding, especially in patients who have previously bled, and worsens both short- and long-term survival (178).

Recidivism is a major risk in all patients at any time after abstinence (179,180). Estimates vary, depending on the time course of follow-up and the definition of recidivism (e.g., any alcohol consumption, vs. moderate-to-harmful drinking), but over the course of 1 year, relapse rates range from 67% to 81% (181). Therefore, several medications have been tried to help sustain abstinence. One of the first agents to be used, disulfiram, was approved by the Food and Drug Administration in 1983. However, a review of the published literature concluded that there was little evidence that disulfiram enhances abstinence (182), and based on its poor tolerability, its use has been largely supplanted by newer agents. Naltrexone, which was approved in 1995 for the treatment of alcoholism, is a pure opioid antagonist and controls the craving for alcohol. However, it has also been shown to cause hepatocellular injury. A Cochrane systematic review of the use of naltrexone and nalmefene (another opioid antagonist) in 29 RCTs concluded that short-term treatment with naltrexone lowers the risk of relapse (183). Acamprosate (acetylhomotaurine) is a novel drug with structural similarities to the inhibitory neurotransmitter gamma aminobutyric acid, and is associated with a reduction in withdrawal symptoms (184). In 15 controlled trials, acamprosate has been shown to reduce withdrawal symptoms, including alcohol craving, but its effects on survival are not yet known (185). Its effect is more pronounced in maintaining rather than inducing remission when used in combination with counseling and support. In detoxified alcoholics, it has been shown to decrease the rate of relapse, maintain abstinence, and decrease the severity of relapse when it occurs. It has not been shown to have a significant impact on alcoholics who have not been detoxified or become abstinent. Whether it has any additional effect in combination with naltrexone is controversial. A recent large randomized controlled clinical trial did not suggest substantial benefit of acamprosate compared with naltrexone or with intensive counseling in maintaining abstinence (186). There is a paucity of data about the use of these interventions in patients with advanced liver disease. One randomized clinical trial in patients with cirrhosis suggested benefit in achieving and maintaining abstinence with the use of baclofen, a gamma aminobutyric acid B receptor agonist (187).

Recommendations:

6. In patients with evidence of alcohol-induced liver disease, strict abstinence must be recommended, because continued alcohol use is associated with disease progression (Class I, level B).
7. Naltrexone or acamprosate may be considered in combination with counseling to decrease the likelihood of relapse in patients with alcohol abuse/dependence in those who achieve abstinence (Class I, level A).

Therapy for AH

The cornerstone of the therapy for AH is abstinence, although even patients who become abstinent have an increased risk of developing cirrhosis. However, the risk of cirrhosis is clearly higher in those who continue to drink (188,189), particularly among women (175,190). Although there are no clear dose–effect data, a threshold exists for the development of AH, with the risk increasing with consumption beyond 40 g of alcohol per day (46,191). Furthermore, after an episode of AH, there is no safe amount of alcohol consumption that can be recommended, as AH can persist or re-develop. There is a significant risk of recidivism in patients who attempt to cut back but not stop drinking altogether (192). Complete abstinence is therefore a reasonable lifetime recommendation.

The need to consider therapy is less urgent in patients with AH who have a low risk of complications as defined by an MDF score of <32, without hepatic encephalopathy, or a low MELD score (e.g., MELD <18), or a Glasgow AH Score of <8. This is particularly true in those whose liver score improves during hospitalization, with a decrease in total bilirubin, as they will likely improve spontaneously with abstinence and supportive care alone. For those with more severe disease and therefore a more dismal prognosis, however, medical treatment should be considered.

Nutrition therapy. The presence of significant protein calorie malnutrition is a common finding in alcoholics, as are deficiencies in a number of vitamins and trace minerals, including vitamins A, D, thiamine, folate, pyridoxine, and zinc (193). In a VA Cooperative study of 363 patients with AH, 100% of patients were found to have protein and/or combined protein calorie malnutrition, based on anthropometric and laboratory testing (194). Moreover, the severity of malnutrition correlated with the disease severity and outcomes (194).

This early finding was the motivation for a number of clinical trials of anabolic steroids, nutritional supplementation, or aggressive enteral feeding. Several of these studies showed an improvement in the biochemical markers of liver function or nutritional parameters, but were unable to show an improvement in short-term survival (195). However, at least in some trials subgroups of patients who achieved nutritional goals and positive nitrogen balance had improved survival compared with those who did not (196). As an example, in one study, the mortality rate was 3.3% in the 30 patients in whom positive nitrogen balance was achieved, but 58% in patients who remained in negative nitrogen balance (196).

The most recent study of nutritional therapy compared the outcomes of 35 patients who were randomized to 1 month of enteral tube feeding of 2,000 kcal/day with 40 mg of prednisone/day (197). No difference in mortality was noted, but the time course of deaths was different, with the patients randomized to enteral feeding dying at a median of 7 days, vs. 23 days in the steroid-treated group. Patients treated with nutritional support who survived past the first month seemed to have a decreased mortality compared with the steroid-treated patients (8% vs. 37%) (197). Although technically a negative study, the similar overall mortality rates in the treatment groups suggests a role for nutritional intervention (198), particularly in light of the relatively benign risk:benefit ratio. Based on these data, other

Table 7. Clinical trials of steroids in patients with alcoholic hepatitis

Author	Date	No. of patients	Intervention	Deaths: placebo	Deaths: steroid
Porter (265)	1971	20	Prednisolone: 40 mg intravenously × 10 days, then tapered: 4 mg/day × 1 week, 2 mg/day × 11 days, then 2 mg every 3rd day × 15 days	7/9	6/11
Helman (266)	1971	37	Prednisolone: 40 mg/day × 4 weeks, then tapered over 2 weeks	6/17	1/20
Campra (267)	1973	45	Prednisone: 0.5 mg kg × 3 weeks, then 0.25 mg/kg × 3 weeks	9/25	7/29
Blitzer (268)	1977	33	Prednisolone: 40 mg/day × 14 days, then 20 mg/day × 4 days; 10 mg/day × 4 days; 5 mg/day × 4 days	5/16	6/12
Lesesne (269)	1978	14	Prednisolone: 40 mg/day × 30 days, then tapered over 2 weeks	7/7	2/7
Shumaker (270)	1978	27	Prednisolone 80 mg/day × 4–7 days, then tapered off over 4 weeks	7/15	6/12
Maddrey (157)	1978	55	Prednisolone 40 mg/day × 30 days	6/31	1/24
Depew (271)	1980	28	Prednisolone 40 mg/day × 28 days, then tapered over 14 days	7/13	8/15
Theodossi (272)	1982	55	Prednisolone: 1 g × 3 days	16/28	17/27
Mendenhall (273)	1984	178	Prednisolone: 60 mg × 4 days; 40 mg/day × 4 days; 30 mg/day × 4 days; 20 mg/day × 4 days; 10 mg/day × 7 days; 5 mg/day × 7 days	50/88	55/90
Bories (274)	1987	45	Prednisolone 40 mg/day × 30 days	2/21	1/24
Carithers (158)	1989	66	Prednisolone 32 mg/day × 28 days, then 16 mg/day × 7 days, then 8 mg/day × 7 days	11/31	2/35
Ramond (275)	1992	61	Prednisolone: 40 mg/day × 28 days	16/29	4/32

societies have recommended oral or parenteral supplements for patients with AH at risk of undernutrition (199).

Steroids. The most extensively studied intervention in AH is the use of steroids, based on 13 clinical trials that date back almost 40 years (**Table 7**).

Most of these trials were small, and therefore had only limited statistical power to detect even moderate treatment effects; five suggested an improvement in outcome, with decreased short-term mortality in steroid-treated patients compared with placebo-treated patients, whereas eight showed no effect. It is important to note, however, that these trials used varying inclusion and exclusion criteria, dosing, and were conducted in a variety of patient populations. Three meta-analyses have analyzed data from these trials, and showed an improvement in survival in the treated patients (200–202); one meta-regression, however, using a different statistical weighting of the varying trials, was unable to show any difference (203). The most recent meta-analysis of these data did not show a statistically significant effect of steroids on mortality among all patients

treated, although it did show an effect of steroids in the subgroup of patients with hepatic encephalopathy and/or an MDF score ≥ 32 (204). The presence of substantial statistical heterogeneity in this subgroup of studies prevented the authors from reporting an overall beneficial effect. The implication of this finding is unclear, as statistical heterogeneity among subgroups is a function of both clinical differences and/or methodological differences among studies, and these analyses may be reflect bias or confounding (205). One potential approach to resolve this is the use of individual patient data across clinical trials, which represents the "gold standard" approach to meta-analysis (206). Although it is impractical to retrieve and combine primary data from all the clinical trials in this field, in which large variation in studies over time exists, this approach was pursued using a combined dataset, using pooled primary data from three placebo-controlled trials in patients with comparable measures of disease severity (i.e., an MDF ≥ 32). The results showed a significant increase in short-term survival among the treated patients compared with the control patients: 84.6% vs. 65% (207). This represents a modest absolute reduction in risk, but a 30% reduction in the relative risk, and translates into a number needed to treat of 5—i.e., five patients need to be treated to avert one death. This last meta-analysis also excluded a recent trial comparing steroids with a combination of anti-oxidants, which showed a similar protective effect of corticosteroids among treated patients (208). Although it is possible that anti-oxidants themselves may be detrimental (209), the doses used seem unlikely to account for the differences in survival, and the consistency of the data suggests a protective effect of steroids.

Although the doses and durations of steroid treatment used in the clinical trials were variable, the best available evidence suggests a dose of prednisolone (40 mg/day for 4 weeks, then tapered over 2–4 weeks, or stopped, depending on the clinical situation) should be used in favor of prednisone (210).

An important issue in all studies of medical therapy, and one that has been recognized for some time in this literature, is the possibility that these therapies may not be effective at an advanced stage of disease. Just as there is a threshold for the use of steroids (i.e., identifying patients at high risk of mortality defined by an MDF score ≥ 32), there may also be a ceiling beyond which medical therapies aimed at decreasing the inflammatory cascade may cause more harm than benefit. One study examined this issue, and suggested that patients with a MDF >54 were at a higher mortality risk from use of steroids than from not being treated (63). This cutoff, however, needs to be confirmed.

One recently derived model used six variables to predict the six-month mortality in patients who were universally treated with steroids (including age, renal insufficiency (serum creatinine >1.3 or creatinine clearance <40), albumin, prothrombin time, bilirubin, and change in bilirubin over 1 week), and showed an improved prognostic ability compared with MDF or GAH scores (211). This model, available on the internet (www.lillemodel.com), may allow identification of patients who are at high risk to be treated with other interventions.

Anti-cytokine therapy. A wealth of evidence suggests that dysregulated cytokines, including tumor necrosis factor alpha (TNF-α) and a host of downstream cytokines have a pivotal role in the pathophysiology of AH. Thus, several agents have been studied that affect the immunologic milieu, targeting specific cytokines, and TNF-α in particular.

Among the first agents to be studied was pentoxifylline, an oral phosphodiesterase inhibitor that also inhibits the production of TNF-α, among other cytokines. A randomized placebo-controlled clinical trial tested pentoxifylline in 101 patients with clinical evidence of severe AH (212). The in-hospital mortality in the treated patients was 40% lower than in the placebo arm, with the bulk of the reduction related to a substantially lower likelihood of developing hepatorenal syndrome. The hepatorenal syndrome was responsible for 50% of the 12 deaths in the treatment arm, compared with 91.7% of the 24 deaths in the placebo group.

Other specific inhibitors of TNF that have been studied include infliximab, a monoclonal chimeric anti-TNF antibody, and etanercept, a fusion protein containing the ligand-binding portion of the human TNF receptor fused to the Fc portion of human IgG1 (213). In the first clinical trial of infliximab, 20 patients with biopsy-proven AH and an MDF score between 32 and 55 (based on the original Maddrey score, which showed an increased mortality at a score >93) were randomized to either 5 mg/kg of infliximab plus 40 mg/day of prednisone ($n=11$) or prednisone alone (214). No substantial difference in overall mortality was found, but substantial decreases in other prognostic markers, including cytokine levels and MDF scores, were seen in patients treated with the combination therapy. Another trial, which was performed at 19 centers in France, randomized 36 patients with biopsy-proven AH and an MDF ≥ 32 to prednisolone (40 mg/day for 4 weeks), vs. prednisolone along with infliximab (10 mg/kg, given at study entry, and again at 2 and 4 weeks after entry) (215). The trial was stopped prematurely after seven deaths had occurred in the infliximab group, compared with three in the prednisolone arm. Four of the seven deaths in the infliximab arm were related to infectious etiologies, compared with one in the prednisolone group. The design, and, in particular, the dose of infliximab chosen in the study, has been criticized as predisposing to these infections (216). The utility of etanercept (given six times over three weeks) was tested in 48 patients with moderate-to-severe AH (MELD score >15); unfortunately, no significant difference in 1-month mortality was seen in the treated patients compared with patients given placebo, and an increased mortality was seen at 6 months (217).

Although a strong rationale remains for the use of anti-TNF therapy in AH, there is also a theoretical basis for minimizing TNF inhibition, as it has a role in liver regeneration as well as apoptosis (218). Thus, in light of the poor clinical outcomes observed in the largest of the infliximab trials and the etanercept study, the use of these parenteral TNF

inhibitors should be confined to clinical trials, and recommendations regarding specific therapy will need to await the results of these trials. There are no substantive clinical data comparing the use of steroids or nutrition with specific anti-TNF therapies.

Combination therapy. Although it is assumed that each of these different treatments may operate through independent mechanisms, there are only minimal data regarding the comparative benefit of sequential therapies or combined approaches. One study tested the use of pentoxifylline in 29 patients with severe AH (MDF ≥ 32) who did not respond to steroids based on a drop in bilirubin level after 1 week of prednisolone treatment. Compared with previously treated patients (who were continued on steroids despite lack of bilirubin response), there was no improvement in 2-month survival—arguing against a two-step strategy with an early switch to pentoxifylline (219). Several older studies had examined the role of anabolic steroids with nutritional interventions (based on the presumption that both interventions acted through a similar mechanism, i.e., by correction of protein calorie malnutrition) (220). One pilot study evaluated the role of steroids in combination with enteral nutrition in 13 patients with severe AH, and found an overall mortality of 15%—possibly an improvement from that expected (221). With the advent of new therapies, it is necessary to reconsider the risk–benefit ratio of medical treatment. It has been suggested that it may be possible to use less toxic therapies at a lower threshold of disease severity (222). However, the exact role of these new therapies—and the threshold for their use—is still undefined.

Other treatments. Many other therapeutic interventions have been studied in AH, but have not been able to show convincing benefit, including trials of anti-oxidants (vitamin E, silymarin, combination anti-oxidants), anti-fibrotics (colchicine), anti-thyroid drugs (PTU), promoters of hepatic regeneration (insulin and glucagons), anabolic steroids (oxandrolone and testosterone), as well as calcium channel blockers (amlodipine), polyunsaturated lecithin, and a number of complementary and alternative medicines (reviewed in O'Shea and McCullough (223)). In addition to medical treatment directed at the underlying pathophysiological abnormalities, several studies have tested other aggressive interventions in patients with AH, such as a molecular adsorbent recirculating system (224). Although the results of early studies were optimistic, with better than predicted outcomes in treated patients, a further case series was less promising (225). Case reports have also described the outcome of patients with severe AH treated with leukocytapharesis after failing to improve substantially on steroids (226,227). These reports are promising, but recommendations regarding their appropriate use must await results of comparative studies of outcomes in these patients.

A proposed treatment algorithm for AH is shown in **Figure 1**.

Recommendations:

8. All patients with AH should be counseled to completely abstain from alcohol (Class I, level B).
9. All patients with AH or advanced ALD should be assessed for nutritional deficiencies (protein-calorie malnutrition), as well as vitamin and mineral deficiencies. Those with severe disease should be treated aggressively with enteral nutritional therapy (Class I, level B).
10. Patients with mild-to-moderate AH—defined as a Maddrey score of < 32, without hepatic encephalopathy, and with improvement in serum bilirubin or decline in the MDF during the first week of hospitalization—should be monitored closely, but will likely not require nor benefit from specific medical interventions other than nutritional support and abstinence (Class III, level A).
11. Patients with severe disease (MDF score of ≥ 32, with or without hepatic encephalopathy) and lacking contraindications to steroid use should be considered for a 4-week course

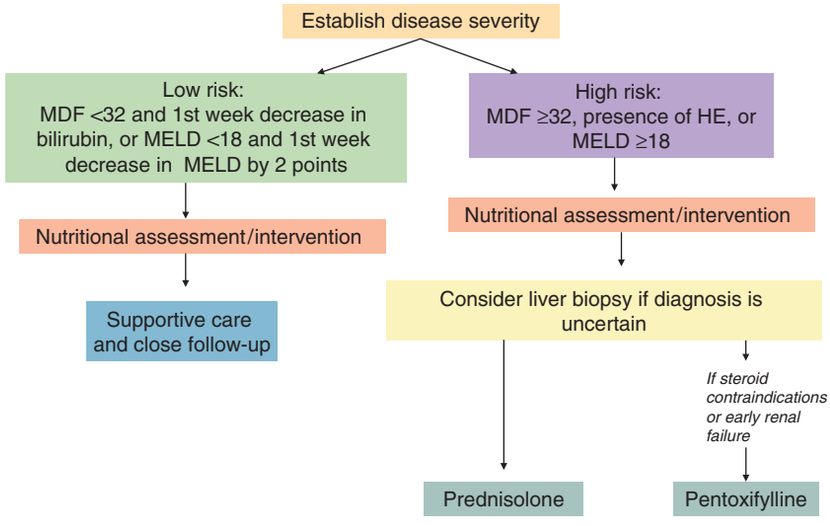

Figure 1. Proposed algorithm for alcoholic hepatitis. MDF, Maddrey discriminant function; MELD, model for end-stage liver disease.

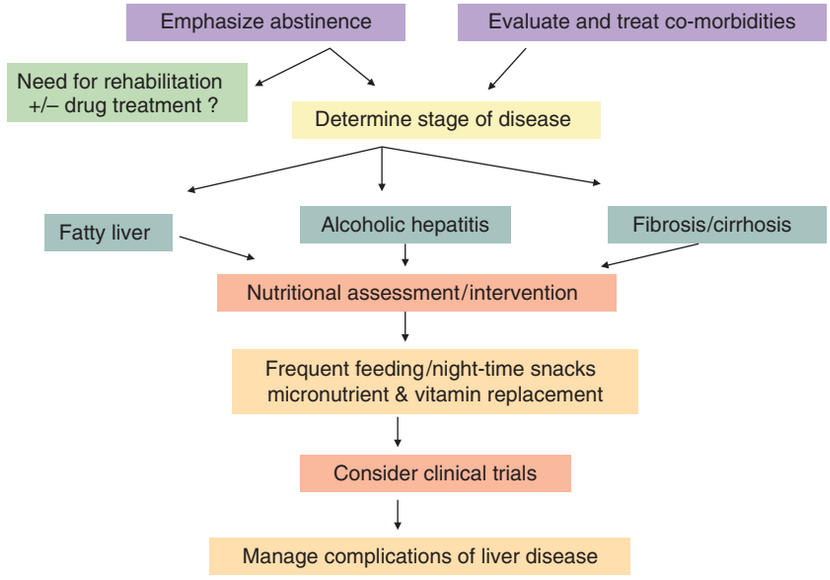

Figure 2. Proposed therapeutic algorithm for the long-term management of alcoholic liver disease.

of prednisolone (40 mg/day for 28 days, typically followed by discontinuation or a 2-week taper) (Class I, level A).

12. Patients with severe disease (i.e., a MDF ≥ 32) could be considered for pentoxifylline therapy (400 mg orally 3 times daily for 4 weeks), especially if there are contra-indications to steroid therapy (Class I, level B).

Long-term management of ALD

A proposed algorithm for the management of ALD is shown in **Figure 2**.

Nutritional therapy. Protein calorie malnutrition is common in ALD, is associated with an increased rate of major complications of cirrhosis (infection, encephalopathy, and ascites), and indicates a poor prognosis (194).

A total of 13 studies (7 randomized and 6 open-label studies) have examined the effect of oral or enteral nutritional supplementation in patients with alcoholic cirrhosis, with interventions that ranged from 3 days to 12 months (reviewed in Stickel *et al.* (228)). Most of these studies are limited by small sample sizes and short durations of therapy. In one study, enteral feeding for 3–4 weeks in 35 hospitalized, severely malnourished, or decompensated patients with alcoholic cirrhosis seemed to improve the survival ($P < 0.065$), hepatic encephalopathy, liver tests, and Child–Pugh score, as compared with controls who received a standard oral diet (197). In longer-term studies, equinitrogenous amounts of dietary branched chain amino acids (BCAA) were compared with casein supplements for 3–6 months in patients with chronic hepatic encephalopathy (229), and shown to improve encephalopathy, nitrogen balance, and serum bilirubin compared with casein. Intake of supplemental protein and of 1,000 kilocalories in decompensated patients with alcoholic cirrhosis has also been shown to reduce hospitalizations for infections over a 1-year period (230).

Long-term aggressive nutritional therapy by the enteral or oral route in patients with alcoholic cirrhosis is supported by studies that have shown improved nutritional status (231,232). Although controversial, this may possibly prevent the complications of cirrhosis (195,233). Multiple feedings, emphasizing breakfast and a nighttime snack, with a regular oral diet at higher-than-usual dietary intakes (1.2–1.5 g/kg for protein and 35–40 kcal/kg for energy) seem beneficial (234,235). Finally, during intermittent acute illness or exacerbations of the underlying chronic liver disease, an above-normal protein intake (1.5 g per kg body weight) and kilocalorie intake (40 kilocalories per kg) improves the protein calorie malnutrition (233), and should be considered in the treatment of these patients.

Recommendation:

13. Patients with alcoholic cirrhosis should receive frequent interval feedings, emphasizing a nighttime snack and morning feeding, to improve the nitrogen balance (Class I, level A).

Medical therapies. A number of other agents have been tested in patients with ALD, including propylthiouracil, which was thought to decrease the hypermetabolic state induced by alcohol (236,237). A Cochrane review of six randomized controlled trials of PTU in ALD, with a total of 710 patients administered either PTU or placebo, did not show any benefit of PTU over placebo on the total or liver-related mortality, complications of liver disease, or liver histology in patients with ALD (238). A possible benefit of supplementation with *S*-adenosyl L-methionine, a precursor to glutathione, has also been studied extensively (239). One trial showed a statistically significant improvement in survival in patients with Childs A and B cirrhosis randomized to *S*-adenosyl L-methionine compared with placebo (240). Despite a strong theoretical rationale, and a number of supportive clinical trials (239,241), a Cochrane

review of published data, based on nine randomized controlled trials with 434 patients in different stages of ALD, did not show any significant benefit of *S*-adenosyl L-methionine on total mortality, liver-related mortality, complications, or liver transplantation (LT) in patients with ALD (242).

Colchicine, which has both anti-inflammatory and antifibrotic properties, has also been tested in alcoholic cirrhosis after several small clinical trials, and has suggested improvement in fibrosis on serial liver biopsies in treated patients (243,244). However, a systematic meta-analysis of 15 randomized trials with 1,714 patients (including patients with alcoholic fibrosis, AH, and/or alcoholic cirrhosis, as well as patients with viral induced or cryptogenic fibrosis and/or cirrhosis) by the Cochrane group (245) showed no benefit of treatment on overall mortality, liver-related mortality, liver tests, or histology. In addition, there was an increased risk of adverse effects related to colchicine therapy.

Emerging data suggest a role for TNF-α-mediated apoptosis in AH, and therapy targeting this cytokine to inhibit apoptosis may be effective (246). Thalidomide, misoprostol, adiponectin, and probiotics have been shown to have anti-cytokine properties in preliminary reports (247–250). Although promising, these treatments cannot be considered as standard treatment for ALD and AH until further evidence of efficacy has been obtained.

Complementary and alternative medicine treatment options. Various alternative treatment options have been tested in the therapy of ALD.

Silymarin, the presumed active ingredient in milk thistle, is postulated to protect patients from ALD on the basis of its antioxidant properties. Six published trials of the use of silymarin in patients with ALD (251) have tested its effects on normalizing liver tests and on improving liver histology. One study suggested a possible survival benefit compared with placebo (252). However, a Cochrane systematic review and a meta-analysis of the 13 published studies of silymarin in ALD and other liver diseases found that the overall methodological quality of the studies was low. Based on the few high-quality trials, it was concluded that milk thistle does not significantly influence the course of patients with ALD (253).

Recommendations:

14. PTU and colchicine should not be used in the treatment of patients with ALD; *S*-adenosyl L-methionine should be used only in clinical trials (Class III, level A).
15. The use of complementary or alternative medicines in the treatment of either acute or chronic alcohol-related liver disease has shown no convincing benefit and should not be used out of the context of a clinical trial (Class III, level A).

LT for ALD

ALD is the second most common indication for LT for chronic liver disease in the Western world (254). Despite this, it is estimated that as many as 95% of patients with end stage liver disease related to alcohol are never formally evaluated for their candidacy for LT (255). This is attributed to perceptions that ALD is self-induced, the possibility of recidivism or non-compliance, and the shortage of organs (179).

A 6-month period of abstinence has been recommended as a minimal listing criterion (256). This time period allows chemical dependency issues to be addressed; in patients with recent alcohol consumption, it may also allow sufficient clinical improvement to make LT unnecessary. This requirement for a fixed abstinence period has not been shown to accurately predict future drinking by alcoholic candidates for LT (257). Despite some data suggesting that patients with ALD were more ill at the time of LT, and were likely to have prolonged intensive care unit stays and increased blood product requirements (258), the overall survival rates are generally similar between alcohol-related and non-alcohol-related LTX recipients (259).

Patients transplanted for ALD are highly likely to drink after transplantation (259). It has been suggested that the consequences of alcohol use are minimal for many recipients, because the amounts consumed are small and infrequent, but there are little reliable data to support this contention. Rates of recidivism between 11–49% (defined as any alcohol consumption after transplantation) at 3–5 years after LT have been reported (179,260). In general, however, only a small fraction of those who undergo LT for ALD revert to heavy alcohol use or abuse (255). Poor follow-up and non-compliance with therapy are observed in only a minority of patients, and graft rejection rates are similar for patients with ALD compared with non-ALD patients (254,259).

An important issue that is still unresolved is the role of LT in patients with AH, who are generally excluded from transplant (256). In one study using retrospective histological analysis of the explanted liver, superimposed AH did not worsen the outcome after LT (261). The availability of living donor transplantation and extended criteria donor LT are likely to heighten the debate on this issue.

Recommendation:

16. The appropriate patients with end-stage liver disease secondary to alcoholic cirrhosis should be considered for LT just as other patients with decompensated liver disease, after a careful evaluation of their medical and psychosocial candidacy. In addition, this evaluation should include a formal assessment of the likelihood of long-term abstinence (Class I, level B).

CONFLICT OF INTEREST

Guarantor of the article: Arthur J. McCullough, MD.
Specific author contributions: Design, literature review, drafting of the manuscript, and manuscript review/revision: Robert O'Shea and Srinivasan Dasarathy; design, manuscript review/revision, and approval of the final version: Arthur J. McCullough.
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Study on Evaluation of Hepatoprotective and Anti-Oxidant Effect of Processed *Glycyrhiza glabra* fortified Ethanol (NTX) in Alcoholics Subjects

Results:

Table 1. Changes in serum ROS levels after alcohol consumption:

Time points	Alcohol (mean value)	NTX (mean value)	SD-Al	SD-NTX	p value (paired t-test)	Significance
0 hr	4.42	4.50	0.83	0.79	0.267	NS
0.5 hr	5.22	5.06	1.65	1.81	0.370	NS
1 hr	5.33	4.81	1.40	0.78	0.030	S
2 hr	5.38	5.11	1.47	1.04	0.159	NS
4 hr	5.30	4.77	1.40	1.07	0.001	S

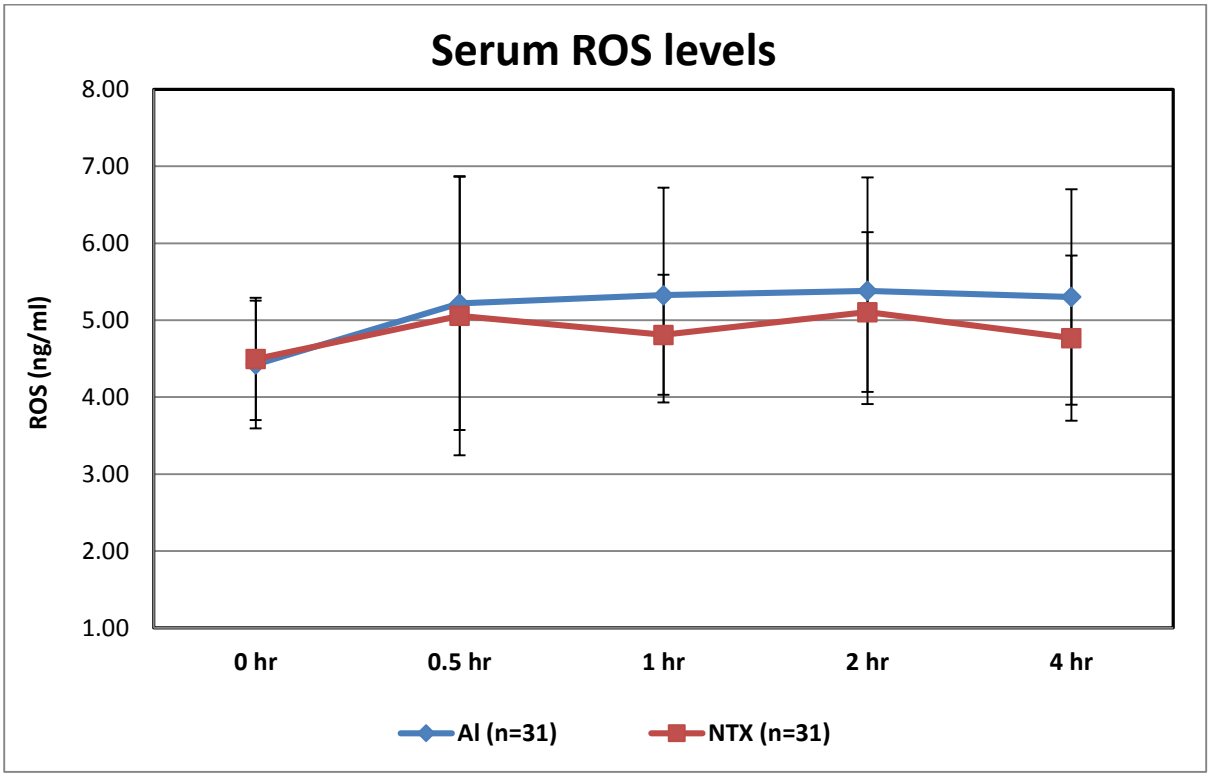

Fig.1: Changes in serum ROS levels at different time intervals after alcohol consumption.

Table 2. Changes in serum derivatives of reactive oxygen metabolites (dROM) levels after alcohol consumption:

Time points	Alcohol (mean value)	NTX (mean value)	SD-Al	SD-NTX	p value (paired t-test)	Significance
0 Hr	75.39	76.41	19.89	16.33	0.664	NS
0.5 Hr	99.17	95.07	31.03	26.19	0.423	NS
1 Hr	98.80	95.04	30.43	32.67	0.423	NS
2 Hr	109.05	93.42	36.39	26.84	0.050	S
4 Hr	89.19	86.41	24.43	19.36	0.550	NS

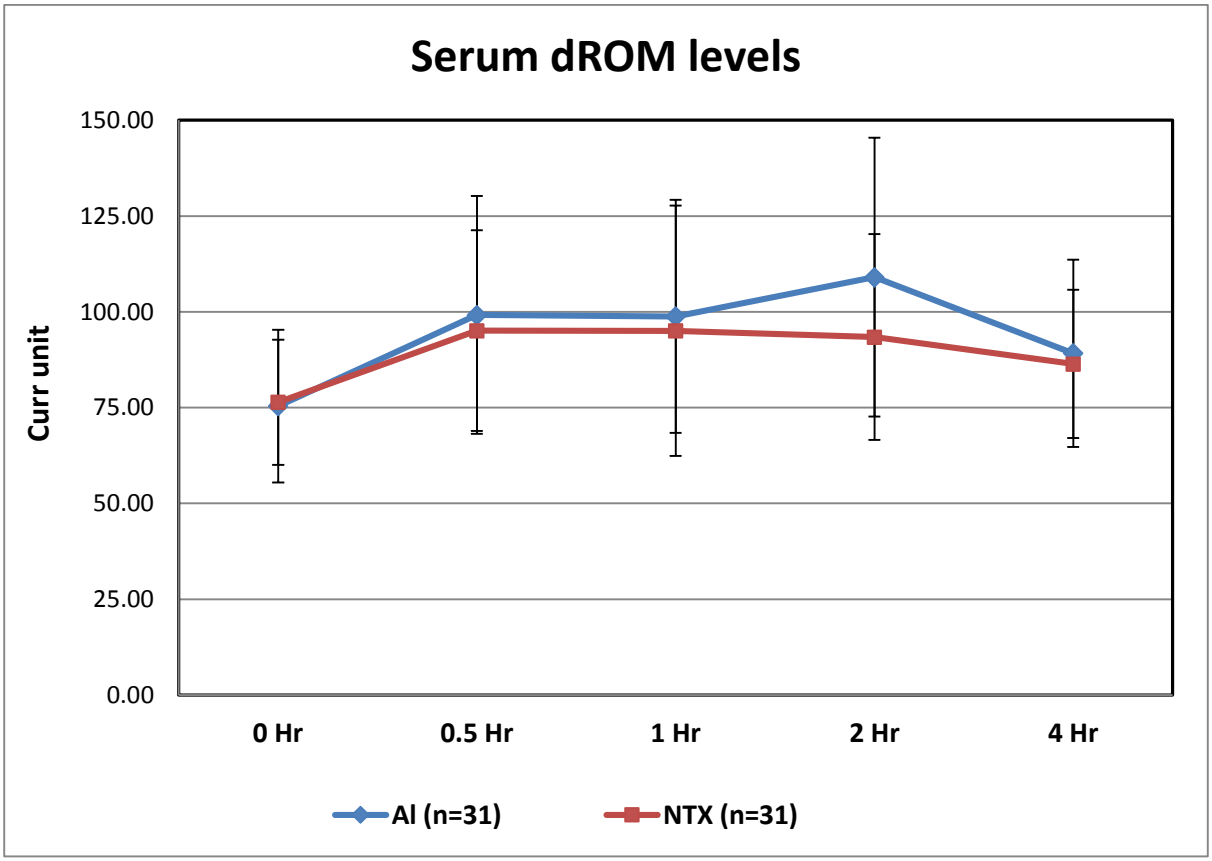

Fig.2: Changes in serum dROM levels at different time intervals after alcohol consumption.

Table 3. Changes in serum total glutathione (GSH) levels after alcohol consumption:

Time points	Alcohol (mean value)	NTX (mean value)	SD-Al	SD-NTX	p value (paired t-test)	Significance
0 hr	4.52	4.49	0.74	0.75	0.518	NS
0.5 hr	4.38	4.63	0.74	0.83	0.029	S
1 hr	4.31	4.65	0.82	0.72	0.009	S
2 hr	4.39	4.38	0.79	0.91	0.966	NS
4 hr	4.27	4.50	0.72	0.87	0.011	S

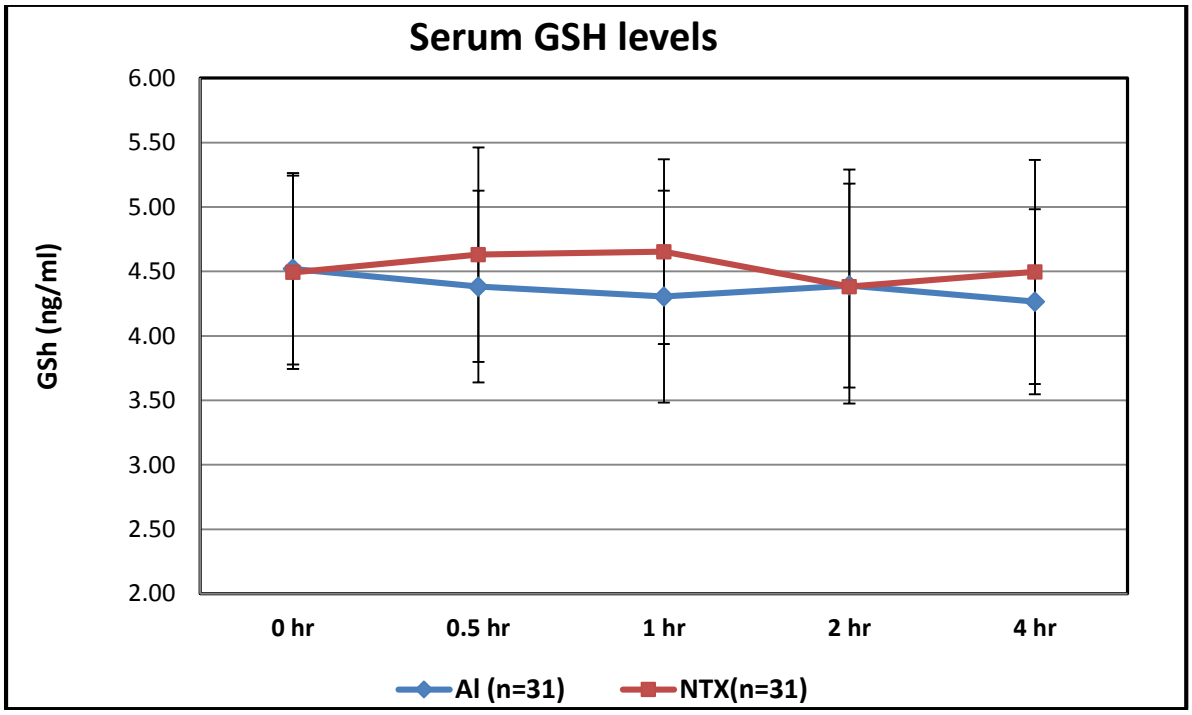

Fig.3: Changes in serum GSH levels at different time intervals after alcohol consumption.

Table 4. Changes in serum 8-Hydroxy-2-deoxyguanosine (8-OHdG) levels after alcohol consumption:

Time points	Alcohol (mean value)	NTX (mean value)	SD-Al	SD-NTX	p value (paired t-test)	Significance
0 hr	58.13	58.13	6.88	6.90	1.00	NS
2 hr	69.41	67.78	17.10	11.54	0.78	NS
4 hr	74.25	64.40	14.80	11.15	0.05	S

n=15 in each arm

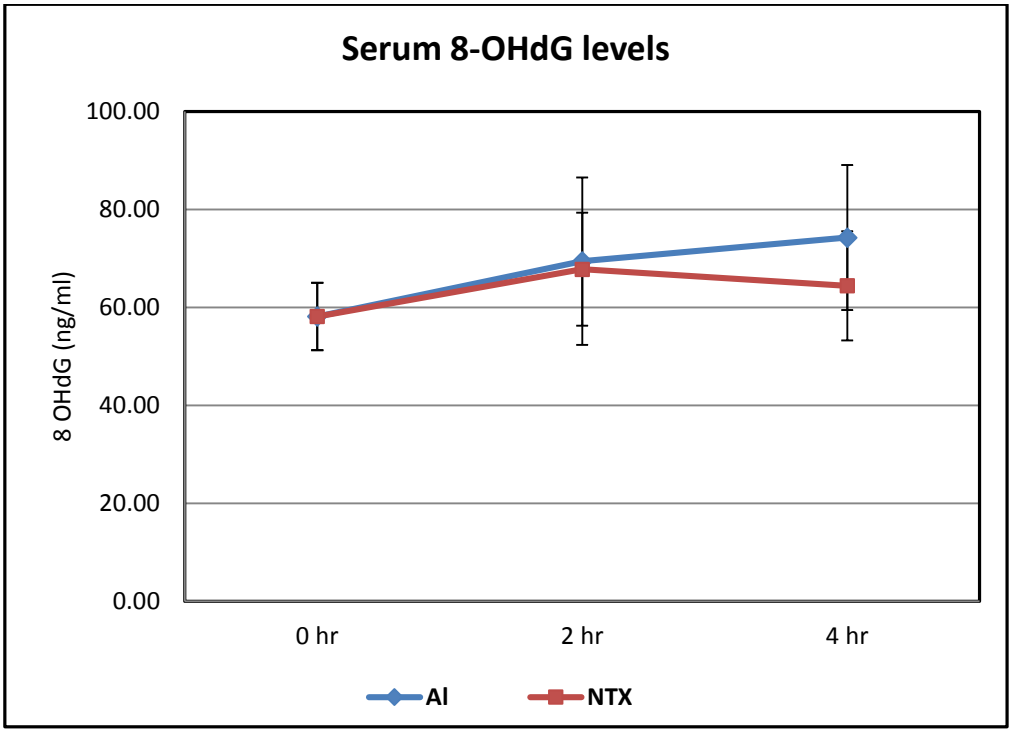

Fig.4: Changes in serum 8-OHdG levels at different time intervals after alcohol consumption.

Table 5. Changes in serum protein carbonyl (PC) levels after alcohol consumption

Time points	Alcohol (mean value)	NTX (mean value)	SD-Al	SD-NTX	p value (paired t-test)	Significance
0 hr	0.692	0.692	0.348	0.35	1	NS
2 hr	1.076	0.834	0.551	0.492	0.24	NS
4 hr	0.982	0.899	0.621	0.602	0.71	NS

n=15 in each arm

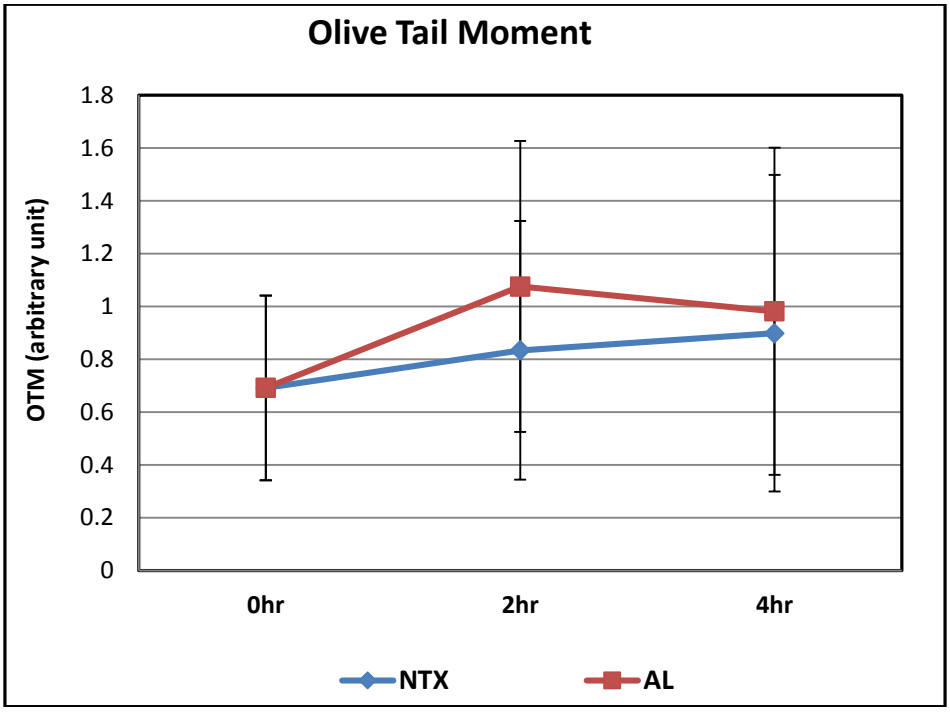

Fig.5: Changes in serum PC levels at different time intervals after alcohol consumption.

- We will receive all CBMN data within 1-2 days and will include in the interim report.
- We will receive some more comet assay scores within 2-3 days and will include in the interim report
- MDA and PC kits are still pending and as soon as these kits are available we will estimate the samples (stored in - 80 °C) and results will be included.

Ultraviolet-B-Induced Oxidative DNA Base Damage in Primary Normal Human Epidermal Keratinocytes and Inhibition by a Hydroxyl Radical Scavenger

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To evaluate the effects of ultraviolet-induced environmental trauma on human skin cells, primary normal human epidermal keratinocytes were exposed to ultraviolet-B radiation (290–320 nm). We found that relatively low doses of ultraviolet-B (62.5–500 mJ per cm^2) caused dose-dependent increases in 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), a biomarker of oxidative DNA damage. Unirradiated normal human epidermal keratinocytes contained 1.49 ($\pm$ 0.11) 8-oxo-dG per 10^6 2′-deoxyguanosine (dG) residues in cellular DNA, which increased linearly to as high as 6.24 ($\pm$ 0.85) 8-oxo-dG per 10^6 dG after irradiation with 500 mJ per cm^2. Further, this oxidative damage was reduced by 60.7% when the cells were pretreated with 1 mM mannitol. As hydrogen peroxide (H_2O_2) is known to be generated during oxidative stress, its accumulation in ultraviolet-B-irradiated normal human epidermal keratinocytes was also assessed and correlated to 8-oxo-dG formation. An ultraviolet-B-induced increase in H_2O_2 was observed in normal human epidermal keratinocytes and its production was inhibited by the addition of catalase. Based on the ability of a neutral molecule like H_2O_2 to permeate membranes, our data indicate that, after ultraviolet-B irradiation, H_2O_2 migrates from the cytosol to the nucleus where it participates in a Fenton-like reaction that results in the production of hydroxyl radicals (OH·), which may then cause 8-oxo-dG formation in cellular DNA. This conclusion is supported by our data showing that OH· scavengers, such as mannitol, are effective inhibitors of oxidative DNA base damage. Although increased levels of 8-oxo-dG were previously found in immortalized mouse keratinocytes exposed to ultraviolet-B radiation, we now report the induction of 8-oxo-dG in normal human skin keratinocytes at ultraviolet-B doses relevant to human skin exposure. ***Key words: antioxidant/free radicals/hydrogen peroxide/8-oxo-7,8-dihydro-2′-deoxyguanosine/ultraviolet.***

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Reactive oxygen species (ROS) are by-products of aerobic life. Although ROS normally account for approximately 1% of the oxidant load in aerobes, these levels can rise to as high as 17% during times of oxidative stress (Fridovich, 1978; 1984) and, in humans, increased levels of ROS have been associated with cancer, atherosclerosis, and diabetes (Sies, 1985; Frenkel, 1992; Halliwell and Gutteridge, 1999). Human skin, which is constantly exposed to environmental stresses, is vulnerable to the effects of ROS generated by exposure to ultraviolet (UV) radiation. Beginning with Pathak and Stratton (1968), who detected melanin radicals in epidermis after exposure to UVB, the effects of UVB on the generation of ROS in skin have been well established. For example, lipid peroxidation was detected in UVB-treated epidermal homogenates by Dixit *et al* (1983), whereas Danno *et al* (1984) inhibited sunburn cell formation by pretreating mice with the superoxide anion radical scavenger superoxide dismutase.

In average Caucasian skin, a UVB dose of approximately 40–100 mJ per cm^2 can induce erythema (Hawk, 1992), an inflammatory response causally related to the generation of ROS (Fuchs and Packer, 1993). In addition to an acute erythemal response, chronic actinic damage, such as immune suppression, has also been associated with oxygen-free-radical-mediated damage (Katiyar and Mukhtar, 2001).

At the nuclear level, DNA is susceptible to oxidative damage (Troll *et al*, 1984; Frenkel, 1992; Marnett, 2000). Of the four DNA bases, 2′-deoxyguanosine (dG) has the lowest ionization potential and thus is the most susceptible to oxidation. One of the positions where oxidation of dG can occur is at the C8 position, giving rise to 8-oxo-dG. The latter oxidized nucleoside exists in tautomeric equilibrium between the enol form 8-hydroxy-2′-deoxyguanosine and the keto form 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), with the oxo form greatly predominating. Although both oxidized forms are present, we shall refer here only to the 8-oxo-dG form. 8-oxo-dG is a useful biomarker of oxidative damage in DNA. It was first assayed by Floyd *et al* (1986) who used high performance liquid chromatography (HPLC) with electrochemical detection for the measurement of 8-oxo-dG coupled with UV detection for the

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Abbreviations: CuDIPS, copper(II)3,5-diisopropyl-salicylate hydrate; DCFdA, 2′,7′-dichlorodihydrofluorescein diacetate; dG, 2′-deoxyguanosine; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; ST DNA, salmon testes DNA.

measurement of the other four major nucleosides. This technique has proved to be an extremely useful tool for measuring oxidative DNA base damage and has been utilized by many workers in the field. For example, Kasai *et al* (1986) found 8-oxo-dG increased in HeLa cell DNA after exposure to oxygen radical generators, such as γ-radiation. This early work helped to establish 8-oxo-dG as a product of oxygen free radical reactions. Later, increased levels of 8-oxo-dG were observed in mouse skin after treatment with tumor promoters by Frenkel *et al* (1991) who then further demonstrated a reduction in these levels by chemopreventive agents including caffeic acid phenethyl ester (Frenkel *et al*, 1993). Additionally, the oxidative potential of UV radiation to induce 8-oxo-dG in HeLa cells was described by Zhang *et al* (1997).

Kuchino *et al* (1987) have shown that, if not repaired, this lesion, and/or the products derived from it (Ravanat *et al*, 1999), may be misread by a polymerase during replication and lead to mispairing with A. Then, during the next round of replication, this A will correctly pair with T leading to a G→T transversion mutation. This replication sequence demonstrates the mutagenic nature of 8-oxo-dG (Moriya and Grollman, 1993) and underscores the importance of protecting cells against oxidative DNA damage. Further, G→T transversions were detected in plasmid-borne yeast after exposure to sunlight and UVB (Kunz and Armstrong, 1998).

Normal human epidermal keratinocytes (NHEK) are the major cells in human epidermis. Nevertheless, 8-oxo-dG has not yet been determined in these cells after UV exposure. Previous work by Beehler *et al* (1992) showed an increase in 8-oxo-dG in mouse keratinocytes after UVB irradiation, and in another study by Stewart *et al* (1996) UVB-induced 8-oxo-dG in mouse keratinocytes was inhibited by Trolox (a synthetic form of tocopherol but without a phytyl side chain) and indicated that antioxidants could inhibit UVB-induced 8-oxo-dG production in epidermal cells.

The objective of this study was to measure oxidative DNA base damage in the form of 8-oxo-dG in NHEK as a result of UVB irradiation and to induce this damage at environmentally relevant doses comparable to normal human exposures. We chose to study NHEK because the presence of 8-oxo-dG has not been investigated yet in normal human keratinocyte cells or at these doses. Further, in order to understand the mechanism of 8-oxo-dG induction, NHEK were pretreated with various ROS scavengers. Additionally, the production of hydrogen peroxide (H_2O_2), which was shown to increase in NHEK after UVB exposure (Peus *et al*, 1998), was correlated to UVB-induced oxidative DNA base damage.

MATERIALS AND METHODS

Chemicals 2′-Deoxyribonucleosides, lithium acetate, chloroform, isoamyl alcohol, mannitol, α-carotene, copper(II)3,5-diisopropyl-salicylate hydrate (CuDIPS), catalase, and thiourea were obtained from Sigma (St. Louis, MO). 8-oxo-dG was purchased from Cayman (Ann Arbor, MI) and Optima grade methanol was obtained from Fisher (Pittsburgh, PA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFdA) was purchased from Molecular Probes (Eugene, OR). Salmon testes DNA (ST DNA) was also from Sigma and used as a positive control at a concentration of 5 μg per ml.

Cells Primary NHEK were obtained from Cascade (Portland, OR) as primary culture cells isolated from fetal foreskin. Cells were cultured in 10 ml EpiLife (calcium-free) medium containing growth factors in 1% Supplemented Serum (Cascade) using T75 Falcon culture flasks with screw-capped HEPA filters. At approximately 50% confluency of the third passage, there were sufficient numbers of cells to perform experiments. In all cases cells were obtained with the relevant institutional approval for experiments handling human material in accordance with the Helsinki Principles.

UV radiation A bank of four Sylvania Sunlamp fs 40 bulbs, which generate UVB radiation (290–320 nm), was used to irradiate cells. Before irradiation, the culture medium was removed and the cells were rinsed in Dulbecco's phosphate-buffered saline (D-PBS). Then, 6 ml D-PBS were added to the T75 flask and the cells were exposed to UVB radiation. Cells were exposed through the top of the flask and, as the flasks were made of polystyrene, the top acted as a filter that eliminated wavelengths below 300 nm. Thus, the wavelengths centered around 313 nm, which are the most erythromogenic for human skin in natural sunlight, were used for irradiation of the cells.

Fluences were measured with an International Light IL1400A radiometer with a UVB probe attachment. A measurement of approximately 0.055 mW per cm^2 was typically made through an empty flask from which the bottom had been removed so that the probe could be positioned to where the cells would receive UVB. After exposure, D-PBS was aspirated, 2 ml Cell Lysing Solution (Gentra) were added, and DNA was extracted as described below.

Isolation of cellular DNA Special care was taken to isolate cellular DNA under nonoxidative conditions and as quickly as possible (Huang *et al*, 2001). Usually, two T75 flasks of cells at 50% confluency, which corresponds to approximately 5.5×10^6 cells, were used for a single sample subjected to 8-oxo-dG analysis. For DNA extraction, cells were rinsed in D-PBS, lyzed with 2 ml of Cell Lysis Solution, and then treated with 125 units of ribonuclease A (Roche) in a 15 ml polypropylene conical screw-capped tube. Proteins were removed by the addition of 120 units proteinase K (Roche) and incubated for 3 h at 55°C. This digestion was followed by a protein precipitation step performed by adding 1.75 ml 2 M sodium acetate, pH 4.5, and keeping on ice for 10 min. The precipitate was then removed by high-speed centrifugation (IEC, MP4R, #809 rotor) at 5000*g* at 5°C for 10 min. After centrifugation, the supernatant was transferred to a new 15 ml polypropylene conical tube and proteins and lipids were extracted with 3 ml chloroform/isoamyl alcohol (24:1) by shaking and then separating the aqueous phase by low-speed centrifugation (Beckman TJ-6, TH-4 rotor at 1200*g*, ambient temperature, 10 min). The upper aqueous phase was carefully transferred to another 15 ml polypropylene tube and treated with 1 μg of RNase (DNase-free) (Roche) at 37°C for 30 min. The sample was then re-extracted with chloroform/isoamyl alcohol (24:1), as before. After partition by centrifugation, however, the upper phase was added to 5 ml of isopropyl alcohol in a new 15 ml polypropylene conical tube and mixed by inversion. In the presence of isopropyl alcohol, DNA was precipitated and collected as a pellet after centrifugation, as before, in the high-speed IEC centrifuge. The pellet was then washed with 2 ml 70% nondenatured ethanol and subjected again to centrifugation at 5000*g* for 5 min. The 70% ethanol was carefully removed and the tube was dried by inversion and removal of liquid droplets with a Dacron-tipped swab and a micropipette tip. The pellet was then resuspended in 100 μl 10 mM Tris–HCl, pH 7.4, at 55°C for a period of 15 min.

Isolation of 8-oxo-dG from DNA DNA was sequentially digested with enzymes to the nucleoside level in preparation for HPLC analysis. 8-oxo-dG was isolated from ST DNA and DNA from NHEK. The following digestion protocol is an adaptation of the work by Frenkel *et al* (1991), Huang *et al* (2001), and Fiala *et al* (1989). DNA samples were dissolved in 10 mM Tris–HCl, pH 7.4, and subjected to the following enzymatic treatments in 1.5 ml polypropylene capped microfuge tubes. Approximately 40 μg DNA were digested with 40 units of deoxyribonuclease I in 10 mM Tris–HCl, pH 7.4, containing 100 mM NaCl and 10 mM $MgCl_2$ (37°C, 30 min). The pH was then lowered by the addition of 30 mM sodium acetate, pH 5.3, and the samples were digested with 1 unit of nuclease P1 (Roche) at 37°C for 60 min. One unit of alkaline phosphatase (Roche) was then added and the pH of the reaction was raised by the addition of 100 mM Tris–HCl, pH 7.4, followed by incubation at 37°C for 60 min.

As it has been reported (Liuzzi and Paterson, 1992) that UV-irradiated DNA is more resistant to digestion, an additional experiment was performed in which UVB-irradiated DNA was digested with and without snake venom phosphodiesterase (Worthington, Lakewood, NJ, USA). Phosphodiesterase (0.05 units) was added and incubated with the samples for an additional 30 min after digestion by alkaline phosphatase. A small increase in the yield of 8-oxo-dG without an effect on the yield of dG was observed in this experiment (data not shown) and phosphodiesterase was added to all subsequent DNA digestions.

After enzymatic hydrolysis, the sample, which had an approximate volume of 100 μl, was transferred into a Millipore ultra-free centrifugal filter with a nominal molecular weight cutoff of 5000 Da in order to remove proteins. The filters were subjected to centrifugation in a microcentrifuge (13,000*g*, 15 min) and a portion of the filtrate was injected into the HPLC system for nucleoside analysis.

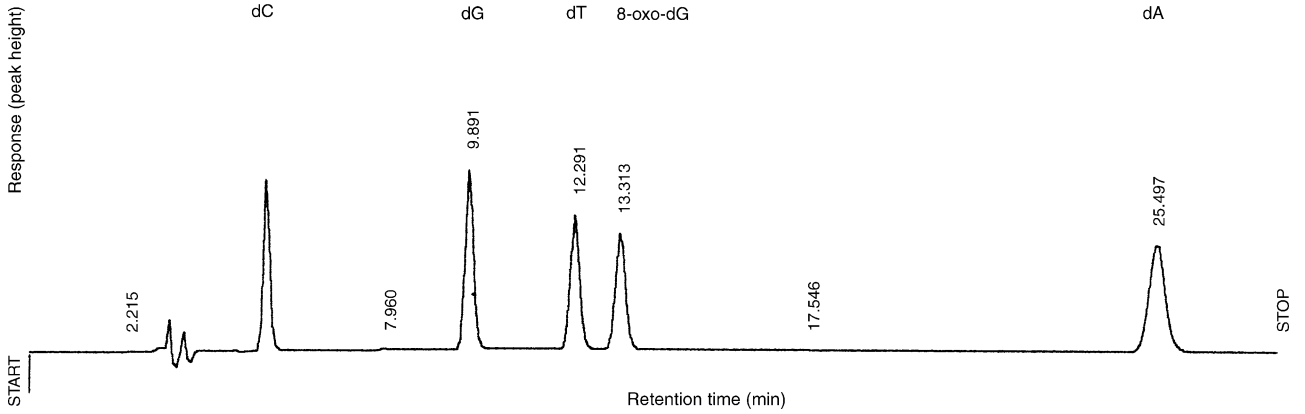

Figure 1. HPLC chromatogram of nucleoside standards. Separation of 2′-deoxyribonucleosides (dC, dG, dT, 8-oxo-dG, and dA) by HPLC/UV at a concentration of 0.1 nmol each. Separation was achieved with a 5 μm bead, C8/C18 reverse phase column, in 100 mM lithium acetate, pH 5.3, 6% methanol at a flow rate of 0.6 ml per min.

8-oxo-dG analysis HPLC was used to separate the four nucleosides and 8-oxo-dG. A Solvent Delivery System 582 (ESA, Chelmsford, MA, USA) delivered a mobile phase that consisted of 100 mM lithium acetate, pH 5.3, in 6% methanol at a flow rate of 0.6 ml per min. An isocratic gradient was pumped through a YMC Basic reverse phase column (4.6 × 150 mm) that was interfaced with a Lamda-Max 481 (Waters, Milford, MA, USA) UV detector to quantitate 2′-deoxycytidine (dC), dG, thymidine (dT), and 2′-deoxyadenosine (dA). Although 8-oxo-dG standards were detected by UV, its levels in cellular DNA are too low for UV detection and therefore electrochemical detection, which is orders of magnitude more sensitive, was used. For electrochemical detection analysis, a CoulArray Electrochemical Detector (ESA) was used in line with the UV detector. Approximately 20 μg of digested DNA were injected manually into a Rheodyne 7125 injector with a glass Hamilton flat-tipped syringe. **Figure 1** shows a chromatogram of nucleoside standards at 0.1 nmol each, using UV detection, whereas **Fig 2(*a*)** shows a chromatogram of 8-oxo-dG at 0.5 pmol using electrochemical detection, as well as 8-oxo-dG from control and treated cells (Fig 2,b,c).

H_2O_2 analysis H_2O_2 was determined by adding DCFdA to cells, which was a modification of the technique used by Bhimani *et al* (1995). For analysis, 2×10^4 cells were plated into the wells of a 96-well microtiter plate because it was determined that this number of cells attained approximately 50% confluence after overnight incubation. The next day, the medium was aspirated and 100 μl of a 10 μM DCFdA solution in D-PBS were added to the cells; they were incubated at 37°C for 15 min. DCFdA was prepared by dissolving 50 μg DCFdA in 100 μl ethanol and then adding 50 μl to 5 ml D-PBS to a final concentration of 10 μM. After incubation with DCFdA, 100 μl 25 mM sodium azide (a catalase inhibitor) were added at room temperature, and cells were incubated at 37°C for 2 h. Fluorescence measurements were made in a fluorescence plate reader (Cytofluor, PerSeptive Systems, Framingham, MA, USA) with a 485/20 nm excitation filter and a 530/25 nm emission filter set at a gain of 75.

Calculations Standard curves for each of the four nucleosides and 8-oxo-dG were calculated using regression analysis. The concentration of test samples was determined compared to the standards. The amount of 8-oxo-dG was expressed as a fraction of either total nucleosides or dG. Statistical significance was determined by a Student's *t* test and by ANOVA.

RESULTS

UVB irradiation of ST DNA In order to determine if UVB-induced increases in 8-oxo-dG could be detected in our analytical system, 100 μl of 5 μg per ml (0.5 mg total) ST DNA were placed on a piece of Parafilm stretched across a 35 mm Petri dish and exposed to UVB. Aliquots of 10 μl were removed at each time point that corresponded to doses between 0 and 500 mJ per cm^2. This range of doses was used because it encompassed a potential human exposure to UVB. The samples were then enzymatically digested to nucleosides and assayed for the presence of 8-oxo-dG. **Figure 3** shows dose-dependent increases in 8-oxo-dG and demonstrates that, under these experimental conditions, oxidative damage is induced in ST DNA by UVB and that an increase in 8-oxo-dG can be observed even at doses as low as 50 mJ per cm^2.

UVB-induced oxidative DNA base damage in NHEK Keratinocyte monolayers at approximately 50% confluency in D-PBS were irradiated in T75 flasks with increasing doses of UVB. Cells were exposed to 0, 62.5, 125, 250, and 500 mJ per cm^2. After each dose of radiation, the cells were harvested, and DNA was extracted, digested, and analyzed for 8-oxo-dG. The results showed significant increases in 8-oxo-dG, as evident from **Table I**.

The average baseline value for NHEK was 1.49 (± 0.11 SEM) 8-oxo-dG per 10^6 dG under these experimental conditions. Further, once the basal levels could be reliably measured, a dose of only 62.5 mJ per cm^2 was found to induce an increase in 8-oxo-dG to 2.07 (± 0.07 SEM). Moreover, a linear increase in 8-oxo-dG values was observed for each successive UVB dose and indicates that ROS are produced in NHEK in response to relatively low levels of UVB. As shown in **Fig 4**, ANOVA of the data demonstrated highly significant increases in 8-oxo-dG with a p-value < 0.001. These data clearly indicate a correlation between low dose UVB irradiation and oxidative DNA damage in human keratinocytes. Having established that UVB is able to induce 8-oxo-dG in normal keratinocytes over a range of doses, the 125 mJ per cm^2 dose was chosen as the dose used in other experiments for two reasons: first, it produced a significant increase in damage and, second, it was a biologically relevant dose that could be related to UVB-generated environmental trauma in human skin easily related to a sunburn in Caucasian skin.

Effect of ROS scavengers on UVB-induced damage in NHEK In order to understand the mechanisms of oxidative DNA damage in normal keratinocytes caused by UVB irradiation and to identify ROS responsible for it, several antioxidants, which could target specific ROS, were incubated with NHEK before UVB exposure. 1 mM and 5 mM mannitol were prepared in growth media and incubated overnight with NHEK during its log phase of growth. The next day media were removed and cells were washed with D-PBS and then treated as described in *Materials and Methods*. In three experiments, the average level of 8-oxo-dG per 10^6 dG increased from 1.54 (± 0.11 SEM) to 3.78 (± 0.36 SEM) after UVB exposure (125 mJ per cm^2), whereas the averages of the 1 mM and 5 mM mannitol-treated samples were 2.41 (± 0.31 SEM) and 1.99 (± 0.13 SEM), respectively, and a 1 mM mannitol-treated unirradiated control was 1.53. Thus, NHEK that were

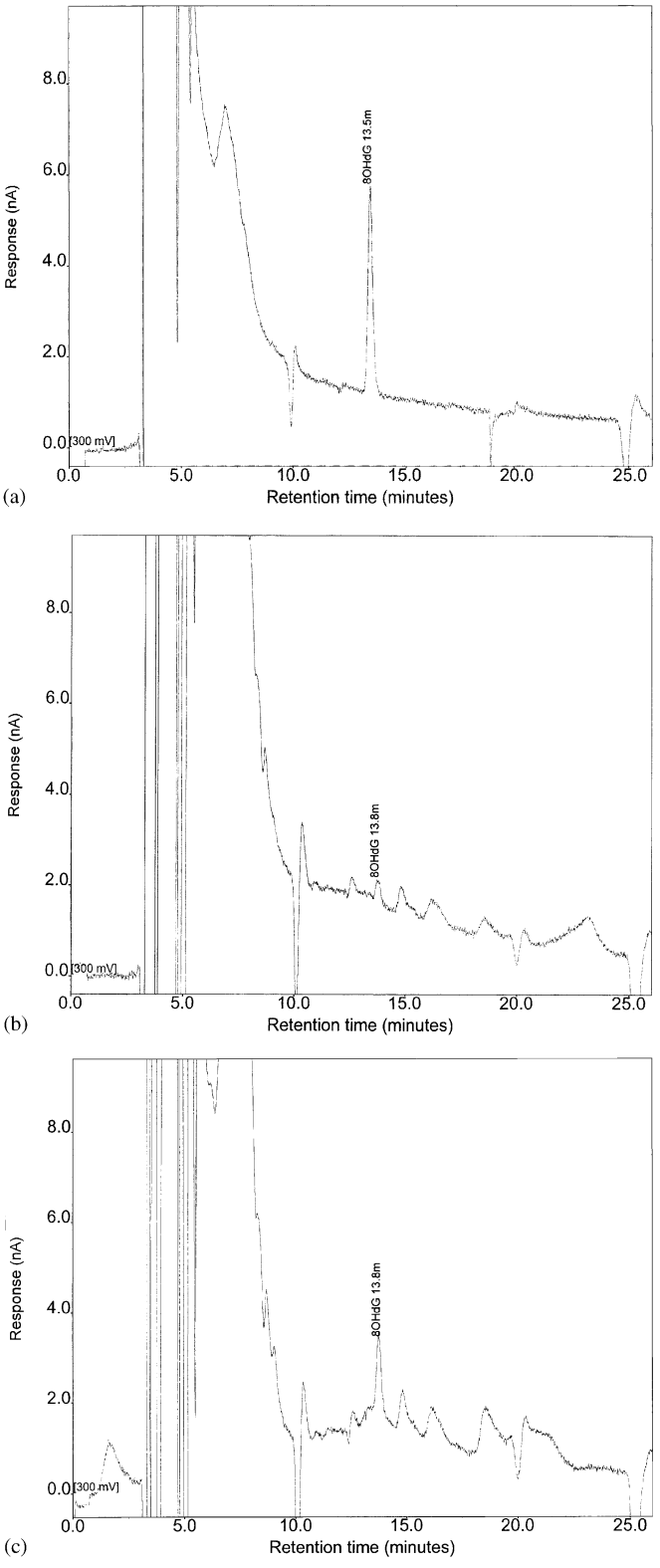

Figure 2. HPLC chromatogram of subpicomole levels of 8-oxo-dG. (*a*) 0.5 pmol of 8-oxo-dG standard was measured by electrochemical detection that was optimized to 300 mV using the same separation conditions as described in **Fig 1**; (*b*) 8-oxo-dG isolated from NHEK; (*c*) 8-oxo-dG isolated from NHEK after UVB irradiation.

pretreated with 1 mM mannitol showed a 60.7% reduction in 8-oxo-dG and the 5 mM treated samples exhibited a 79.9% decrease. These data are shown in **Table II**. As mannitol

Table I. UVB-induced increases in 8-oxo-dG in NHEK

UVB dose (mJ per cm^2)		8-oxo-dG per 10^6 dG (±SE)	Δ	% increase
0	($n=11$)	1.49 (±0.11)	0	100
62.5	($n=2$)	2.07 (±0.07)	0.58	138
125.0	($n=9$)	3.82 (±0.30)	2.33	256
250.0	($n=5$)	4.95 (±1.15)	3.46	332
500.0	($n=4$)	6.24 (±0.85)	4.75	418

Delta (Δ) values indicate a net increase in 8-oxo-dG per 10^6 dG in UVB-irradiated NHEK.

scavenges hydroxyl radicals (Halliwell and Ahluwalia, 1976), this indicates that UVB may have initiated a reaction that led to the generation of hydroxyl radicals in NHEK. To further strengthen these findings, thiourea, another hydroxyl radical scavenger (Cederbaum *et al*, 1979), was tested under the same experimental conditions. Similarly to mannitol, 1 mM thiourea also reduced the level of 8-oxo-dG in UVB-irradiated NHEK by an average of 74.1%.

Other ROS scavengers were also utilized under the same conditions. When β-carotene, which scavenges singlet oxygen (Sies, 1989), was incubated with the cells, no inhibition was detected. There may have been solubility problems, however, because even though 1 mM β-carotene was shaken into media over a 2 h period, some precipitation in the flasks was observed the next day. NHEK were also preincubated with CuDIPS, a superoxide dismutase mimetic (Leuthauser *et al*, 1981). At 0.1 mM, no changes in 8-oxo-dG levels were apparent; however, at 1 mM, 8-oxo-dG increased about 100-fold to 120.2 8-oxo-dG per 10^6 dG. This dramatic rise in 8-oxo-dG was similar to results obtained when H_2O_2 was added to cells (data not shown). Thus, any superoxide anion radicals that may have been generated by UVB exposure may have been converted to H_2O_2 by this superoxide dismutase mimetic molecule. As a result, the increased level of H_2O_2 may have overwhelmed the endogenous catalase and glutathione peroxidase enzymes, thus allowing oxidative damage to occur. Alternatively, the presence of a transition metal like Cu, which may have been liberated from the CuDIPS, may have also acted as a catalyst for hydroxyl radical formation. These results are summarized in **Table II**.

Effect of UVB on H_2O_2 levels in cells In order to correlate UVB-induced oxidative DNA damage in NHEK with H_2O_2 levels, the effect of UVB on H_2O_2 formation was assessed. NHEK were exposed to a range of UVB from 20 to 100 mJ per cm^2 and then subjected to DCFdA and fluorescence analysis. As can be seen in **Fig 5**, doses as low as 20 mJ per cm^2 were sufficient to increase H_2O_2 formation. Additionally, in order to show that H_2O_2 was released from NHEK as a result of UVB irradiation, 10 units per ml catalase were added to the media after irradiation and then measured for fluorescence. **Figure 6** shows a marked decrease in fluorescence in those samples treated with catalase and indicates that the increased fluorescence observed in the UVB-exposed cells was due to H_2O_2.

DISCUSSION

Chronic exposure to sunlight is a major risk factor for photocarcinogenesis and photoaging in human skin (Matsui and DeLeo, 1995; Armstrong and Kricker, 2001). As ROS are generated in skin as a result of UVB irradiation, our goal was to measure the effects of ROS in normal human keratinocytes at doses comparable to human exposure. As DNA damage may lead to an aberrant expression or dysregulation of gene products, we focused our efforts on 8-oxo-dG because much literature exists regarding it as a biomarker for oxidative DNA damage and its biologic effects

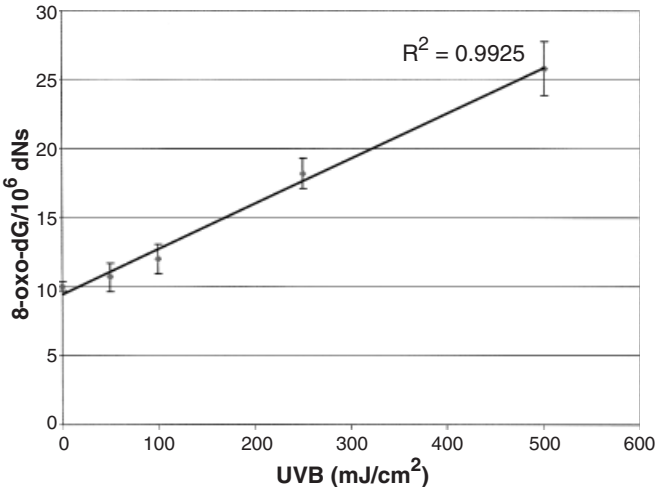

Figure 3. UVB-mediated dose-dependent increase in 8-oxo-dG in ST DNA. A linear increase in 8-oxo-dG was observed when naked ST DNA was exposed to increasing levels of UVB. Data expressed as mean ± SEM; $n = 2$ for 50, 250, 500 mJ per cm^2, $n = 3$ for 100 mJ per cm^2.

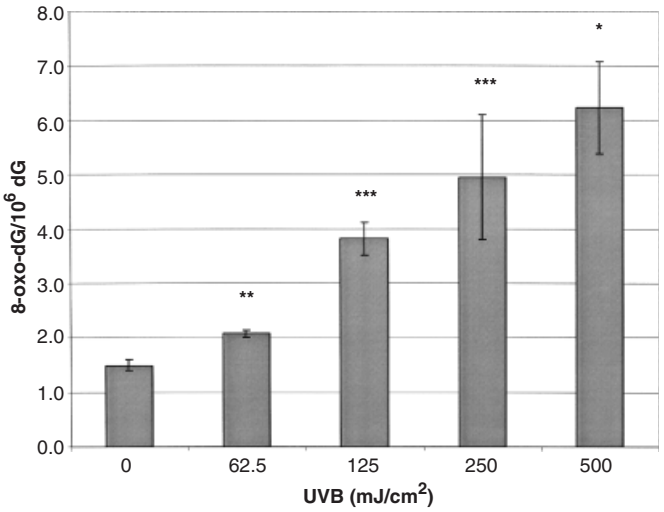

Figure 4. UVB-induced 8-oxo-dG formation in NHEK. A dose-dependent increase in 8-oxo-dG was observed in DNA extracted from NHEK after exposure to UVB. ANOVA $p < 0.001$; *$p < 0.01$, **$p < 0.002$, ***$p < 0.001$. Data expressed as mean ± SEM; $n = 11$, 2, 9, 5, and 4 for 0, 62.5, 125, 250, and 500 mJ per cm^2, respectively.

(Kasai, 1997; Halliwell, 1999). To analyze 8-oxo-dG, we used an HPLC technique with electrochemical detection that could sensitively measure subpicomole levels. Additionally, we carefully and quickly extracted DNA under nonoxidative conditions to ensure reproducible basal values.

In a first series of experiments, we tested our system by exposing naked ST DNA to UVB. We found a dose-dependent increase in 8-oxo-dG, which demonstrated the viability of our analysis as well as the ability of UVB to induce oxidative DNA base damage at low levels of exposure. Although the mechanism for this increase is unknown, perhaps the presence of iron in commercially prepared DNA in an oxygenated, aqueous microenvironment contributed to the formation of 8-oxo-dG. One possibility may be a photo-oxidative reaction that generates

Table II. Effects of antioxidants on 8-oxo-dG levels induced by UVB irradiation in NHEK DNA

	% UVB-induced DNA damage
UVB	100.0
UVB + 1 mM mannitol	39.3 (±8.2% SE)
UVB + 5 mM mannitol	20.1 (±3.4% SE)
UVB + 1 mM thiourea	25.9 (±25.8 SE)
UVB + 1 mM β-carotene	100.0
UVB + 0.1 mM CuDIPS	100.0
UVB + 1 mM CuDIPS	10,000.0

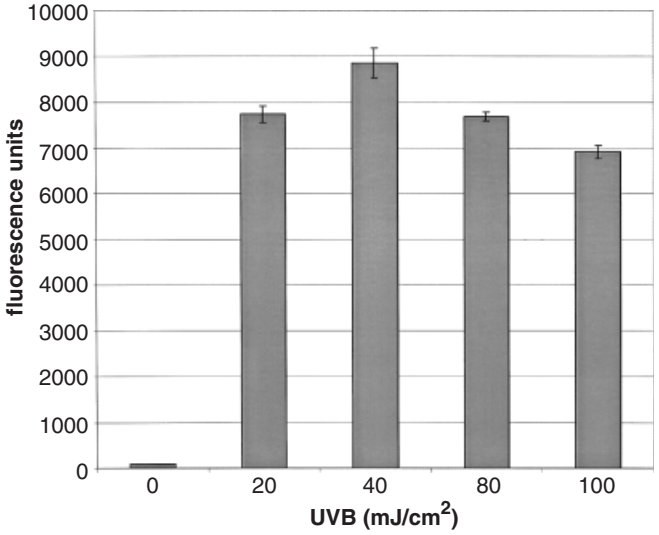

Figure 5. UVB-induced H_2O_2 increase in NHEK. Increases in H_2O_2 were observed after UVB irradiation of NHEK (± %SEM, $n = 10$); ANOVA $p < 0.006$.

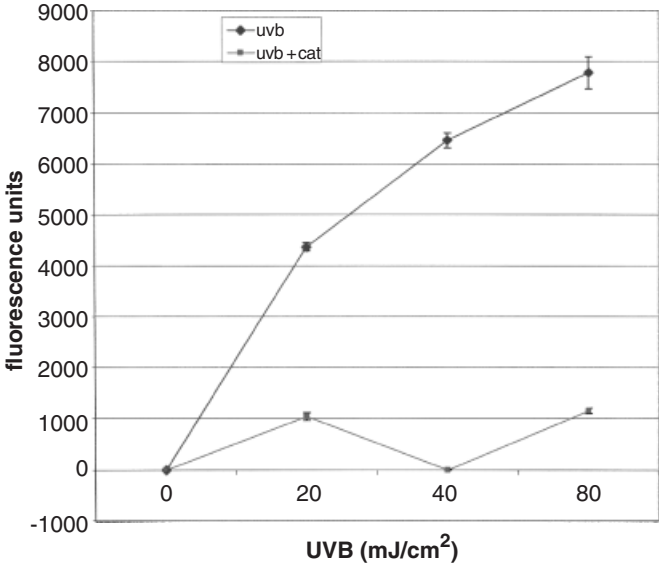

Figure 6. Catalase-mediated inhibition of UVB-induced H_2O_2 formation in NHEK. Increases in H_2O_2 after UVB irradiation of NHEK were inhibited in the presence of 10 units per ml catalase (± %SEM, $n = 3$).

guanine peroxyl radicals, as shown by Hildenbrand and Schulte-Frohlinde (1990), which might then lead to guanine hydration and subsequent generation of 8-oxo-dG as proposed by Kasai *et al* (1992). In our next series of experiments, we evaluated oxidative DNA base damage at the cellular level and observed a linear increase in 8-oxo-dG when NHEK were exposed to increasing doses of UVB. Although it has been shown before that UVB could induce 8-oxo-dG in mouse keratinocytes, this is the first demonstration of such an effect in normal human keratinocytes. Moreover, this is the first evidence of 8-oxo-dG damage induced by UVB at environmentally relevant levels (62.5–500 mJ per cm^2). These results are significant because they show the potential for oxidative DNA base damage in UVB-exposed human skin. Further, they extend our understanding of UV-associated pathologies in human skin for two reasons. First, murine keratinocytes may respond differently to UVB radiation than human cells because mice are nocturnal and normally have fur and, as a result, may not have developed a response to UVB exposure that is similar to humans. Second, these data show that significant oxidative DNA damage can occur in cells at exposures as low as 62.5 mJ per cm^2 UVB and thus UVB, at a dose sufficient to cause a minimum erythemal response in average Caucasian skin, may represent a significant health hazard to human skin DNA. This is supported by Heenen *et al* (2001) who correlated erythema in human skin with unscheduled DNA synthesis, as well as sunburn cell development.

NHEK were also pretreated with various ROS scavengers to evaluate their ability to modulate UVB-induced damage. After overnight treatment, 1 and 5 mM mannitol significantly inhibited the production of 8-oxo-dG by 60.7% and 79.9%, respectively. Thiourea also had a similar effect and inhibited 8-oxo-dG formation by 74.1%. As mannitol and thiourea scavenge $OH\cdot$, these results indicate hydroxyl radical involvement in UVB-induced formation of 8-oxo-dG in NHEK. Nevertheless, the possibility also exists that guanine peroxyl radicals may have contributed to 8-oxo-dG formation. Another scenario might involve singlet oxygen as 8-oxo-dG increased in cellular DNA when an endoperoxide of N,N′-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide, which generates singlet oxygen, was added to cells (Ravanat *et al*, 2000).

Additionally, an increase in H_2O_2 was measured in NHEK after UVB irradiation. Thus, as oxidative stress in cells can lead to the formation of H_2O_2, it is possible that H_2O_2, being a neutral species, can permeate the nuclear membrane, react with DNA-associated transition metals, and participate in a Fenton reaction leading to hydroxyl radical production and oxidative DNA base damage. Our data support the findings of Imlay and Linn (1988) who ascribed H_2O_2 toxicity in bacteria, at least in part, to the Fenton reaction. Although it has been reported (Buchko *et al*, 1995) that singlet oxygen is responsible for UV-mediated production of 8-oxo-dG, the experiments that were done to support this finding used UVA (320–400 nm) in the presence of a photosensitizer, which preferentially produces singlet oxygen. Nevertheless, generation of singlet oxygen by UVA cannot be ruled out as a generalized mechanism for 8-oxo-dG production solely by UV radiation (Zhang *et al*, 1997). This possibility is further underscored by the work of Bishop *et al* (1994) who demonstrated, *in vitro*, the ability of triplet excited states of dinucleotides to generate singlet oxygen, which was then quenched by guanine and itself oxidized in the process. Low doses of UVB without added photosensitizers, however, may give more realistic information regarding oxidant generation in keratinocytes. Additionally, determining the oxidant conditions of the nucleus by measuring 8-oxo-dG may help to provide information regarding the redox state of the nucleus, which is critical for transcriptional regulator activity (Klein *et al*, 1998) and which, in turn, contributes to cell maintenance and survival. In conclusion, these data show UVB-induced generation of 8-oxo-dG in normal human keratinocytes and describe a possible oxidative mechanism involving hydroxyl radicals.

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· 临床论著 ·

甘草酸二铵治疗慢性乙型肝炎 3201 例荟萃分析

秦刚 施光峰 宋艳艳 陈明泉

【摘要】 **目的** 根据现有临床研究系统评价甘草酸二铵治疗慢性乙型肝炎的疗效及安全性。**方法** 从 1995 至 2004 年中国生物医学光盘数据库和 1995 至 2004 年中国期刊网数据库，检索以慢性乙型肝炎为研究对象，比较甘草酸二铵治疗与其他护肝药物治疗效果的随机对照试验(RCTs)文献，并对 RCT 进行 meta 荟萃分析。**结果** 共 24 项 RCT 3201 例患者入选。甘草酸二铵组有效率高于对照组，两组比较差异有统计学意义($P<0.01$)；甘草酸二铵组有效率高于甘草酸单铵组，两组比较差异有统计学意义($P<0.01$)；甘草酸二铵联合丹参治疗慢性病毒性肝炎有明显抗纤维化功效。**结论** 甘草酸二铵具有较强的护肝、退黄、抗肝纤维化等作用，未发现明显不良反应。

【关键词】 甘草次酸；肝炎，乙型，慢性

Meta-analysis of document on diammonium Glycyrrhizinate in treatment of patients with chronic hepatitis B

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【**Abstract**】 **Objective** In order to evaluate the efficacy of Diammonium Glycyrrhizinate in the treatment of chronic hepatitis B. **Methods** The randomized clinical trials (RCTs) that compared the efficacy of Diammonium Glycyrrhizinate and other kind of treatment in chronic hepatitis-B were chosen from CBM disks from 1995 to 2004 and CNKI from 1995 to 2004. A meta-analysis was employed to evaluate the results of these therapies. **Results** Twenty-four RCTs including 3201 cases were analyzed. Compared with control group, the total RR of efficiency rate of Diammonium Glycyrrhizinate group were 1.378(95%CI 1.243～1.529), showing significant difference ($P<0.01$). There was significant difference between the efficiency rate of Diammonium Glycyrrhizinate group and that of Glycyrrhizin group ($RR=1.273$(95%CI 1.172～1.383), $P<0.01$). Combining use of Diammonium Glycyrrhizinate and Danshen could exert better antifibrosis effect. **Conclusions** Diammonium Glycyrrhizinate was superior to both supportive drugs and Potenlini in treatment of chronic hepatitis-B. It could reduce ALT and TB and ameliorate fibrosis as well.

【**Key words**】 Glycyrrhetinic acid; Hepatitis, B Chronic

甘草酸二铵(商品名：甘利欣)是目前临床最常用的保肝、抗炎药物之一，多年临床应用发现其有较强的抗炎、免疫调节、保护肝细胞膜、改善胆红素代谢、抗肝纤维化等作用，有关甘草酸二铵疗效的临床研究不少，但大样本的系统分析尚未见报道。应用 meta 分析荟萃多项小样本、不同研究结论的资料，定量分析各研究结果数据，可提高研究结论的可信性。为了更加客观地评价甘草酸二铵治疗慢性乙型肝炎作用，现对甘草酸二铵临床 RCT 资料进行 meta 分析。

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材料与方法

一、材料

1995～2004 年中国生物医学文献数据库(CBM)光盘、中国期刊网数据库(CNKI)。

二、文献检索方法

采用关键词和主题词(全部扩展树)检索病毒性肝炎,乙型;自由词检索甘草酸二铵/甘利欣、随机、对照研究、对比研究、分组研究,特征词检索人类。查找了 2 项数据库:中国生物医学文献光盘(CBMdisc),检索年限为 1995 年至 2004 年;中国期刊网数据库(CNKI,网上检索),检索年限为 1995 年至 2004 年。并根据查阅文献的参考文献查缺补漏。

三、文献纳入标准

①主要研究目的为比较甘草酸二铵和其他护肝药物(包括西药和中药)及甘草酸单铵(商品名:强力宁)对照治疗慢性乙型肝炎的临床随机对照试验;为了避免遗漏其中真正的随机对照临床试验,增加文献检索敏感性,论文中凡具有"随机"字样者,均纳入 RCTs 范围内;②试验前治疗组和对照组的基线情况进行统计学检验证实无明显差异,以确定组间均衡性和可比性;③治疗时间≥2 个月。

四、统计学处理

两名研究者独立进行文献选择和资料提取工作,而后进行交叉核对。对入选 RCTs 用 Stata 8.2 进行统计分析。观察指标为计量资料时选用加权均数差值(weighted mean differences, WMD)。计数资料时采用 Peto 方法,计算相对危险度(RR),两者均计算 95% 的可信区间(CI),而后进行异质性检验,当试验结果的异质性无统计学意义($P>0.05$)时,选择固定效应模型作 Meta 分析,当试验结果的异质性有统计学意义($P<0.05$)时,选择随机效应模型,最后对研究结果进行敏感性分析。

结　果

一、文献入选情况

共有 24 项 RCT 3201 例慢性乙型肝炎患者入选,所有试验中,有 13 项试验[1-13]为甘草酸二铵与其他常见基础护肝药物(如丹参、门冬氨酸钾镁、硫普罗宁、垂盆草、复方益肝灵片、维生素 E 等)的疗效比较;5 项试验[14-18]为甘草酸二铵与甘草酸单铵治疗的比较;2 项试验[19,20]为甘草酸二铵加复方丹参治疗与复方丹参治疗的抗纤维化疗效比较;4 项试验[21-24]为甘草酸二铵加复方丹参/丹参治疗与基础治疗的比较。

所有病例的诊断均依据 1995 年北京第 5 次全国传染病和寄生虫学术会议修订的标准[25],或 2000 年西安第 10 次全国传染病和寄生虫学术会议修订的《病毒性肝炎防治方案》诊断标准[26]。各组在性别、年龄、病程及病变程度上具有可比性。所有试验甘草酸二铵的使用初始剂量为每日 1 次,150～200 mg 静脉滴注,总疗程≥2 个月。

二、甘草酸二铵护肝、退黄作用

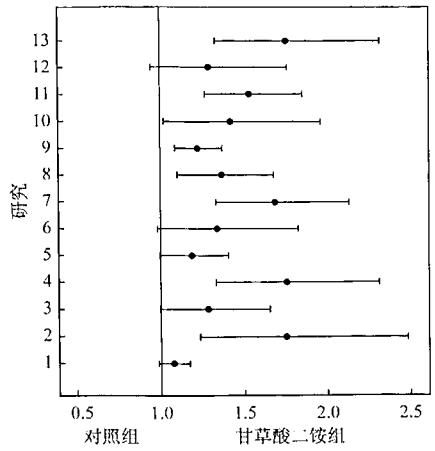
疗程为 2 个月。临床疗效判断分为显效、有效、无效。显效:临床症状消失,丙氨酸转氨酶(ALT)、总胆红素(SB)完全恢复正常;有效:临床症状消失,ALT、SB 基本恢复正常;无效:临床症状无改善,ALT、SB 达不到上述标准。

(一)甘草酸二铵治疗组与对照组的治疗有效率比较　13 项 RCT 对照组治疗包括丹参/复方丹参组 2 组、丹参/复方丹参+门冬氨酸钾镁 2 组、垂盆草 2 组、硫普罗宁 1 组,其他为复方益肝灵、肌苷、维生素 E 等的综合支持治疗。比较了两种疗法的有效率,甘草酸二铵组有效率高于对照组,两组比较差异有统计学意义,见表 1。

表 1　甘草酸二铵组与对照组的治疗有效率比较

文献作者	甘草酸二铵组			对照组		
	例数	有效例数	有效率(%)	例数	有效例数	有效率(%)
尹玉学等[1]	100	96	96	76	68	89.47
陈清春[2]	62	56	90.32	31	16	51.61
林海秋[3]	50	44	88	32	22	68.75
靳虹等[4]	58	54	93.1	47	25	53.19
陈殿双等[5]	180	146	81.11	70	48	68.57
景玉焕等[6]	30	25	83.33	32	20	62.5
林述龙等[7]	64	59	92.19	62	34	54.84
邹桂舟等[8]	86	74	86.05	60	38	63.33
陈祖涛等[9]	160	146	91.25	112	84	75
陈厚皎等[10]	35	30	85.71	28	17	60.71
徐德先等[11]	102	87	85.29	102	57	55.88
庄婷婷等[12]	63	43	68.25	49	26	53.06
刘爱民等[13]	58	54	93.1	47	25	53.19
合计	1048	914	87.21	748	480	64.17

同质性检验结果为 $Q=38.878$,$P<0.01$。因此采用随机效应模型,Meta 分析结果为 $RR=1.378$,其 95% 可信区间为(1.243～1.529),$P<0.01$,见图 1。



注：研究 1～13 与参考文献 1～13 对应

图 1 甘草酸二铵组与对照组的治疗有效率比较（RR 值及 95%可信区间）

（二）甘草酸二铵治疗组与甘草酸单铵治疗组的治疗有效率比较　甘草酸单铵组剂量、疗程均与甘草酸二铵组相同，即甘草酸单铵 150～200 mg，每日 1 次，静脉滴注。

5 项研究[14-18]比较了两种疗法的有效率，甘草酸二铵组有效率高于甘草酸单铵组，两组比较差异有统计学意义，见表 2。

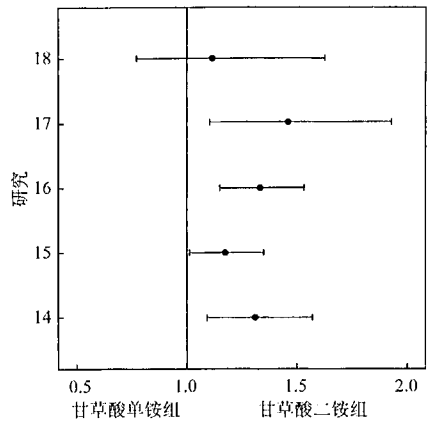
表 2 甘草酸二铵组与甘草酸单铵组的治疗有效率比较

文献作者	甘草酸二铵组			甘草酸单铵组		
	例数	有效例数	有效率(%)	例数	有效例数	有效率(%)
龙桂华[14]	60	55	91.67	60	42	70
朱其守[15]	135	122	90.37	65	50	76.92
邹刚等[16]	100	92	92	100	69	69
崔志雄等[17]	40	35	87.5	40	24	60
金学慧等[18]	40	29	72.5	20	13	65
合计	375	333	88.89	285	198	69.47

同质性检验结果为 $Q=3.076$，$P=0.545$。因此采用固定效应模型，Meta 分析结果为 $RR=1.273$，其 95%可信区间为(1.172～1.383)，$P<0.01$，见图 2。

三、甘草酸二铵抗肝纤维化作用

疗程为 2 个月。治疗前后各进行一次血清肝纤维化指标如透明质酸(HA)、Ⅳ型胶原(Ⅳ-C)检查，部分 RCT 还检测Ⅲ型前胶原(PⅢP)、层粘蛋白(LN)等。



注：研究 14～18 与参考文献 14～18 对应

图 2 甘草酸二铵组与甘草酸单铵组的治疗有效率比较（RR 值及 95%可信区间）

2 项 RCT[19,20]比较发现甘草酸二铵联合复方丹参治疗效果优于单用复方丹参，以 HA 改善最为显著，说明两药的配伍可起协同抗肝纤维化作用。

4 项 RCT[21,21]证实，血清 HA、Ⅳ-C 在甘草酸二铵联合丹参/复方丹参治疗前明显高于正常值，经治疗后明显下降，以 HA 改善更明显，见表 3、表 4。对 HA 指标进行同质性检验：甘草酸二铵联合丹参/复方丹参组的结果为 $Q=62.679$，$P<0.01$，采用随机效应模型，治疗前后 HA 指标之差值为 116.052，其 95%可信区间为(40.991～191.114)，$P=0.002$；对照组的结果为 $Q=19.397$，$P<0.01$，治疗前后 HA 指标之差值为 46.613，其 95%可信区间为(−1.635～94.862)，$P=0.058$。对Ⅳ-C 指标进行同质性检验，甘草酸二铵联合丹参/复方丹参组的结果为 $Q=73.884$，$P<0.01$，采用固定效应模型，治疗前后Ⅳ-C 指标之差值为 38.809，其 95%可信区间为(5.996～71.623)，$P=0.02$；对照组的结果为 $Q=3.488$，$P<0.322$，治疗前后 HA 指标之差值为 9.826，其 95%可信区间为(0.861～18.790)，$P=0.032$。

四、不良反应

15 项 RCT 对不良反应进行了观察和具体说明，其结论为甘草酸二铵在临床试验中不良反应轻微，少数病例有口干、头晕等感觉或轻微胃肠道反应，无需治疗，均可耐受。2 项研究显示甘草酸单铵对照组少数病例出现一过性高血压、水肿等不良反应。

表 3　甘草酸二铵联合丹参/复方丹参治疗前后血清 HA 变化(μg/L, $\bar{x}\pm s$)

文献作者	甘草酸二铵联合丹参/复方丹参组			对照组		
	病例数	治疗前 HA	治疗后 HA	病例数	治疗前 HA	治疗后 HA
郭辛[21]	45	323.03±90.12	156.10±81.20	38	321.15±80.97	261.08±73.87
林耀怀[22]	72	118.32±34.13	76.22±21.31	38	125.29±16.34	130.42±34.45
陈晶[23]	44	263.71±181.64	133.54±113.40	41	281.54±172.80	207.21±162.63
邹桂舟[24]	92	264.72±182.65	133.56±113.41	92	282.55±173.81	207.83±162.65

表 4　甘草酸二铵联合丹参/复方丹参治疗前后血清Ⅳ-C 变化(μg/L, $\bar{x}\pm s$)

文献作者	甘草酸二铵联合丹参/复方丹参组			对照组		
	病例数	治疗前Ⅳ C	治疗后Ⅳ C	病例数	治疗前Ⅳ C	治疗后Ⅳ C
郭辛[21]	45	131.09±31.21	62.18±13.31	38	134.15±27.42	123.21±31.21
林耀怀[22]	72	43.17±32.48	35.41±27.37	38	76.44±36.93	82.18±50.39
陈晶[23]	44	90.41±72.8	51.60±31.22	41	87.94±77.43	70.64±51.68
邹桂舟[24]	92	91.42±72.9	51.61±31.23	92	88.96±76.45	70.65±51.69

讨　论

甘草酸二铵的主要化学成分为 α 体的甘草酸二铵盐，有较强的抗炎、保护肝细胞膜、改善胆红素代谢、抗肝纤维化等作用，还能诱导干扰素的产生，激活巨噬细胞等功能，在抗病毒、抗肿瘤及免疫调节等方面起重要作用。我们对甘草酸二铵临床 RCT 资料 3201 例进行了 meta 分析。

甘草酸二铵能抑制肝脏对皮质激素的还原代谢，从而类固醇激素的作用增强，显示抗炎、抗毒作用，但无皮质激素样的不良反应。药理实践证明，小鼠口服本品能减轻因四氯化碳、硫代乙酰胺和 D-氨基半乳糖引起的血清 ALT 升高，并能明显减轻 D-氨基半乳糖对肝脏的形态损伤和改善免疫性因子对肝脏形态的慢性损伤。甘草酸二铵亲脂性强，在体内极易与载体蛋白结合，与毛细胆管膜蛋白-类脂质受体复合物结合后，使其通透性减低，胆汁成分经肝细胞入血减少，改善胆红素代谢功能。

本组 13 项 RCT 显示甘草酸二铵治疗慢性乙肝有效率高于基础治疗组，体现为临床症状改善明显，降酶、退黄作用可靠、迅速，能有效控制肝脏炎症。5 项 RCT 显示甘草酸二铵治疗慢性乙肝优于甘草酸单铵，治疗有效率高，不良反应少。

在临床及实验研究中发现丹参和甘草酸二铵均具有抗肝纤维化作用。甘草酸二铵的作用机制，一方面可能是通过抗炎作用来减轻肝脏炎性损伤，消除纤维增生的诱发因素，从而降低肝星状细胞(HSC)的活性；另一方面也可能直接抑制纤维生成细胞的增生与细胞外基质的产生。脂质过氧化产物可直接刺激胶原基因转录，而甘草酸二铵具有良好的抗过氧化作用，产生抗肝纤维化效应。肝穿刺活检病理检查是诊断肝纤维化的金标准，但因其属于创伤性检查，在不同时期对同一患者做多次肝穿尚未被广泛接受，因而其应用受限，不利于动态观察。近几年观察到有关血清学指标亦可相对地反映肝纤维化程度，并与病理结果有较好的一致性，其中，HA、Ⅳ-C 等均是反映肝纤维化较好的血清学指标，对估计慢性肝炎的病情进展及观察药物抗纤维化的治疗效果有重要的实际意义。本组 2 项 RCT 比较发现甘草酸二铵＋复方丹参两药合用效果优于单用复方丹参，以 HA 改善最为显著，说明两药的配伍可起协同抗肝纤维化作用；4 项 RCT 证实，血清 HA、Ⅳ-C 在甘草酸二铵联合丹参/复方丹参治疗前明显高于正常值，经治疗后明显下降；此外，刘斌等[27]通过肝穿刺活组织检查发现甘草酸二铵对 S3 期以前的轻、中度肝纤维化效果较好；上述结果提示甘草酸二铵联合丹参/复方丹参治疗慢性病毒性肝炎有明显抗纤维化功效，从而为临床治疗慢性肝炎及抗肝纤维化提供新途径。我们从提取的临床随机对照试验中发现，部分试验未描述随机化方法、随机分配方案及失访病例。大多数仅叙述采用随机分组，而未给予足够的信息证实是否做到了真正的随机。仅有 5 篇研究报道停药 3 个月后的随访情况，多数文献无随访报告，这有待于在今后进一步的临床研究中加

以完善。

总之，通过总结近十年来甘草酸二铵治疗慢性乙型肝炎的临床 RCT 资料 3201 例，分析结果表明甘草酸二铵具有较强的护肝、退黄、抗肝纤维化等作用，临床应用安全而有效，其停药后的随访观察还有待于进一步的研究。

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Relationship between DDR2 and alcoholic liver fibrosis and response of compound glycyrrhizin tablets on them: an experimental study.

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Objective: To investigate the relationship between DDR2 and alcoholic liver fibrosis as well as the response to compound glycyrrhizdn tablets treatment on them. Methods: 48 rats were randomly divided into 6 groups (8 in each group): the control group, the model group, the Alc disc group, the Alc contin group, the CGT+Alc disc group and the CGT+Alc contin group. After 16-week intragastric administration with alcohol, rats in the model group were sacrificed, at the same time rats in the control group were killed. The rest having been treated by intrastric alcoholic for 16 weeks in the Alc disc group (stopped alcohol intragastric), the CGT+Alc disc group (stopped alcohol intragastric and treated by CGT), the Alc contin group (continued alcohol intragastric without treatment of CGT) and the CGT+Alc contin group (continued alcohol intragastric with treatment of CGT simultaneously) were sacrificed at the end of the 20th week. Levels of serum hyaluronic acid (HA), laminin (LN), procollagen type III (PC III) and collagen type IV (C IV) were determined. Pathologic changes of the liver were observed by HE staining and Masson staining. Expressions of DDR2 mRNA and protein were determined by RT-PCR and Western blot, respectively. Results: Pathological liver fibrosis was obviously found in rats with alcoholic intragastric administration for 16 weeks. Area-density percentage of collagen fibrosis, levels

VV600 - Non-communicable Human Diseases and Injuries

VV810 - Human Toxicology and Poisoning, (New March 2000)

Organism descriptor

rats

Descriptor

alcohol intake

antiinflammatory properties

glycyrrhizin

hepatic fibrosis

histopathology

laboratory animals

liver

liver diseases

receptor protein-tyrosine kinase

Identifier

alcohol consumption

anti-inflammatory properties

protein-tyrosine kinase

Broad term

Muridae

rodents

mammals

vertebrates

Chordata

animals

eukaryotes

CAS registry number

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of serum HA, LN, PC III and C IV, and levels of DDR2 mRNA and protein in the liver were significantly increased in the model group compared with those in the control group (P<0.01). Moreover, all these indexes were decreased in the CGT+Alc disc group compared with the Alc disc group and in the CGT+Alc contin group compared with the Alc contin group (P<0.05). Conclusion: CGT had certain therapeutic effects on alcoholic liver diseases, which may relate to down-regulation of DDR2 expression.

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ALCOHOLIC LIVER DISEASE (ALD);

IMPLICATION OF OXIDATIVE STRESS & EXTRAPOLATIVE FACTORS IN PATIENTS SUFFERING, UPDATE FROM LOCAL POPULATION OF PUNJAB-PAKISTAN

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ABSTRACT… Objective: The aim of the present study was to investigate the changes in biochemical parameters including lipid profile, liver and kidney profile as well as oxidative stress profile, particularly in patients suffering from alcoholic liver disease (ALD). **Study design:** Fifty chronic alcoholics admitted for treatment to the in-patient wards at Jinnah Hospital, Lahore-Pakistan. Chronic alcoholics with alcohol abuse for more than four to five years, and with or without clinical complications, were included. Apparently twenty healthy individuals served as control. **Period:** 2012-2013. **Materials and methods:** Various circulating biochemical biomarkers including renal profile, hepatic and lipid profile were evaluated. Moreover, stress markers (MDA. SOD, GSH and catalase) were also investigated. **Results:** A very strong direct and indirect correlation of ALP was found with TB, MDA and GSH (r=.950**, r=.929** and r=-.967** respectively, P<0.01). MDA was observed having very strong indirect correlation with GSH and catalase (r=-.909** and r=-.777** respectively, P<0.01). **Conclusion:** All parameters in combinations may be useful indicator or may be good and reliable biochemical markers for identification and determination of severity of alcoholic liver diseases (ALD). The damaging of hepatocytes due to the consumption of alcohol disturbs almost all types of biochemical coordination in the biological system.

Key words: Alcoholic Liver Disease (ALD), Total Bilirubin, lipid peroxidation, MDA, ALT, AST, ALP

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INTRODUCTION

The liver is one of the largest organs in the body, weighting 3.3 pounds. It has a distinctive property that liver cells can be regenerated themselves. Because of this, damage to the liver cells cannot appear until or unless the damage caused is considerable and extensive.[1] It's a very important organ in the body as its functions are very essential to life. Liver primarily filters circulating blood and thus removing and destroying toxic substances. The major function of liver is to secrete bile in small intestine which helps in digestion and absorption of fats. The protein products manufactured as a result of metabolism are also converted by liver into urea so that they can be excreted via kidney. Liver also regulates blood clotting mechanisms. Because of the regenerative ability of liver this important organ can survive harsh condition during life time.[2]

Hepatitis C is much considered as main cause for liver cirrhosis. Fat deposition in liver can be occurred in almost all heavy drinkers. This fatty liver can also be seen in nonalcoholic that drinks casually. Fatty liver is seemed to be reversible and this cannot leads towards serious disease.[1] Liver transplantation is one of the effective therapies available to some patient.[3] Liver cirrhosis is indolent disease, most of the patient show asymptomatic disease till the onset of last stage.[4] Liver cirrhosis is pathological disease that is characterized by irreversible chronic injury of hepatic cells in association of vast fibrosis.[5]

Objective

The aim of the present study was to investigate the changes in biochemical parameters in patients with alcoholic liver disease (ALD).

MATERIALS AND METHODS

	Groups	(n)
A	Non-Alcoholics (Served as control)	20
B	Alcoholics (01-05 Yrs Exposure time)	4
C	Alcoholics (06-10 Yrs Exposure time)	41
D	Alcoholics (11 Yrs and above Exposure time)	5

Table-I. Study Design

SOURCE OF DATA

Fifty chronic alcoholics admitted for treatment to the in-patient wards at Jinnah Hospital, Lahore-Pakistan. Detailed history of alcohol intake was collected including clinical complications. Apparently twenty healthy individuals served as control. Chronic alcoholics with alcohol abuse for more than four to five years, and with or without clinical complications, were included. Alcoholics with smoking, occasional drinkers and with any systemic illness were excluded from the control group.

BIOCHEMICAL ASSAYS

The estimation of AST, ALT and ALP were estimated by following principle by using commercially available Bio Meraux and Randox kits. Hemoglobin concentration was determined using cyanmeth reagent.[6] Urea in serum was estimated by the kinetic method.[7] Creatinine level was estimated by rate of change in absorbance using alkaline picrate.[8] Total Bilirubin levels of serum were measured by the method of Jendrassik and Groff.[9]

Liver GSH was estimated according to the method of Ellman.[10] Catalase was assayed according to the method of Aebi.[11] Lipid peroxidation in liver tissues was estimated calorimetrically by measuring thiobarbituric acid reactive substances (TBARS) by the method of Ohkawa.[12] Superoxide dismutase (SOD) activity was determined by the method of Kakkar.[13]

STATISTICAL ANALYSIS

ANOVA was applied to check statistically significant ($P<0.05$) difference among the groups. Data was represented by mean±SD.

RESULTS

Hematological Profile of Alcoholics vs. Non Alcoholics

The results regarding hemoglobin and RBC profile in Table-II demonstrating statistically highly significant differences and consistent decreasing (hemoglobin) and increasing (RBC) pattern between and within the alcoholics and non-alcoholics subjects (P=.05, .01and .001 respectively). The lowest value (10.30%) of hemoglobin was recorded in alcoholics in group D (11 Yrs and above Exposure time) and highest (14.90%) in group A (served as control). A slightly decreasing trend was observed in other groups B-C (11.30%, 10.78%) respectively. The highest value (5.97) of RBC count (x10) was found in non-alcoholics. Increasing trend of RBC count (x10) was observed from groups B-D (3.75, 4.02 and 4.26) respectively but this increasing trend presenting lower values as compared to group A (served as control).

PARA-METERS	GROUPS	MEAN±SD	(n)	P-VALUE
Hb	A	14.90±0.99	20	.000*
	B	11.30±1.39	4	
	C	10.78±1.20	41	
	D	10.30±1.69	5	
RBC	A	5.97±0.49	20	.000*
	B	3.75±0.57	4	
	C	4.02±0.43	41	
	D	4.26±0.04	5	

Table-II. Hematological Profiles of Alcoholics vs. Non Alcoholics

Blood Urea and Creatinine Profile of Alcoholics vs. Non Alcoholics

The data regarding blood urea and creatinine profile in Table-III reflecting highly significant differences but inconsistent increasing and decreasing pattern between and within the alcoholics and non-alcoholics subjects (P=.05, .01and .001 respectively). The lowest value (0.93 mg/dl) of

blood creatinine (Normal range, 0.8-1.7 in Males) was recorded in non-alcoholics and highest (1.96 mg/dl) in group B (01-04 Yrs Exposure time). A slightly decreasing trend was observed in other groups (C and D). Increasing trend of blood urea was noted from groups A-C (20.73, 22.49 and 24.37 mg/dl) respectively with a slightly decreasing value (23.94 mg/dl) respectively.

PARA-METERS	GROUPS	MEAN±SD	(n)	P-VALUE
Urea	A	20.73±1.48	20	.000*
	B	22.49±2.20	4	
	C	24.37±2.12	41	
	D	23.94±2.57	5	
Creatinine	A	0.96±0.19	20	.000*
	B	1.96±0.06	4	
	C	1.91±0.25	41	
	D	1.94±0.45	5	

Table-III. Renal Profiles of Alcoholics Vs Non Alcoholics

Hepatic Profile of Alcoholics vs. Non Alcoholics

PARA-METERS	GROUPS	MEAN±SD	(n)	P VALUE
ALT	A	24.00±5.69	20	.000*
	B	36.75±19.73	4	
	C	52.04±21.16	41	
	D	62.60±33.90	5	
AST	A	20.25±5.21	20	.000*
	B	48.50±9.32	4	
	C	79.17±31.09	41	
	D	138.40±25.89	5	
ALP	A	207.83±6.28	20	.000*
	B	369.16±13.53	4	
	C	389.25±23.20	41	
	D	393.07±36.83	5	
TB	A	0.59±0.07	20	.000*
	B	2.00±0.02	4	
	C	2.05±0.20	41	
	D	2.09±0.15	5	

Table-IV. Hepatic Profiles of Alcoholics vs. Non Alcoholics

ALT, AST, ALP and total bilirubin exhibited highly significant (P=.05, .01and .001 respectively) differences among the groups (Table-IV). The higher values of AST as compared to ALT were also recorded in alcoholics. Likewise, the ratio of AST/ALT in the studied groups was reflecting the progression of cirrhosis in preponderance alcoholics. The exposure duration/time in alcoholics also shed light in the development of alcoholic liver disease. The highest values of AST and ALT (138.40 IU/L and 62.60 IU/L) were observed in group D (11 Yrs and above Exposure time) followed by groups C (05-10 Yrs Exposure time) and B (01-04 Yrs Exposure time) as in comparison with that of non-alcoholics (Group A served as control). The same patterns were also noted in case of alkaline phosphatase (ALP) and total bilirubin levels between and within the studied groups (Table-IV).

Circulating Stress Biochemical Markers Profile of Alcoholics Vs Non Alcoholics

PARA-METERS	GROUPS	MEAN±SD	(n)	P VALUE
MDA	A	1.36±0.03	20	.000*
	B	7.78±1.70	4	
	C	8.28±1.68	41	
	D	8.45±1.63	5	
SOD	A	0.73±0.25	20	.016*
	B	0.06±0.05	4	
	C	0.12±0.10	41	
	D	0.30±0.22	5	
GSH	A	9.77±1.17	20	.000*
	B	2.24±0.94	4	
	C	2.04±0.85	41	
	D	2.16±0.97	5	
Catalase	A	4.27±0.73	20	.000*
	B	0.77±0.83	4	
	C	0.74±1.36	41	
	D	0.47±0.25	5	

Table-V. Stress Biomarkers Profiles of Alcoholics vs. Non Alcoholics

The consistent increasing trend in MDA levels (1.36, 7.78, 8.28 and 8.45 nmol/ml) were recorded in different groups (A-D) (Table-V). The consisting decreasing trends in case of Glutathione from groups A-C were recorded (9.77, 2.24 and 2.04 μg/dl) with a slight increase in group D (2.16 μg/dl). Catalase levels were also shows the consisting decreasing trend (4.27, 0.77, 0.74 and 0.47 μmol/mol of protein) in different studied groups (A-D) by the passage of exposure time on alcohol (Table-V). The lowest value (0.06 ng/ml) of SOD was recorded in group B (01-04 Yrs Exposure

time) and highest in case of non-alcoholics (0.73 ng/ml). An increasing trend from 0.13-0.30 ng/ml of SOD was recorded in groups C (05-10 Yrs Exposure time) and D (11 Yrs and above Exposure time) respectively demonstrating and stabilizing trend with the passage of exposure time on alcohol.

Lipid Profile of Alcoholics Vs Non Alcoholics

The results depicted in Table-VI reflecting that the lipid profile (TCh, Tg, LDL and HDL) of alcoholic versus non-alcoholics differed significantly (P=.05, .01and .001 respectively). In case of blood TCh and HDL levels a decreasing trend was recorded as compared to control between and within the studied groups. But reverse is true for Tg and LDL blood levels (Table 06). The consisting pattern of decreasing and increasing trends of blood TCh and Tg levels were recorded within the studied groups (A-D). The highest value (4.44 mmol/L) of TCh was recorded in group A (Non-alcoholics) followed by groups B (3.60 mmol/L), C (3.18 mmol/L) and D (2.72 mmol/L) respectively (Table-VI). But in case of Tg the highest value was recorded in group D (1.94 mmol/L) followed by groups C (1.85 mmol/L), B (1.81 mmol/L) and A (1.24 mmol/L) respectively. Inconsistent pattern of increasing and decreasing trends of blood LDL and HDL levels were recorded within the studied groups (A-D).

PARA-METERS	GROUPS	MEAN±SD	(n)	P VALUE
TCh	A	4.44±0.37	20	.000*
	B	3.60±0.41	4	
	C	3.18±0.49	41	
	D	2.72±0.56	5	
TG	A	1.24±0.15	20	.000*
	B	1.81±0.11	4	
	C	1.85±0.29	41	
	D	1.94±0.24	5	
LDL	A	2.31±0.15	20	.000*
	B	3.18±0.52	4	
	C	2.88±0.44	41	
	D	2.81±0.66	5	
HDL	A	1.73±0.17	20	.000*
	B	1.18±0.04	4	
	C	1.22±0.19	41	
	D	1.48±0.49	5	

Table-VI. Lipid Profiles of Alcoholics vs. Non Alcoholics

Significant levels < 0.05

DISCUSSION

Toxicity may be enhanced by malnutrition and malabsorption of nutrients and vitamins. In the development of alcohol-related pathology, certain bodily systems and circulating biochemical markers are markedly more vulnerable than others.

Hemoglobin (Hb) was observed to has direct and strong correlation (Table-VII) with RBC and GSH (r=.716** and r=.832** respectively) and was statistically significant (P<0.01). There is an increase of total bilirubin (TB) level of serum of moderate and heavy alcoholic patients, respectively (Table-IV). Creatinine was found to be as indirectly correlated (Table-VII) with Hb and RBC (r=-.769** and r=-.778** respectively; P<0.01), on the other hand, as directly strongly correlated with ALP and TB (r=.831** and r=.847** respectively; P<0.01). The bilirubin level in association with urea and creatinine may be used as markers in combination for ALD.

Hepatic enzymes leak into the circulation when hepatocytes or their cell membranes are damaged. Although these aminotransferases are sensitive indicators of liver cell damage, neither alone is an ideal marker. A very strong direct and indirect correlation (Table-VII) of ALP was found with TB, MDA and GSH (r=.950**, r=.929** and r=-.967**) and were statistically significant (P<0.01).

Alcohol abuse is one of the most causes of acute and chronic liver disease in worldwide. In western countries 50 percent of end stage liver diseases have alcohol as a major causative factor. The prognosis of liver cirrhosis is more than other common types of cancer for example breast, prostate and colon cancer. Unfortunately food and drug administration (FDA) has not yet designed any widely applicable drug therapy for alcoholic liver cirrhosis.[14] Most of the liver cancers are of epithelial cell origin but very rare can be of nonepithelial origin. Hepatocellular carcinoma is most common type occurring overall the world.

Long term intake of alcohol recognized as a major

cause of liver diseases specially hepatocellular carcinoma.[15] There are two mechanisms, direct (Genotoxic) and indirect mechanisms by which alcohol is involved in the development of hepatocellular carcinoma (HCC). Indirect mechanism is the most common pathway in the development of liver carcinogenesis in developed countries.[16] Alcoholic liver cirrhosis is a main globular health problem which results in fatty liver and inflammation and also leads to cirrhosis and hepatocellular carcinoma. It increases gut permeability which is associated with translocation of bacteria and their products. Lipopolysaccharide (LPS) is a major component of gram negative bacterial cell wall and by the action of parenchymal and nonparenchymal cells it is detoxified in the liver.[17] Some aspects of the relation between HCC and heavy alcohol drinking are still unsettled that is, the effect of consumption of alcoholic beverages, the age at which alcohol drinking start and the time while quitting. Hepatitis B and hepatitis C infection along with alcohol increase the risk of HCC and this is suggested by epidemiologic and pathological studies.[18,19]

Alcoholic liver diseases (ALD) are developing seriously in a large population of heavy drinkers. Alcoholic cirrhosis and alcoholic hepatitis appear to be predisposed by gender, diet, hereditary and occurrence of liver illness. Most of the liver damage is attributed to alcohol metabolism. Chronic alcohol consumption is a main factor and has a potent effect of liver disease on human health. ALD is a major and leading cause of liver cirrhosis throughout the world. Alcohol induced liver injury may also has some spectrums

	Hb	RBC	ALT	AST	ALP	TB	TCH	Tg	LDL	HDL	Urea	Creatinine	MDA	SOD	GSH	CAT
Hb	1	.716**	-.530**	-.665**	-.854**	-.847**	.676**	-.628**	-.484**	.524**	-.557**	-.769**	-.821**	.290*	.832**	.694**
		.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.015	.000	.000
RBC		1	-.452**	-.598**	-.841**	-.851**	.711**	-.638**	-.522**	.695**	-.470**	-.778**	-.756**	.282*	.858**	.754**
			.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.018	.000	.000
ALT			1	.727**	.585**	.559**	-.379**	.586**	.570**	-.256*	.406**	.457**	.545**	-.200	-.525**	-.378**
				.000	.000	.000	.001	.000	.000	.032	.000	.000	.000	.097	.000	.001
AST				1	.695**	.671**	-.643**	.543**	.412**	-.434**	.444**	.578**	.666**	-.209	-.665**	-.495**
					.000	.000	.000	.000	.000	.000	.000	.000	.000	.082	.000	.000
ALP					1	.950**	-.746**	.788**	.545**	-.630**	.667**	.831**	.929**	-.366**	-.967**	-.806**
						.000	.000	.000	.000	.000	.000	.000	.000	.002	.000	.000
TB						1	-.750**	.744**	.567**	-.654**	.616**	.847**	.899**	-.385**	-.940**	-.773**
							.000	.000	.000	.000	.000	.000	.000	.001	.000	.000
TCH							1	-.475**	-.262*	.564**	-.398**	-.630**	-.676**	.241*	.745**	.638**
								.000	.028	.000	.001	.000	.000	.045	.000	.000
Tg								1	.561**	-.454**	.505**	.591**	.744**	-.276*	-.760**	-.607**
									.000	.000	.000	.000	.000	.021	.000	.000
LDL									1	-.130	.363**	.552**	.532**	-.254*	-.544**	-.422**
										.283	.002	.000	.000	.034	.000	.000
HDL										1	-.323**	-.571**	-.530**	.212	.661**	.530**
											.006	.000	.000	.078	.000	.000
Urea											1	.516**	.658**	-.135	-.669**	-.529**
												.000	.000	.264	.000	.000
Creatinine												1	.800**	-.434**	-.835**	-.754**
													.000	.000	.000	.000
MDA													1	-.312**	-.909**	-.777**
														.008	.000	.000
SOD														1	.276*	.295*
															.021	.013
GSH															1	.793**
																.000
CAT																1

Table-VII. Pearson's Correlation Matrix of Circulating Biomarkers between Alcoholics and Non Alcoholics

*** Significant level (0.01)* *** Significant level (0.05)*

like steatosis, steatotohepatitis, cirrhosis and hepatocellular carcinoma. A mechanism that has a significant role in the development and progression of disease is epigenetic mechanism. This mechanism is involved in the parenchymal and nonparenchymal cells of liver. Its function is the initiation of inflammation and fatty liver. It also contribute towards hepatocytes necrosis and apoptosis.[20, 21]

In case of chronic and/or acute condition of autoimmune hepatitis, viral or drug-induced hepatitis ALD, the concentrations of aminotransferases in serum is moderately raised.[22] The diagnosis of some liver diseases is achieved by using the ratio of AST/ALT. The group of ALD with heavy intake of alcohol represented significant increase in AST/ALT ratio as compared to the group of ALD with lesser intake of alcohol. A necessary coenzyme referred as pyridoxal-5'-phosphate for both AST and ALT. The deficiency of this coenzyme is observed in ALD. Such deficiency leads to the decrease in hepatic ALT to a greater extent as compared to AST.[23,24] In the present study, the serum ALP level was significantly higher in group B (170%) group C (187%) and group D (189%) in comparison to non-alcoholics.

Plasma levels of MDA, SOD, catalase and GSH were expressed in Table-V. Statistically significant elevation was observed in MDA levels among the alcoholics as compared to the non-alcoholics. In comparison with group C and D, the decreased levels of MDA were observed in B group because chronic consumption of alcohol is associated with elevation in lipid peroxidation (LPO).[25] The pathogenesis of ALD has been associated with the peroxidation of polyunsaturated fatty acids (PUFA). GSH is responsible for the protection of cellular components from the damaging free radicals and also play an important role in redox balance. In comparison to group C and D the high alcohol intake leads to alcoholic liver disease (ALD). MDA was observed having very strong indirect correlation (Table VII) with GSH and catalase ($r=-.909^{**}$ and $r=-.777^{**}$ respectively, $P<0.01$). Decrease in hepatic GSH levels by the chronic consumption of alcohol induces oxidative stress.[22,25] Moreover, hepatic GSH has an important association with LPO due to its ability to bind with free radicals that may responsible for peroxidation.[26] Patients with alcoholic liver disease have lower hepatic GSH levels, which appear to be independent of nutritional status and probably reflect increased oxidative stress.[27] Several factors contribute to the fall in hepatic GSH level in alcoholic liver disease. A direct strong association (Table VII) between GSH and catalase was found ($r=.793^{**}$, $P<0.01$).

CONCLUSION:

All parameters in combinations may be useful indicator or may be good and reliable biochemical markers for identification and determination of severity of alcoholic liver diseases (ALD). The damaging of hepatocytes due to the consumption of alcohol disturbs almost all types of biochemical coordination in the biological system.

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"Contentment is the greatest wealth."

Shuja Tahir

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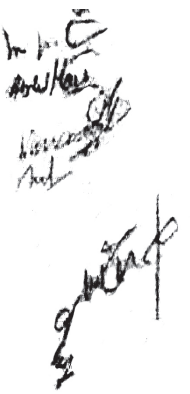
1	Dr. Muhammad Saeed Qureshi		
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Chemopreventive activity of glycyrrhizin on lead acetate mediated hepatic oxidative stress and its hyperproliferative activity in Wistar rats

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Abstract

Lead is a pervasive environmental pollutant with no beneficial biological role and its toxicity continues to be a major health problem due to its interference with natural environment. In the present study we have evaluated the chemopreventive effect of glycyrrhizin on lead acetate mediated hepatic oxidative stress, toxicity and tumor promotion related alterations in rats. Lead acetate (100 mg/kg bwt., i.p.) enhanced lipid peroxidation with concomitant reduction in glutathione, glutathione reductase, glutathione-*S*-transferase and glutathione peroxidase activities. There was an increase in the levels of transaminase enzymes and LDH. Lead acetate treatment also enhanced ornithine decarboxylase (ODC) activity and [^{3}H] thymidine incorporation into hepatic DNA. Pretreatment of rats orally with glycyrrhizin (150 and 300 mg/kg bwt., orally) resulted in a significant decrease in hepatic microsomal lipid peroxidation ($P < 0.001$) and increase in the level of GSH content ($P < 0.001$) and its dependent enzyme. There was significant reduction in the levels of SGPT, SGOT and LDH ($P < 0.001$). A significant inhibition in ODC activity and DNA synthesis ($P < 0.001$) was also observed. On the basis of the above results it can be hypothesized that glycyrrhizin is a potent chemopreventive compound against lead acetate mediated hepatic oxidative stress, toxicity and tumor promotion related responses in rats.

Keywords: Glycyrrhizin; Lead acetate; Oxidative stress; Tumor promotion

1. Introduction

Humans are exposed to various hazardous substances. Lead is one of the common environmental and industrial pollutants that has been found in the environment and the biological system. The persistence of lead in the animals and humans is now associated with health risk [1]. Lead has been found to produce wide range of biochemical and physiological dysfunctions in humans and laboratory animals [2]. The major source of lead is from industries where lead and lead based components are used, such as lead acid battery manufacturing, cable and wire products industries, rubber and plastic industries, soldering activities, in-foundry work such as casting, forging and grinding activities [3]. Pregnant ladies, infants and young children are mostly affected by lead exposure [4]. A pregnant lady can transfer her body burden of lead to the growing foetus as there is no placental barrier for a heavy metal like lead [5]. Most of the environmen-

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tal exposure occurs through inhaling air containing lead dust, drinking water supplied through leaded pipelines and consuming processed, preserved and stored food [6]. Studies suggest that one of the important mechanisms associated with toxic effects of lead is oxidative stress caused by disrupted prooxidant/antioxidant balance in animals including humans. Reduced glutathione (GSH) levels and glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities in tissues or in blood are most commonly used to evaluate lead induced oxidative damage. The oxidative stress has also been implicated to contribute to lead associated tissue injury in the liver, kidneys, brain and other organs [7,8]. Lead has been shown to increase hydrogen peroxide production in cells [9]. Another mechanism of free radical generation and adduct formation may involve aminolevulinic acid (ALA), the heme precursor whose levels are elevated by lead exposure through feedback inhibition of the enzyme ALA synthase [10]. ALA can generate free radicals and has been shown to cause oxidative damage to DNA in Chinese hamster ovary cells in vitro through the formation of 8-OHdG adducts [11]. The oxidative damage to DNA in human lead toxicity has been observed by a recent study of 7,8-dihydro-9-oxoguanine adducts in lymphocytes collected from persons exposed environmentally to metals, including lead, chromium, cadmium and nickel. Lead at high doses can induce proliferation, there is some evidence that oxygen radicals, produced by lead (especially in the presence of hydrogen peroxide) may induce DNA adducts [12]. Reactive oxygen species are important cytotoxic and signalling mediators in the pathophysiology of inflammatory liver diseases [13]. Recent studies indicate that compounds with antioxidant or anti-inflammatory properties can inhibit tumor initiation, promotion and progression in experimental models [14]. The antioxidant system may be composed of several endogenous and/or dietary antioxidant compounds and enzymes that interact with and inactivate ROS [15].

Natural components from numerous plants are used to halt or retard the carcinogenic process. Glycyrrhizin (Fig. 1) is the major biologically active component of liquorice. Glycyrrhizin is the calcium and potassium salt of glycyrrhizinic acid and upon hydrolysis, the glycoside loses its sweet taste and is converted to the aglycone glycyrrhetinic acid plus two molecules of glucuronic acid. Glycyrrhetinic acid has been shown to inhibit the activity of tumor promoter in mouse skin [16].

Glycyrrhizin exhibits a number of pharmacological effects, including anti-inflammation, anti-ulcer, anti-allergy, anti-carcinogenesis and immunomodulation [17–20]. It is also used as a potential therapeutic agent for several viral diseases, including chronic hepatitis, acquired immunodeficiency syndrome and herpes infection [21].

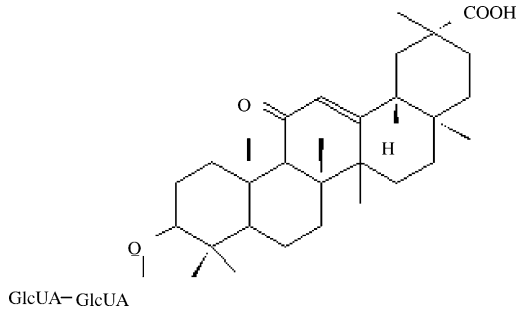

Fig. 1. Molecular structure of glycyrrhizin.

The present study is designed to evaluate the antioxidative and anti-proliferative activity of glycyrrhizin against lead acetate induced early biomarkers of hepatic tumor promotion in animal model.

2. Materials and methods

2.1. Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), nicotinamide adenine dinucleotide phosphate reduced (NADPH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), bovine serum albumin (BSA), 1,2,dithio-bis-nitrobenzoicacid (DTNB), 1-chloro,2,4-dinitrobenzene (CDNB), glutathione reductase, ethylenediamine tetra acetic acid (EDTA), pyridoxal phosphate, phenyl methylsulfonylfluoride (PMSF), 2-mercaptoethanol, dithiothreitol, Tween 80, Brij 35, ethanolamine, methoxyethanol, citric acid and glycyrrhizin were obtained from Sigma Chemicals Co. (St. Louis, MO). Ascorbic acid, hydrogen peroxide, ferric chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate and sodium hydroxide was purchased from E. Merck, India. DL-[^{14}C] ornithine and [^{3}H] thymidine was procured from Amersham Biosciences, Chennai, India. All other chemicals were of the highest purity commercially available.

2.2. Animals

Female albino Wistar rats weighing 150–200 g were procured from the Central Animal House Facility, Jamia Hamdard and used throughout the experiment. The animals were housed in an air-conditioned room (24 ± 2 °C) and had access to a basic diet and water.

2.3. Experimental protocol

To study the effect of pretreatment with glycyrrhizin on lead acetate mediated hepatic oxidative stress and serological studies, thirty female albino Wistar rats were randomly allocated to five groups of six rats each. Group I served as vehicle control and received only saline. Group III received pretreatment with glycyrrhizin by gavage (orally) once daily for 5 days at a dose of 150 mg/kg body weight and groups IV and V received pretreatment with glycyrrhizin by gavage once daily for 5 days at a dose of 300 mg/kg body weight. Half-an-hour after the last treatment with glycyrrhizin, the animals of the groups III and IV received only a single intraperitoneal injection of lead acetate at a dose of 100 mg/kg body weight. The animals of group II received single i.p. dose of lead acetate (dose of 100 mg/kg body weight) only on the fifth day. After 24 h of i.p. injection of lead acetate, the animals of all the groups were sacrificed by cervical dislocation. Just before sacrifice, blood for separation of serum was collected from these animals by retro-orbital sinus puncture and stored at 4 °C for estimation of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and lactate dehydrogenase (LDH).

For ODC activity study, the treatment regimen for the animals was similar as in case of oxidative stress. After 24 h of treatment with lead acetate (100 mg/kg body weight, i.p.), the animals were sacrificed by cervical dislocation and processed for subcellular fractionation.

For [^{3}H] thymidine incorporation study, similar experimental regimen was followed and the animals were administered [^{3}H] thymidine (25 μCi/0.2 ml saline/100 g of animal, i.p.) after 24 h of lead acetate treatment. Two hours after the injection of thymidine the animals were sacrificed. Liver sections were excised and thoroughly rinsed with ice-cold saline and then processed for the quantification of [^{3}H] thymidine incorporated into hepatic DNA.

2.4. Biochemical estimations

2.4.1. Estimation of lipid peroxidation

The assay of lipid peroxidation was done according to the method of Wright et al. [22]. The reaction mixture consisted of 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsome, 0.2 ml ascorbic acid (100 mM) and 0.02 ml ferric chloride (100 mM) in a total of 1 ml. This reaction mixture was then incubated at 37 °C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1 ml of TCA (10%). Following addition of 1.0 ml TBA (0.67%), all the tubes were placed in a boiling water bath for a period of 20 min. The tubes were shifted to ice bath and then centrifuged at 2500 × *g* for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm. The results were expressed as the nmol MDA formed/h/g tissue at 37 °C by using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

2.4.2. Estimation of glutathione reduced

The reduced glutathione (GSH) in liver was determined by the method of Jollow et al. [23]. A 1.0 ml postmitochondrial supernatant fraction (PMS) (10%) was mixed with 1.0 ml sulphosalicylic acid (4%). The samples were incubated at 4 °C for at least 1 h and then centrifuged at 1200 × *g* for 15 min at 4 °C. The reaction mixture contained 0.4 ml of the filtered sample, 2.2 ml phosphate buffer (0.1 M, pH 7.4) and 0.4 ml DTNB (4 mg/ml) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm on spectrophotometer (Milton Roy Model-21 D). The reduced glutathione concentration was calculated as nmol GSH/g tissue.

2.4.3. Assay for glutathione-S -transferase activity

Glutathione-*S*-transferase (GST) activity was assayed by the method of Habig et al. [24]. The reaction mixture consisted of 2.5 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml GSH (1 mM), 0.2 ml CDNB (1 mM) and 0.1 ml of the cytosolic fraction (10%) in a total volume of 3.0 ml. Changes in absorbance were recorded at 340 nm and enzymatic activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of $9.6 \times 10^3\ M^{-1}\ cm^{-1}$.

2.4.4. Assay for glutathione reductase activity

Glutathione reductase activity was assayed by the method of Carlberg and Mannervik [25]. The reaction mixture consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml PMS (10%) in a total volume of 2.0 ml. Enzyme activity was quantities at 25 °C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using a molar extinction coefficient of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$.

2.4.5. Assay for catalase activity

Catalase activity was assayed by the method of Claiborne [26]. Briefly, the reaction mixture consisted of 2.0 ml phosphate buffer (0.1 M, pH 7.4), 0.95 ml hydrogen peroxide (0.019 mM) and 0.05 ml

PMS (10%) in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated as nmol H_2O_2 consumed/min/mg protein.

2.4.6. Assay for glutathione peroxidase activity

Glutathione peroxidase activity was measured by the method of Mohandas et al. [27]. The reaction mixture consisted of 1.44 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 0.05 ml glutathione reductase (1 IU/ml), 0.05 ml reduced glutathione (1 mM), 0.1 ml NADPH (0.2 mM) and 0.01 ml H_2O_2 (0.25 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. The disappearance of NADPH at 340 nm was recorded at 25 °C. Enzyme activity was calculated as nmol NADPH oxidized/min/mg protein using a molar extinction coefficient of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$.

2.4.7. Serum oxaloacetate and pyruvate transaminase activity (SGOT and SGPT)

SGOT and SGPT activity were determined by the method of Reitman and Frankel [28]. Each substrate (0.5 ml) (2 mM α-ketoglutarate and either 200 mM α-L-alanine or L-aspartate) was incubated for 5 min at 37 °C in a water bath. Serum (0.1 ml) was then added and the volume was adjusted to 1.0 ml with 0.1 M, pH 7.4-phosphate buffer. The reaction mixture was incubated for exactly 30 and 60 min at GPT and GOT, respectively. Then to the reaction mixture, 0.5 ml of 1 mM DNPH was added and left for another 30 min at room temperature. Finally, the color was developed by addition of 5.0 ml of NaOH (0.4N) and product read at 505 nm.

2.4.8. Lactate dehydrogenase (LDH) activity

LDH activity was estimated in serum by the method of Kornberg et al. [29]. The assay mixture consisted of serum, 0.02 M NADH, 0.01 M sodium pyruvate, 0.1 M, pH 7.4-phosphate buffer and distilled water in a total volume of 3 ml. Enzyme activity was recorded at 340 nm and activity was calculated as nmol NADH oxidized/min/mg protein.

2.4.9. Assay for ornithine decarboxylase activity

ODC activity was determined using 0.4 ml hepatic 105,000 × *g* supernatant fraction per assay tube by measuring the release of CO_2 from DL-[^{14}C] ornithine by the method of O'Brien et al. [30]. The liver was homogenized in Tris–HCl buffer (pH 7.5, 50 mM) containing EDTA (0.4 mM), pyridoxal phosphate (0.32 mM), PMSF (0.1 mM), 2-mercaptoethanol (1.0 mM), dithiothreitol (4.0 mM) and Tween 80 (0.1%) at 4 °C using a polytron homogenizer (Kinematica AGPT 3000). In brief, the reaction mixture contained 400 μl enzymes and 0.095 ml co-factor mixture containing pyridoxal phosphate (0.32 mM), EDTA (0.4 mM), dithiothreitol (4.0 mM), ornithine (0.4 mM), Brij 35 (0.02%) and DL-[^{14}C] ornithine (0.05 μCi) in total volume of 0.495 ml. After adding buffer and co-factor mixture to blank and others tubes, the tubes were closed immediately with a rubber stopper containing 0.2 ml ethanolamine and methoxyethanol mixture (2:1) in the central well and kept in water bath at 37 °C. After 1 h of incubation, the enzyme activity was arrested by injecting 1.0 ml citric acid solution (2.0 M) along the sides of glass tubes and the solution was continued for 1 h to ensure complete absorption of CO_2. Finally, the central well was transferred to a vial containing 2 ml ethanol and 10 ml toluene based scintillation fluid. Radioactivity was counted in liquid scintillation counter (LKB-Wallace-1410). ODC activity was expressed as pmol CO_2 released/h/mg protein.

2.4.10. Quantitation of hepatic DNA synthesis

The isolation of hepatic DNA and incorporation of [^{3}H] thymidine in DNA was done by the method employed by Smart et al. [31]. The liver tissues were quickly excised from the animal, cleaned free of extraneous material and homogenate (20%) was prepared in ice cold water. The precipitate thus obtained was washed with cold trichloroacetic acid (5%) and incubated with cold perchloric acid (10%) at 4 °C for 24 h. After the incubation it was centrifuged and the precipitate was washed with cold perchloric acid (5%). Then the precipitate was dissolved in warm perchloric acid (5%) followed by incubation in boiling water bath for 30 min and filtered through a Whatman 50 paper. The filtrate was used for [^{3}H] thymidine counting in liquid scintillation counter (LKB-Wallace-1410) by adding the scintillation fluid. The amount of DNA in the filtrate was estimated by diphenylamine method of Giles and Mayers. The amount of [^{3}H] thymidine incorporated was expressed as dpm/μg DNA.

2.4.11. Protein estimation

The protein concentration in all samples was determined by the method of Lowry et al. [32].

2.4.12. Statistical analysis

The level of significance between different groups is based on analysis of variance test followed by Dunnett's *t*-test. The *P*-values of less than 0.05 have been considered significant.

Table 1
Effect of prophylactic treatment of animals with glycyrrhizin on lead acetate mediated hepatic glutathione and glutathione-*S*-transferase in Wistar rats

Treatment groups	Reduced glutathione (nmol GSH/g tissue)	Glutathione-*S*-transferase (nmol CDNB conjugate formed/min/mg protein)
Saline (control)	0.54 ± 0.012	135 ± 9.97
Lead acetate (100 mg/kg body weight)	0.31 ± 0.006$^{###}$	84 ± 4.12$^{###}$
Glycyrrhizin (150 mg/kg body weight) + lead acetate (100 mg/kg body weight)	0.34 ± 0.008**	88 ± 3.63*
Glycyrrhizin (300 mg/kg body weight) + lead acetate (100 mg/kg body weight)	0.42 ± 0.013***	109 ± 1.79*
Only glycyrrhizin (300 mg/kg body weight)	0.56 ± 0.009	128 ± 4.20

Each value represents mean ± S.E. of six animals. Saline treated group served as control.
* Differ significantly from the corresponding value for rats treated with lead acetate treated control ($P < 0.05$).
** Differ significantly from the corresponding value for rats treated with lead acetate treated control ($P < 0.01$).
*** Differ significantly from the corresponding value for rats treated with lead acetate treated control ($P < 0.001$).
$^{###}$ Differ significantly from the corresponding value for saline treated control ($P < 0.001$).

3. Results

Table 1 shows the effect of glycyrrhizin on levels on lead acetate mediated hepatic glutathione and glutathione-*S*-transferase in Wistar rats. Lead acetate caused 56% decrease in GSH and 62% decrease in GST when compared with control group. Treatment with glycyrrhizin (150 and 300 mg/kg bwt., orally) caused 7–21% and 3–18% elevation in levels of GSH and GST, respectively, when compared with lead acetate group.

Table 2 shows the effect of glycyrrhizin on levels on lead acetate mediated hepatic glutathione peroxidase and glutathione reductase in Wistar rats. Lead acetate caused 67% and 79% reduction in GPx and GR, respectively, when compared with saline treated group. Treatment with glycyrrhizin (150 and 300 mg/kg bwt., orally) caused 7–11% and 8–15% elevation in levels of GPx and GR, respectively, when compared with lead acetate group.

Table 3 shows the effect of glycyrrhizin on levels on lead acetate mediated hepatic catalase and lipid peroxidation in Wistar rats. Lead acetate caused 30% reduction in catalase and 295% elevation in LPO when compared with saline treated group. Treatment with glycyrrhizin (150 and 300 mg/kg bwt., orally) caused 13–39% elevation in levels of catalase and 92–116% decrease in LPO when compared with lead acetate group.

Table 4 shows the effect of pretreatment of animals with glycyrrhizin on lead acetate induced liver damage markers. Lead acetate caused 237%, 273% and 147% elevation in the levels of SGOT, SGPT and LDH, respectively, when compared saline group. Treatment with glycyrrhizin (150 and 300 mg/kg bwt., orally) caused 34–65%, 37–103% and 16–28% reduction in the lev-

Table 2
Effect of prophylactic treatment of animals with glycyrrhizin on lead acetate mediated hepatic glutathione peroxidase and glutathione reductase in Wistar rats

Treatment groups	Glutathione peroxidase (nmol NADPH oxidized/min/mg protein)	Glutathione reductase (nmol NADPH oxidized/min/mg protein)
Saline (control)	308 ± 9.87	228 ± 5.11
Lead acetate (100 mg/kg body weight)	207 ± 11.73$^{###}$	181 ± 2.64$^{###}$
Glycyrrhizin (150 mg/kg body weight) + lead acetate (100 mg/kg body weight)	229 ± 9.46*	199 ± 5.25*
Glycyrrhizin (300 mg/kg body weight) + lead acetate (100 mg/kg body weight)	243 ± 8.77**	214 ± 5.27***
Only glycyrrhizin (300 mg/kg body weight)	294 ± 8.42	240 ± 8.53

Each value represents mean ± S.E. of six animals. Saline treated group served as control.
* Differ significantly from the corresponding value for rats treated with lead acetate treated control ($P < 0.05$).
** Differ significantly from the corresponding value for rats treated with lead acetate treated control ($P < 0.01$).
*** Differ significantly from the corresponding value for rats treated with lead acetate treated control ($P < 0.001$).
$^{###}$ Differ significantly from the corresponding value for saline treated control ($P < 0.001$).

Table 3
Effect of prophylactic treatment of animals with glycyrrhizin on lead acetate mediated hepatic catalase and lipid peroxidation in Wistar rats

Treatment groups	Catalase (nmol H_2O_2 consumed/min/mg protein)	Lipid peroxidation (nmol malondialdehyde formed/h/g tissue)
Saline (control)	24 ± 1.78	10 ± 0.077
Lead acetate (100 mg/kg body weight)	7 ± 0.70###	31 ± 0.082###
Glycyrrhizin (150 mg/kg body weight) + lead acetate (100 mg/kg body weight)	10 ± 1.34*	21 ± 0.181***
Glycyrrhizin (300 mg/kg body weight) + lead acetate (100 mg/kg body weight)	17 ± 1.23***	19 ± 0.11***
Only glycyrrhizin (300 mg/kg body weight)	18 ± 0.98	11 ± 0.092

Each value represents mean ± S.E. of six animals. Saline treated group served as control.
* Differ significantly from the corresponding value for rats treated with lead acetate treated control ($P < 0.05$).
*** Differ significantly from the corresponding value for rats treated with lead acetate treated control ($P < 0.001$).
Differ significantly from the corresponding value for saline treated control ($P < 0.001$).

els of SGOT, SGPT and LDH on comparison with lead acetate treated group.

Fig. 2 shows the effect of pretreatment of rats with glycyrrhizin on lead acetate mediated induction of hepatic ODC activity and enhancement in the incorporation of [^{3}H] thymidine into hepatic DNA. Lead acetate treatment resulted in 588% induction in the ODC activity as compared with saline treated control. Treatment with glycyrrhizin (at 150 and 300 mg/kg bwt., orally) caused 172–335% reduction in the ODC activity when compared with lead acetate group.

Lead acetate treatment resulted in 175% increase in the incorporation of [^{3}H] thymidine into hepatic DNA as compared with saline treated controls. Treatment with glycyrrhizin (at 150 and 300 mg/kg bwt., orally) caused 22–59% reduction in the incorporation of [^{3}H] thymidine into hepatic DNA when compared with lead acetate group.

4. Discussion

Glycyrrhizin has been shown to possess anti-inflammatory [33], anti-viral [34] and anti-carcinogenic [35] activities. It has been reported that glycyrrhizin shows corticosteroid like action, anti-viral action, inhibition of prostaglandin E_2 and suppression of superoxide and hydroperoxides in macrophages [36]. Glycyrrhizin also exerts inhibitory effect on neutrophils' migration to the site of inflammation [37].

Lead is similar in chemical structure to calcium and competes with it for absorption in the gastrointestinal tract and deposition in bone. Previous studies have suggested that some of the toxic effects of lead may be due to its interference with calcium in activation of PKCs or through production of reactive oxygen species (ROS). PKCs play an important role in signal transduction, cell growth and differentiation [38,39].

Table 4
Effect of prophylactic treatment of animals with glycyrrhizin on lead acetate mediated liver damage markers in serum

Treatment groups	SGPT (IU/l)	SGOT (IU/l)	LDH (nmol NADH oxidized/min/mg protein)
Saline (control)	28 ± 3.37	34 ± 4.26	311 ± 4.25
Lead acetate (100 mg/kg body weight)	77 ± 3.31###	81 ± 2.29###	458 ± 3.77###
Glycyrrhizin (150 mg/kg body weight) + lead acetate (100 mg/kg body weight)	66 ± 4.25*	69 ± 4.16**	409 ± 3.31***
Glycyrrhizin (300 mg/kg body weight) + lead acetate (100 mg/kg body weight)	48 ± 4.26***	58 ± 3.38***	371 ± 4.24***
Only glycyrrhizin (300 mg/kg body weight)	42 ± 2.77	43 ± 2.39	323 ± 3.31

Each value represents mean ± S.E. of six animals. Saline treated group served as control.
* Differ significantly from the corresponding value for rats treated with lead acetate treated control ($P < 0.05$).
** Differ significantly from the corresponding value for rats treated with lead acetate treated control ($P < 0.01$).
*** Differ significantly from the corresponding value for rats treated with lead acetate treated control ($P < 0.001$).
Differ significantly from the corresponding value for saline treated control ($P < 0.001$).

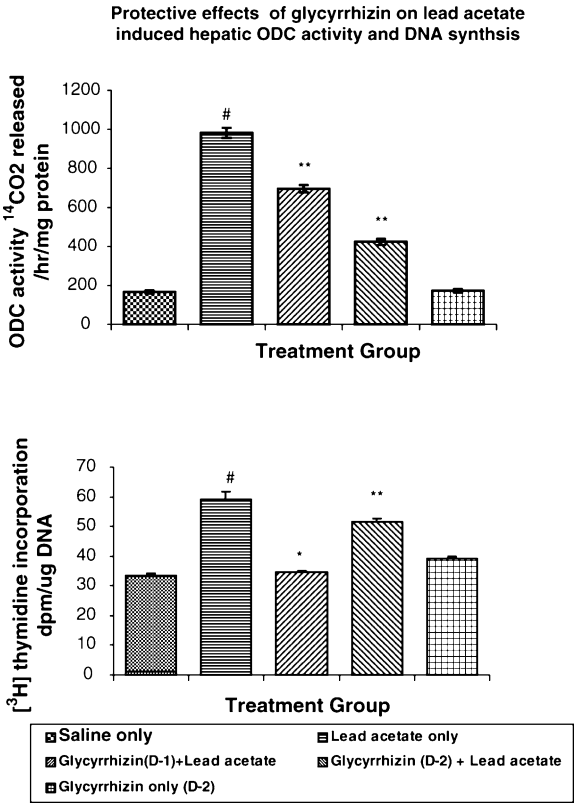

Fig. 2. Protective effects of glycyrrhizin on lead acetate induced hepatic ODC activity and DNA synthesis. Each value represents mean ± S.E. of six animals. Saline treated group served as control. Values marked with daggers differ significantly from the corresponding value for Saline treated control ($^{\#}P < 0.001$). Values marked with asterisks differ significantly from the corresponding value for rats treated with Lead acetate treated control ($^{*}P < 0.05$ and $^{**}P < 0.001$).

Induction of ODC and [^{3}H] thymidine incorporation into the DNA are integral events of tumor promotion and widely used as biochemical markers to evaluate tumor-promoting activity of an agent. In our study the elevation of hepatic ODC activity and ultimately the incorporation of [^{3}H] thymidine in DNA emphasize the proliferative and tumor-promoting activity of lead acetate. However, glycyrrhizin dose-dependently inhibited the induction of ODC activity and [^{3}H] thymidine incorporation. Several inhibitors of ODC and DNA synthesis have been reported to inhibit tumor promotion in various organs [40].

Upon oral administration, glycyrrhizin is partly hydrolysed by glucuronidase originating from intestinal microbes to give glycyrrhetinic acid that is absorbed easily [36]. In one of the studies glycyrrhetinic acid has been shown to compete with 12-O-tetradecanoyl phorbol-13-acetate for its membrane receptor resulting in anti-tumor-promoting activity in vivo [41].

Several in vitro studies have shown lead to be a co-carcinogen, which interferes with DNA repair process and takes part in the Fenton reaction to generate reactive oxygen species, which induce oxidative stress [42]. The involvement of oxidative stress has been associated invariably with all the stages of cancer formation including tumor promotion.

The pretreatment of animals with glycyrrhizin significantly ameliorated GSH content with concomitant decrease in MDA. It can be suggested that glycyrrhizin has established antioxidant properties that might have counteracted the toxic effects of lead acetate by effective scavenging and blocking the conjugation of reactive intermediates to GSH as evident from ameliorated GSH content and decreased MDA formation in a dose dependent manner. Lead acetate administration resulted in concomitant decrease in GPx, GR and GST activities. Pretreatment with glycyrrhizin and subsequent exposure of rats with lead acetate led to significant increase in above mentioned enzymes. It is evident from the results that glycyrrhizin induces variety of enzymes, viz., phase II enzyme, involved in the detoxification and excretion of carcinogenic or toxic substances and other antioxidant enzymes responsible for maintaining a balanced state between free radicals/oxidants and the antioxidants within the cellular environment. Studies conducted earlier with glycyrrhizin have shown to counteract CCl_4-induced hepatotoxicity with their anti-oxidative potential [36].

We suggest the possible mechanism of action of glycyrrhizin could be that it binds lead thus producing these protective effects. There is another possibility that glycyrrhizin might have converted into glycyrrhetinic acid and this glycyrrhetinic acid might also have a role in binding with lead. Besides, certain other study points out that glycyrrhetinic acid provides maximum conjugation with free radicals [43] and that might be responsible for the protective action of glycyrrhizin.

In our study though the only glycyrrhizin treated group showed an elevation in the levels of the markers of hepatotoxicity in the higher dose, nonetheless surprisingly the values with the group pretreated with glycyrrhizin (high dose) followed by lead acetate were significantly lower as compared to the only lead acetate treated group. Contradictory to the fact that the compound exhibited some toxicity with the markers, it showed fairly protective properties with the parameters of proliferation and oxidative stress.

Based on the present data we suggest that glycyrrhizin can be an effective chemopreventive agent against lead acetate mediated oxidative stress and hyperproliferative activity in rats.

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Reduction Therapy of Alanine Aminotransferase Levels Prevent HCC Development in Patients with HCV-associated Cirrhosis

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Abstract. *Background: To find a way to prevent the development of hepatocellular carcinoma (HCC) from hepatitis C virus-associated liver cirrhosis (HCV-LC), an analysis of the HCV-LC patients who had received reduction therapy of the alanine aminotransferase (ALT) levels was performed. Patients and Methods: Seventy-four consecutive HCV-LC patients of Child Stage A were followed for >10 years for the development of HCC. They were divided into two groups: in group A, the reduction therapy for the ALT levels was aggressively performed, while in group B, the reduction therapy was not performed aggressively. The patients were subdivided into three sub-groups according to their serum ALT levels. In groups A and B, the high ALT group was comprised, respectively, of nine and five patients whose annual average serum ALT levels were persistently high (≥80 IU), while the low ALT group was comprised of 19 and 20 patients whose annual average serum ALT levels were persistently low (<80 IU). The remaining eleven and ten patients had annual average serum ALT levels which fluctuated and were unclassified (unclassified group). Results: In group B, 65.7% of the patients had developed HCC in 13 years, in contrast to only 41.0% of group A (p=0.039). In group A, the median HCC development time was 12.8 years, in contrast to only 3.8 years in group B (p=0.0013). Multivariate analysis demonstrated that the mode of reduction therapy and ALT levels were the significant factors affecting HCC development. Conclusion: The chances of surviving for more than ten years without developing HCC for HCV-LC patients of Child Stage A were far more favorable in group A than group B. These results suggest that aggressive reduction therapy for ALT levels in HCV-LC patients could significantly prevent HCC development.*

Approximately 30 million people worldwide are estimated to have liver cirrhosis (LC) associated with the hepatitis C virus (HCV) (1). In addition, patients with HCV-associated liver cirrhosis (HCV-LC) have a high risk of developing hepatocellular carcinoma (HCC), with HCC actually developing in 6-8% of all patients annually with HCV-LC in Japan (2-4). Thus, 60-80% of the patients may develop HCC in 10 years. So, prevention of HCC development in such patients represents an urgent problem.

In a series of studies on patients with HCV-LC, we reported that the incidence of HCC in the high alanine aminotransferase (ALT) group was greater than in the low ALT group, that the incidence of multiple nodule development in the high ALT group was greater than in the low ALT group, and that the survival period, the interval free from recurrence and the number of recurrences after hepatectomy were better in the low ALT group (5-9). Aggressive reduction therapy has been conducted for patients with HCV-LC since 1989.

In view of our reports (5-9), aggressive reduction therapy is presumed to suppress HCC development in patients with HCV-LC. Our reduction therapy to suppress the incidence of development into HCC was investigated retrospectively.

Patients and Methods

Seventy-four consecutive post-hepatitic cirrhosis patients [Child Stage A (10)] were either HCV antibody-positive (C-100 antibody or the second generation tests) or HCV-RNA-positive (polymerase chain reaction). The patients were confined to Child Stage A patients because long-term follow-up was required to observe HCC occurrence. Habitual alcohol drinkers were omitted from the study; patients who took more than 40 g of ethanol daily and more than

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three days a week were also excluded. Patients who had been previously exposed to the hepatitis B virus, as well as HBs antigen-positive, HBc antibody-positive and HBs antibody-positive patients were also excluded. Further, patients who had undergone prior hepatic resection for HCC were excluded. All the patients had been hospitalized at the Kanagawa Cancer Center Hospital, Japan, between June 1, 1985 and May 31, 2000. The diagnosis of cirrhosis was made by liver biopsy in all cases. This study was performed according to the Declaration of Helsinki; all the subjects were informed and consented to participate in the study and the study protocol was approved by the Human Research Review Committee of Kanagawa Cancer Center Hospital.

Therapeutic procedures. Sho-saiko-to (11), juzen-taiho-to and stronger-neo minophagen C [SNMC; glycyrrhizin (12)] are herbal medicines used in Japan to treat chronic viral liver diseases, working by reducing inflammatory processes and controlling ALT levels. Ursodeoxycholic acid (UDCA) and protoporphyrine are also used to suppress elevated ALT levels in some cases. Suzuki *et al.* (12) demonstrated a significant decrease in serum ALT levels obtained by intravenous injection of SNMC for patients with chronic hepatitis and Hirayama *et al.* (13) showed a significant decrease in serum ALT levels obtained by oral administration of Sho-saiko-to to patients with chronic active hepatitis. Bellentani *et al.* (14) demonstrated a significant decrease in serum ALT levels obtained by the oral administration of UDCA in the long-term treatment of patients with chronic hepatitis. Recently, a significant decrease in serum ALT levels after the use of combination therapy with these drugs was demonstrated in patients with HCV-LC (15).

In group A, the patients were initially administered one of the following agents: UCDA (300-600 mg/day), Sho-saiko-to (7.5 g/day) or SNMC glycyrrhizin (≈60-100 mL, 2-3 times/week) (11, 12). When the average ALT level was successfully maintained at <80 IU, the single drug treatment was continued. Each drug was given for 2 months. If the average ALT level did not remain <80 IU, then the following combinations of two agents were administered: a) UDCA and SNMC (≈60-100 mL, 2-3 times/week), b) Sho-saiko-to and UCDA, c) Sho-saiko-to and SNMC (≈60-100 mL, 2-3 times/week), or d) protoporphyrine (PPP) (≈60-120 mg/day) and SNMC (≈60-100 mL, 2-3 times/week). The treatment was maintained when the ALT level dropped to <80 IU. If the average ALT level could not be maintained at <80 IU with a combination of two agents, then a combination of three agents was administered: Sho-saiko-to, UDCA and SNMC (≈60-100 mL, 2-3 times/week), or Juzen-taiho-to, UDCA and SNMC. Attempts were made to find the best therapeutic regimen for each patient. When the ALT levels could not be maintained at <80 IU by any means, the regimen that lowered the ALT level the most was adopted. This regimen was adopted since the ALT levels in many patients could not be maintained at <80 IU using only a single drug.

The group B patients either received no drug or only a single drug, such as UDCA, Sho-saiko-to, juzen-taiho-to or SNMC. When the average ALT level became maintained at <80 IU, no drug treatment was given. If the average ALT level could not be maintained at <80 IU, then only a single drug was administered. Each drug was given for 3 months. The treatment was maintained when the ALT level dropped to <80 IU. If the average ALT level could not be maintained at <80 IU with a single agent, then another type of drug was given.

Table I. *Subjects.*

	group A (n=39)	group B (n=35)	*p*-value
Males/Females	20/19	17/18	$p=1.000$
Age (y/o)	61.2±6.7	60.5±7.4	$p=0.67$
Plt ($x10^4/mm^3$)	10.9±3.0	9.6±3.3	$p=0.93$
Alb (g/dl)	4.0±0.3	4.1±0.4	$p=0.131$
AST (IU/l)	99.4±51.2	78.3±39.3	$p=0.058$
ALT (IU/l)	110.3±61.7	88.1±62.5	$p=0.130$

group A: received aggressive reduction therapy of ALT.
group B: received non-aggressive reduction therapy of ALT.
Plt: platelets, Alb: albumin, AST: asparate aminotransferase, ALT: alanine aminotransferase.

The period (years) from the time of histological diagnosis of cirrhosis to the time of detection of HCC by imaging modalities was calculated in for the patients who developed HCC.

The ALT and aspirate aminotransferase (AST) levels in group A were higher than those in group B. Between groups A and B, there were no significant differences in patient gender, age or ALT, AST, platelet (Plt), or albumin (Alb) levels (Table I).

Classification of ALT groups. It is possible that if the serum ALT levels remained high for many years, then the necrosis of hepatocytes might be severe and, conversely, if the ALT levels were very low for many years, then the necrosis of hepatocytes would subside. Actually, Zelman and Wang (16) demonstrated that elevations in serum aminotransferase levels are a marker of liver cell necrosis. Moreover, Schmidt *et al.* (17) demonstrated the close correlation between elevated transaminases and histological necroinflammation in biopsied specimens of chronic liver disease, including liver cirrhosis. Subsequently, Baier *et al.* (18) studied the relationship between the histological necroinflammation in the biopsied specimens and the elevation of transaminase levels in chronic liver diseases and found that, in liver cirrhosis, the histological findings of necroinflammation coincided with a significant elevation of transaminases in 53 cases out of 64 (83%), although the tendency was not so prominent in chronic hepatitis. This group also reported that, in chronic liver disease cases with subsided inflammation in liver biopsy specimens, almost normal transaminase levels were recognized in the majority of cases.

In place of repeated liver biopsy, the time-course of serum ALT levels was monitored and it was assumed that, if the annual average serum ALT level was persistently high (≥80 IU), then the level of liver cell necrosis would be severe and, conversely, if it was persistently low (<80 IU), then the level of liver cell necrosis would be slight. Eighty IU were adopted as a cut-off level because the annual average serum ALT level of HCV-LC patients with high DNA synthesis activity of hepatocytes, estimated by BrdU uptake (19) *in vitro* (BrdU labeling index ≥1.5%) and shown in our previous study (20) to have a high risk of developing HCC, was >80 IU in all the patients (5, 7, 21).

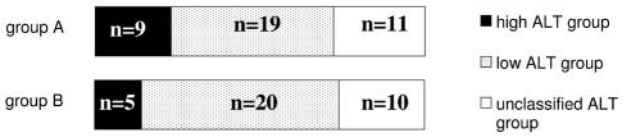

Figure 1. *High, low and unclassified ALT groups in groups A and B. Groups A and B were subdivided into three groups (high ALT group, low ALT group and unclassified ALT group). There was no significant difference in the proportion of patients in the three groups between groups A and B (p=0.606). ALT: alanine aminotransferase.*

The annual average serum ALT levels, starting from the time of liver biopsy, were calculated each year for all the patients. In calculating the annual average of these levels, twelve measurements were usually included. The details are reported in a previous study (5). Groups A and B were subdivided into three groups, respectively: those in whom the high ALT levels were highly predominant (high ALT group), those in whom the low ALT levels were very predominant (low ALT group), and those in whom the high and low ALT levels were mixed (unclassified ALT group). There was no significant difference in the proportion of patients in the three groups between groups A and B (Figure 1). The initial ALT level was defined as the peak value at 3 months before biopsy. The effect of the reduction therapy on ALT levels was investigated in groups A and B.

Statistical analyses. The Student's *t*-test (Welch method), the Chi-square test (Fisher's exact probability), the Kaplan-Meier method (log-rank test) and the multivariate analysis were used for the statistical analyses, with a risk ratio less than 0.05 considered significantly different.

Results

The initial ALT levels were higher in group A than in group B, but there was no significance. After reduction therapy, the ALT levels were significantly high in group A in the first year. However, there were no significant differences between the ALT levels in groups A and B after the second year (Figure 2).

The incidence of HCC in groups A and B was, respectively, 77.8% and 60.0%, in the high ALT group, 26.3% and 55.0% in the low ALT group, and 36.4% and 90.0% in the unclassified ALT group. There was a significant difference only in the unclassified ALT group ($p=0.024$) (Figure 3). The incidence of HCC within 3 years of groups A and B was 11.1% and 40.0% in the high ALT group, 5.3% and 25.0% in the low ALT group and 0% and 40.0% in the unclassified ALT group. There was a significant difference only in the unclassified ALT group ($p=0.004$) (Figure 4).

In total, 39 patients out of 74 (52.7%) developed HCC within 13 years from the beginning of the study, although 35 patients did not develop HCC in the same observation

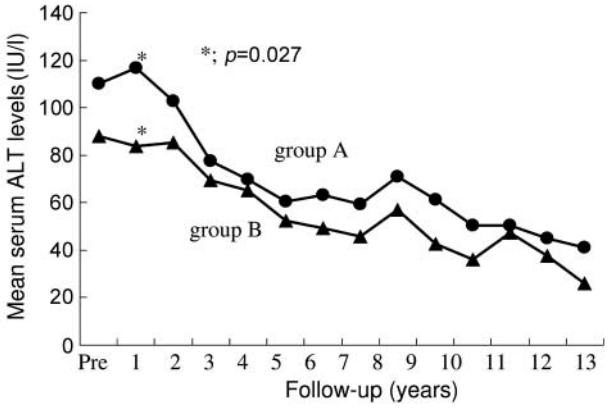

Figure 2. *Time-course of mean ALT serum levels in groups A and B. After reduction therapy, the ALT level was significantly high in group A in the first year. However, there were no significant differences between the ALT levels in groups A and B after the second year. ALT: alanine aminotransferase.*

period. The incidence of HCC development in group B [65.7% (23/35)] was significantly higher than in group A [41.0% (16/39)] ($p=0.039$). The median HCC development time in group A (12.8 years) was significantly longer than in group B (3.8 years) ($p=0.0013$) (Figure 5).

Multivariate analysis demonstrated that reduction therapy and ALT levels were the significant factors affecting HCC development. The incidence of HCC development in group B was 5.8 times higher than in group A. The high and unclassified ALT groups were 4.6 and 2.2 times, respectively, higher than in the low ALT group (Table II).

Discussion

It is generally accepted that approximately 70% of patients with HCV-LC will develop HCC within 10 years of the diagnosis of LC. Preventing the development of HCC in these patients is, therefore, important. For that purpose, the establishment of a low-risk group for HCC development in HCV-LC patients seems to be essential.

Among the many hypotheses proposed to explain the pathogenesis of carcinoma, one is that repeated inflammation and the resulting increased proliferation (mitotic activity) of tissue cells correlate with the development of carcinoma, presumably by chromosomal instability, an increased rate of random mutations (22, 23) and promotion (24, 25). We previously examined the correlation between DNA synthesis of hepatocytes in liver biopsy specimens and the development of HCC in patients with HCV-associated cirrhosis using bromodeoxyuridine (BrdU, a thymidine analog) (19) uptake *in vitro* (20, 26). HCC tended to develop or became detectable when DNA synthesis of hepatocytes increased (20, 26). Subsequently,

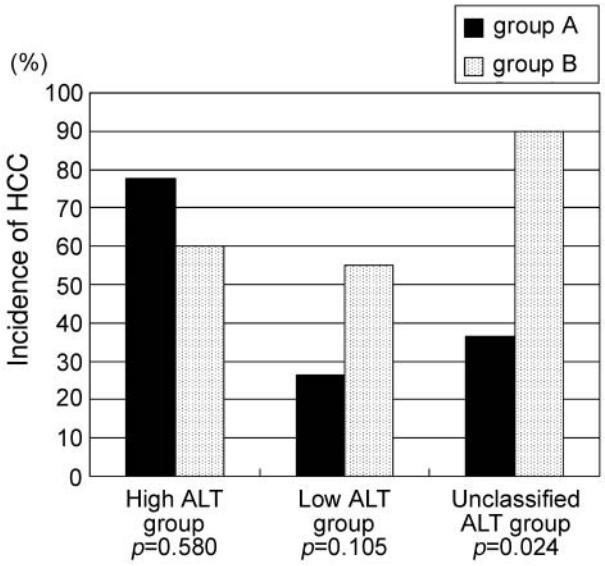

Figure 3. *Incidence of HCC in groups A and B that were subdivided into the high, low and unclassified ALT groups. The incidence of HCC of groups A and B was, respectively, 77.8% and 60.0% in the high ALT group, 26.3% and 55.0% in the low ALT group, and 36.4% and 90.0% in the unclassified ALT group. There was significant difference only in the unclassified ALT group (p=0.024). HCC: hepatocellular carcinoma. ALT: alanine aminotransferase.*

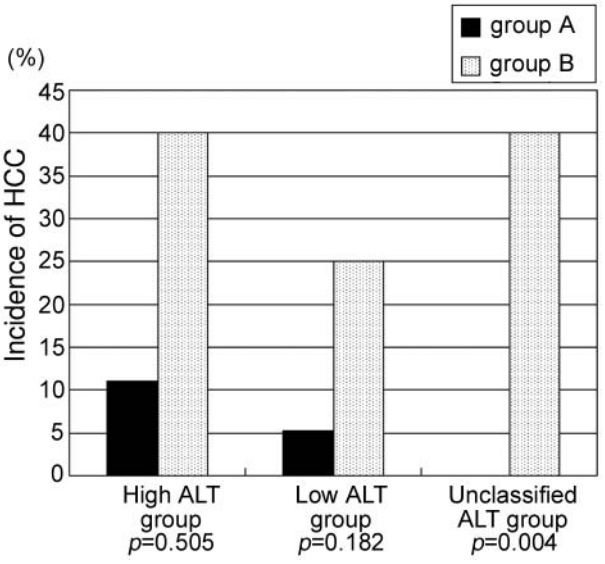

Figure 4. *Incidence of HCC <3 years in groups A and B that were subdivided into high, low and unclassified ALT groups. The incidence of HCC within 3 years in groups A and B was 11.1% and 40.0% in the high ALT group, 5.3% and 25.0% in the low ALT group, and 0% and 40.0% in the unclassified ALT group, respectively. There was a significant difference only in the unclassified ALT group (p=0.004). HCC: hepatocellular carcinoma. ALT: alanine aminotransferase.*

we found that the increased DNA synthesis activity of hepatocytes also acted as an accelerating factor in the recurrence of HCC in hepatectomized patients with

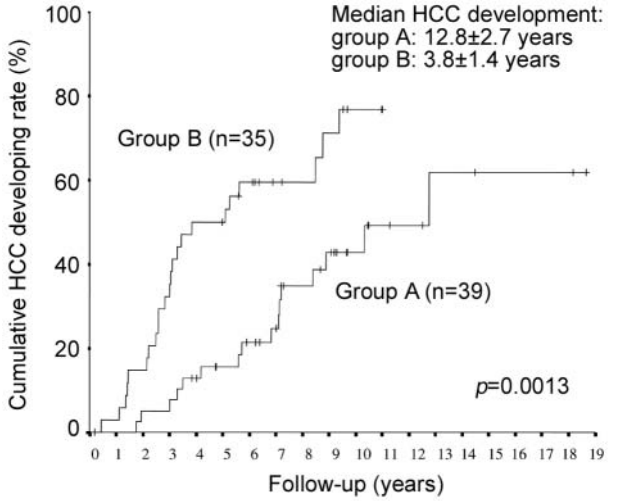

Figure 5. *Cumulative HCC developing rate in groups A and B. The incidence of HCC development in group B [65.7% (23/35)] was significantly higher than in group A [41.0% (16/39)] (p=0.039)]. The median HCC developing time in group A (12.8 years) was significantly longer than in group B (3.8 years) (p=0.0013). HCC: hepatocellular carcinoma.*

Table II. *The results of multivariate analysis using proportional hazard analysis for developing hepatocellular carcinoma (HCC).*

Item	*p*-value	Hazard ratio	95%CI
Group A/B	<0.001	5.802	2.696-12.484
ALT groups			
low group	0.004		
high group	0.001	4.645	1.815-11.882
unclassified group	0.031	2.286	1.076-4.855

CI: confidence interval;
ALT: alanine aminotransferase.

HCV-LC and HCC (27). We assume that increased DNA synthesis in hepatocytes, which has been proven to be an accelerating factor in HCC development, is a result of sustained inflammatory necrosis of the hepatocytes.

Recently, Adachi *et al.* suggested a correlation between postoperative intrahepatic recurrence and proliferating cell activity or serum ALT levels, which represent liver cell necrosis (28). More recently, we demonstrated a correlation between the recurrence of HCC and ALT levels in hepatectomized patients with HCV-LC and HCC (6, 7, 29). These findings suggest a strong correlation between the development of HCC and liver cell necrosis. To demonstrate this hypothesis, annual biopsies of cirrhotic liver could provide accurate information regarding the status of inflammatory necrosis; however, these biopsies are not allowed on ethical grounds. In place of liver biopsy, in this study we monitored the time-course of serum ALT

levels, a well-known marker of liver cell necrosis, over a number of years, and whether the HCV-LC patients with persistently low serum ALT levels belonged to the low-risk group for HCC development was examined.

In our previous study, HCV-LC patients of Child Stage A who had not developed HCC for ten years were analyzed. We reported that in the high ALT group, HCC developed in 71.4% of patients compared with 25.0% in the low ALT group over the observation period ($p<0.005$). The expected interval between the diagnosis of cirrhosis and the development of HCC was 6.0 years in the high ALT group and 12.7 years in the low ALT group ($p<0.001$) (5). There was a significant difference between the high ALT group and the low ALT group in the incidence of developing HCC ($p<0.001$) and developing multiple nodules ($p=0.006$) (7). Concerning this phenomenon, it would be expected that patients with elevated ALT levels might have a faster progression to the end-stage of liver cirrhosis, and that more advanced cirrhosis is more likely to correlatete with carcinogenesis.

On the grounds of a previous study, we presume that a reduction of ALT level prevents development of HCC and we performed aggressive reduction therapy of the ALT levels. The aim of this study was to clarify whether this reduction therapy can suppress the incidence of development HCC.

We reported that the incidence of developing HCC was 81.8% in the high ALT group, 29.3% in the low ALT group and 42.1% in the unclassified ALT group (7). In the present study, the incidence of developing HCC in the high and unclassified ALT groups was higher than that in the low ALT group. In any case, in spite of aggressive reduction therapy, the developing HCC cannot be suppressed and the period of developing HCC in the high ALT group cannot be prolonged. Some other new therapy is needed for the high ALT group patients.

In the low and high ALT groups, there was no significant difference between aggressive reduction therapy and non-aggressive reduction therapy regarding the incidence of HCC. However, in the unclassified ALT group, aggressive reduction therapy suppressed the developing HCC and prolonged the period of HCC development significantly. The significant difference in incidence and period of developing HCC between the two therapies is important, in spite of the lack of significant difference in the proportion of the high, low and unclassified ALT groups. Differences were observed in the total incidence of HCC and the incidence of HCC within 3 years between the two therapies in the unclassified group. From these results, it can be deduced that our aggressive therapy for ALT reduces the ALT level and suppresses HCC development, even if the therapy does not reduce the ALT level sufficiently. In the low ALT group, there was no significant incidence of HCC within 3 years. In the non-aggressive reduction therapy patients the incedence of HCC was higher than in the aggressive reduction therapy patients. From the results of the low and unclassified ALT group, it can be concluded that ALT reduction therapy could suppress carcinogenesis.

Conclusion

The results demonstrated that aggressive reduction therapy cannot suppress ALT levels, but can clearly prevent HCC development. Our protocol of reduction therapy can prevent the development of HCC, even if in some cases of the high ALT group, the aggressive reduction therapy was not effective.

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Effects of antioxidants on quercetin-induced nuclear DNA damage and lipid peroxidation

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Summary

The effects of catalase, superoxide dismutase, mannitol, glutathione, and diallyl sulfide on quercetin-induced DNA damage and lipid peroxidation were investigated in a model system of isolated rat-liver nuclei under aerobic conditions and in the presence of equimolar iron or copper. Mannitol produced a small but significant inhibition of the concurrent nuclear DNA damage and lipid peroxidation induced by quercetin in the presence of iron or copper. Catalase significantly decreased quercetin-induced nuclear DNA damage only in the presence of iron and had no significant effect on lipid peroxidation. Superoxide dismutase showed no significant effect on nuclear DNA damage, but stimulated the quercetin-induced lipid peroxidation only in the presence of copper. Glutathione significantly inhibited the nuclear lipid peroxidation but enhanced the DNA damage. Diallyl sulfide significantly enhanced the nuclear DNA damage but stimulated the lipid peroxidation only in the presence of iron. These results suggest that the reactive oxygen species, especially the hydroxyl radicals, are responsible for the concurrent lipid peroxidation and DNA damage induced by quercetin in the presence of iron or copper in isolated rat-liver nuclei.

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Keywords: quercetin; antioxidants; flavonoids; DNA damage; lipid peroxidation; oxygen radicals

Introduction

Ionizing radiation and carcinogens known to produce oxygen free radicals induce tumors [5], and antioxidants show anticarcinogenic activity against certain types of cancer [29]. These observations support the hypothesis that the reactive oxygen species are involved in carcinogenesis.

Flavonoids, a group of polyphenol quinoids, are widely distributed in edible plants and are therefore widespread in the human diet. The estimated daily intake of flavonoids in the average American diet is approximately 1 g per person per day [3]. Quercetin, a naturally occurring flavonoid, is a direct-acting mutagen under aerobic conditions in short-term bacterial and mammalian systems [3,25,27,34].

Carcinogenicity studies of quercetin have

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yielded conflicting results. Two studies reported that quercetin produces intestinal, bladder and liver cancer in rats [10,28]. However, studies from other laboratories [15,16,34,35] have failed to repeat this observation. Of hundreds of flavonoids present in the human diet, only a few have been tested for carcinogenicity. Even for quercetin, which has been tested, there are discrepancies in the results of carcinogenesis studies.

The molecular mechanism of quercetin mutagenicity and possible carcinogenicity is not known. However, the observations that quercetin is mutagenic only under aerobic conditions and its mutagenicity is affected by antioxidants [27] strongly suggest a role for active oxygen. Quercetin is a polyphenol, and polyphenols produce reactive oxygen species by autoxidation in aqueous medium. Pyrogallol, a polyphenol, produces superoxide radicals by autoxidation [23]. Hydroquinone and catechol, two other polyphenols, generate hydrogen peroxide and superoxide radicals at physiological pH [26]. Therefore, the mutagenicity of quercetin may be due to the oxygen radicals produced by its autoxidation [13].

We have shown that quercetin induces DNA damage and lipid peroxidation in a model system of isolated rat-liver nuclei; the oxidative damage is enhanced by iron or copper [31]. The purpose of the present study was to determine the effects of certain antioxidants on nuclear DNA degradation and lipid peroxidation.

Materials and Methods

Chemicals

Catalase, cupric chloride, ethidium bromide, ferric chloride, Folin-Ciocalteu's phenol reagent, glutathione, mannitol, orcinol and superoxide dismutase were purchased from Sigma Chemical Co. (St. Louis, MO). Diallyl sulfide, diphenylamine, thiobarbituric acid and trichloroacetic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Isolation of rat-liver nuclei

Nuclei were isolated from the livers of untreated male Sprague–Dawley rats (200–300 g; 2–3 months of age; Charles River Laboratories, Wilmington, MA) according to the method of Cox et al. [7] as modified by Taningher et al. [36]. The nuclei were characterized by their DNA, RNA and protein contents.

Treatment of nuclei

To 1-ml aliquots of nuclei suspension, containing approximately 0.2 mg of DNA, we added 50 μl of 2 mM quercetin, 50 μl of 2 mM $FeCl_3$ or 2 mM $CuCl_2$ and 20 μl of catalase (2 mg/ml), superoxide dismutase (SOD) (2 mg/ml), mannitol (0.5 M), glutathione (0.25 M), or diallyl sulfide (0.5 M). The antioxidants were added to the nuclei suspension before quercetin and metal ions. After incubation (30 min at 37°C in a shaking water bath), the control and treated nuclei were analyzed immediately to determine DNA damage and lipid peroxidation.

Determination of DNA damage

DNA damage was determined by fluorometric analysis of the DNA unwinding technique [2] as modified by Taningher et al. [36]. The assay was performed in triplicate on ice under dim light. The percentage of double-stranded DNA remaining after the unwinding process was calculated according to Birnboim and Jevcak [2] by the ratio: (unwound DNA fluorescence – denatured DNA fluorescence)/(native DNA fluorescence – denatured DNA fluorescence).

Determination of lipid peroxidation

Lipid peroxidation was determined by the method of Wilber et al. [39] as modified by Wills [40].

Determination of protein, RNA and DNA

The protein, RNA and DNA contents of the isolated nuclei were determined by the methods of Lowry et al. [22], Ceriotti [4] and Dische [9], respectively.

Statistical analysis of the data

Data generated from this study were statistically analyzed by Dr. Curtis Barton (Division of Mathematics, Food and Drug Administration, Washington, DC). The statistical significance of differences between the control and treated nuclei was assessed by the paired *t*-test, with $P < 0.05$ as the level of significance.

Results and Discussion

We have reported that the incubation of isolated rat-liver nuclei with quercetin results in concentration-dependent concurrent DNA damage and lipid peroxidation, both of which are enhanced by iron or copper ions [31]. Quercetin at a concentration of 100 μM reduced the double-stranded nuclear DNA content from the control value of 81.3% to 52.9% and 50.0% in the presence of equimolar iron or copper, respectively. Similarly, 100 μM quercetin increased the nuclear lipid peroxidation from the control value of 7.2 nmol to 9.4 nmol and 14.9 nmol per gram of liver tissue in the presence of equimolar iron or copper, respectively. These results indicated that the reactive oxygen species generated by quercetin in the presence of iron or copper might be responsible for DNA strand breaks and nuclear lipid peroxidation in the isolated nuclei. To find evidence of active oxygen participation, in the present study we sought to determine the effects of catalase (a scavenger of hydrogen peroxide), superoxide dismutase (a scavenger of superoxide radicals), mannitol (a scavenger of hydroxyl radicals), glutathione and diallyl sulfide on the nuclear DNA damage and lipid peroxidation induced by 100 μM quercetin in the presence of equimolar iron or copper.

The effects of antioxidants on quercetin-induced nuclear DNA damage and lipid peroxidation in the presence of iron or copper are given in Tables I and II, respectively. Mannitol showed a small but significant inhibition of the concurrent DNA damage and lipid peroxidation that was induced by quercetin in the presence of iron or copper. Catalase significantly decreased the quercetin-induced nuclear DNA damage only in the presence of iron and had no effect on lipid peroxidation. Superoxide dismutase had no significant effect on DNA damage but significantly stimulated the nuclear lipid peroxidation only in the presence of copper. Glutathione significantly inhibited the nuclear lipid peroxidation but stimulated the DNA damage. Diallyl sulfide significantly enhanced nuclear DNA damage but stimulated lipid peroxidation only in the presence of iron. These observations support the involvement of reactive oxygen in quercetin-induced concurrent nuclear DNA damage and lipid peroxidation in the presence of iron or copper.

Conclusions similar to ours also have been reported by other investigators. The superoxide radical has been implicated in the

Table I. Effects of antioxidants on DNA damage induced by quercetin in the presence of iron or copper in isolated rat-liver nuclei. Each value, expressed as the percent of double-stranded DNA, is the mean ± S.D. of results obtained from different animals (six for mannitol, four for GSH, and three for all other antioxidants).

Antioxidants[†]		Nuclear DNA content after treatment with	
		Iron-quercetin	Copper-quercetin
Catalase	−	50.1 ± 2.5	54.4 ± 1.8
	+	54.5 ± 3.0*	49.3 ± 4.4
SOD	−	52.6 ± 2.4	49.2 ± 1.4
	+	53.4 ± 2.6	49.1 ± 4.7
Mannitol	−	50.6 ± 2.7	49.9 ± 3.3
	+	58.7 ± 39*	57.7 ± 7.2*
GSH	−	46.5 ± 6.7	42.4 ± 4.3
	+	34.4 ± 2.4*	31.3 ± 3.7*
DAS	−	47.0 ± 4.0	51.9 ± 2.0
	+	34.1 ± 5.8*	42.3 ± 1.6*

SOD = superoxide dismutase; GSH = glutathione; DAS = diallyl sulfide.

[†]Without antioxidant treatment = −; with antioxidant treatment = +.

*Significantly different from the control value.

Table II. Effects of antioxidants on lipid peroxidation induced by quercetin in the presence of iron or copper in isolated rat-liver nuclei. Each value, expressed as nanomoles of lipid peroxides per gram of liver, is the mean ± S.D. of results obtained from different animals (six for mannitol, four for GSH, and three for all other antioxidants).

Antioxidants		Nuclear lipid peroxides after treatment with	
		Iron-quercetin	Copper-quercetin
Catalase	−	7.7 ± 0.4	15.2 ± 0.7
	+	7.7 ± 0.5	15.0 ± 0.7
SOD	−	8.1 ± 0.2	16.2 ± 0.2
	+	7.9 ± 1.1	17.9 ± 0.4*
Mannitol	−	8.8 ± 0.2	16.0 ± 0.7
	+	7.8 ± 0.2*	15.2 ± 0.7*
GSH	−	6.8 ± 0.3	14.4 ± 1.0
	+	6.2 ± 0.4*	8.9 ± 0.5*
DAS	−	7.3 ± 0.2	15.6 ± 1.0
	+	10.7 ± 0.2*	16.2 ± 0.2

SOD = superoxide dismutase; GSH = glutathione; DAS = diallyl sulfide; − = without antioxidant treatment; + = with antioxidant treatment.
*Significantly different from the control value.

increased mutagenicity of quercetin by rat-liver cytosol in the Ames test [27]. Myricetin and quercetagetin, two flavonoids with structures similar to quercetin, produce hydrogen peroxide and superoxide radicals by their autoxidation when treated with beef heart mitochondria [17]. Polyphenolic 2-hydroxyemodine, a direct-acting mutagen like quercetin, generates reactive oxygen species and induces DNA strand breaks [20]. Gossypol, a polyphenol, induces DNA strand breaks in human lymphocytes [6]. The polyphenolic compounds catechol and hydroquinone are genotoxic [8] and react with DNA to produce 8-hydroxydeoxyguanosine [21].

One of the biological effects of garlic is the inhibition of tumor development in experimental animals. Diallyl sulfide, which is present at 30 − 100 μg/g of garlic [41], demonstrates anticarcinogenic activity [11]. Garlic inhibits colon cancer in mice [37], liver cancer induced by 1,2-dimethyl-hydrazine in rats [14], esophageal cancer induced by nitrosomethyl benzylamine in rats [38] and neoplasia of the forestomach induced by benzo[*a*]pyrene in mice [33]. Garlic oil inhibits tumor promotion induced by 12-*O*-tetradecanoylphorbol-13-acetate [1]. Aqueous extracts of garlic produce antimutagenic effects when exposed to ionizing radiation, peroxides, adriamycin and *N*-methyl-*N'*-nitro-nitrosoguanidine [19]. The mechanisms of antimutagenic and anticarcinogenic properties of garlic are not known; however, Sparnins et al. [33] showed that the unsaturated allyl groups and linearly connected sulfur atoms are essential for activity of organosulfur compounds extracted from garlic and onion. It is possible that the oxygen radicals react with the unsaturated allyl groups linearly connected with a sulfur atom to provide radical-trapping properties to these compounds.

Generally, glutathione is a cellular antioxidant. Its antioxidant properties may be due to its ability to scavenge oxygen radicals, especially the hydroxyl radicals [18]. However, glutathione can also act as a pro-oxidant. In our studies, glutathione significantly stimulated the DNA damage induced by quercetin in the presence of iron or copper. Diallyl sulfide also had a stimulatory effect on quercetin-induced DNA damage in the presence of iron or copper, similar to the pro-oxidant properties of glutathione. Rowley and Halliwell [30] showed that glutathione in the presence of iron forms superoxide-dependent hydroxyl radicals. The pro-oxidant properties of glutathione and diallyl sulfide in the presence of iron or copper that were seen in our studies may be caused by superoxide-dependent hydroxyl radicals.

Although glutathione and diallyl sulfide have anticarcinogenic properties, our results indicate that they also have potential for inducing DNA damage. Thiols and disulfides produce oxygen free radicals during their autoxidation in the presence of oxygen and

transition metals, such as iron and copper [24]. Ajoene, a natural component of garlic, is cytotoxic to human fibroblasts, hamster kidney cells, and a tumorigenic lymphoid cell line derived from a Burkitt lymphoma [32]. Thus, sulfur compounds such as glutathione and diallyl sulfide may have a dual role in carcinogenesis. They could have both toxic and beneficial effects, depending on their biological environment. Therefore, the interactions of various dietary components such as flavonoids, sulfur compounds, iron and copper are important factors for carcinogenesis.

Little is known about the susceptibility of cell nuclei to lipid peroxidation and possible damage to DNA. Nuclear DNA is associated with certain regions of the nuclear membrane [12]. The proximity of nuclear DNA to the nuclear membrane may facilitate the interaction of DNA with the peroxyl radicals and other reactive intermediates formed during membrane lipid peroxidation. It is possible that the DNA damage induced by quercetin in our studies may be caused by peroxyl radicals.

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DNA Repair

Oxygen radical-induced single-strand DNA breaks and repair of the damage in a cell-free system

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Abstract

Ferric nitrilotriacetate (Fe^{3+}-NTA) catalyzes hydrogen peroxide-derived production of hydroxyl radicals, which are known to cause DNA damage. In the present work, Fe^{3+}-NTA plus hydrogen peroxide-induced single-strand DNA breaks and repair of the DNA damage were studied in vitro by monitoring DNA damage- and DNA repair-dependent conformational changes of pUC18 plasmid DNA. Single-strand DNA breaks were induced in the pUC18 DNA by Fe^{3+}-NTA plus hydrogen peroxide in a dose-dependent fashion. Induction of the DNA damage was inhibited by deferoxamine mesylate (an iron chelator) and by hydroxyl radical scavengers such as dimethyl sulfoxide (DMSO), D-mannitol and ethanol indicating that the DNA damage was caused by hydroxyl radicals which were generated by reaction of Fe^{3+}-NTA with hydrogen peroxide. The oxygen radical-induced single-strand DNA breaks were repaired partly (more than 50%) by incubating the damaged DNA at 37° C for 3 h with a partially purified preparation of APEX nuclease (a multifunctional DNA repair enzyme), DNA polymerase β, four deoxyribonucleoside triphosphates, T4 DNA ligase and ATP. Analyses of the partially purified preparation of APEX nuclease revealed that a 45-kDa protein as well as APEX nuclease in the preparation were involved in the repair of the single-strand DNA breaks. APEX nuclease was suggested to initiate the repair by removing 3′ termini blocked by the nucleotide fragments and also by incising the 5′ side of AP sites. The 45-kDa protein was suggested to be required for removal of the 5′ tags such as 5′-terminal deoxyribose phosphate residues produced by the action of APEX nuclease on AP sites.

Keywords: Radical DNA damage; Ferric nitrilotriacetate; Hydrogen peroxide; DNA repair; APEX nuclease

1. Introduction

Oxygen free radicals are formed in tissue cells by many endogenous and exogenous causes such as metabolism, chemicals and ionizing radiation. Oxygen radicals may attack DNA at either sugars or bases, giving rise to a large number of damaged products (Imlay and Linn, 1988). Iron is known to be involved in the generation of active oxygen species and in the formation of highly toxic hydroxyl radicals from other active oxygen

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species such as hydrogen peroxide (H_2O_2) (Cross et al., 1987; Inoue and Kawanishi, 1987; Imlay and Linn, 1988; Aruoma et al., 1989).

To study the effects of the oxygen radical on DNA, ferric nitrilotriacetate (Fe^{3+}-NTA) plus H_2O_2 has been used frequently as an oxygen radical source. Nitrilotriacetate (NTA) is a synthetic chelating agent that has been used in various countries as a constituent of detergents (Mottola, 1974; Anderson et al., 1985). It forms water-soluble chelate complexes with several metal cations at neutral pH. Fe^{3+}-NTA, a chelate complex with iron, was reported to be able to induce hemochromatosis in mammals (Awai et al., 1979), and renal tubular necrosis and adenocarcinoma (Okada and Midorikawa, 1982; Ebina et al., 1986; Li et al., 1987). Inoue and Kawanishi (1987) proposed that hydroxyl radicals could be formed by reaction of Fe^{3+}-NTA with H_2O_2 and that the radical might be responsible for the toxic and carcinogenic actions of Fe^{3+}-NTA in vivo. Various types of DNA damage, including single- and double-strand breaks (Toyokuni and Sagripanti, 1993), and base modifications (Aruoma et al., 1989), are reported to be induced on DNA by Fe^{3+}-NTA plus H_2O_2 treatment. Free radical scavenging reaction and repair of radical-damaged DNA are cellular defenses against oxygen radicals.

In this paper, we studied DNA damage caused by Fe^{3+}-NTA and H_2O_2, and in vitro repair of the damaged DNA using repair enzymes purified from mouse ascites sarcoma cells.

2. Materials and methods

Materials

Ferric chloride and sodium bicarbonate were obtained from Katayama Chemicals, Osaka, nitrilotriacetic acid (NTA), D-mannitol and ethidium bromide from Nakarai Tesque Inc., Kyoto, 31% hydrogen peroxide (H_2O_2) from Mitsubishi Gas Co., Ltd., Tokyo, and ribonucleotides (NTPs) and deoxyribonucleotides (dNTPs) were from Seikagaku Kogyou Co., Ltd., Tokyo. Deferoxamine mesylate (Desferal®) was kindly provided by Japan Ciba Geigy Co., Ltd., Takarazuka, Osaka. The other reagents used were obtained as described previously (Seki and Oda, 1988). Distilled, deionized water was used throughout the experiments. Mouse ascites sarcoma (SR-C3H/He) cells were obtained and maintained as described previously (Seki and Oda, 1977).

Preparation of pUC18 DNA

Growth of JM-109 cells transformed with pUC18 plasmid, amplification of the plasmid, harvesting and alkaline lysis of the bacteria, and purification of pUC18 DNA were conducted by the large-scale isolation procedure as described by Sambrook et al. (1989).

Preparation of Fe^{3+}-NTA solution

Fe^{3+}-NTA solution was prepared by the method of Awai et al. (1979). Ferric chloride was dissolved in 1.0 N HCl solution to make 0.1 M iron stock solution. NTA solution (50 mM) was made by dissolving disodium nitrilotriacetate in distilled water. The NTA solution was added to the diluted iron stock solution at the 1:5 molar ratio of iron to NTA, and then the solution was adjusted to pH 7.4 by adding sodium bicarbonate powder with constant stirring.

Preparation of Fe^{3+}-NTA-H_2O_2-damaged pUC18 DNA

Superhelical pUC18 DNA (60 μg) was incubated at 37° C for 30 min with 100 μM Fe^{3+}-NTA and 100 μM H_2O_2 in a total volume of 450 μl under ambient pO_2. The reaction was stopped by addition of 10 mM deferoxamine mesylate, and the mixture was dialysed at 4° C against a solution (pH 8.0) containing 1 mM NaCl, 1 mM EDTA and 1 mM Tris-HCl. The treated DNA was used for repair assay.

Preparation of partially purified and highly purified APEX nuclease

APEX nuclease was extracted from permeable mouse ascites sarcoma cells with a 0.2 M potassium phosphate buffer (pH 7.5), and partially purified by sequential chromatographies with DEAE-cellulose and phosphocellulose columns essentially as described previously (Seki et al., 1991b). Recombinant APEX nuclease was highly

purified by sequential chromatographies with DEAE-cellulose, phosphocellulose and Toyopearl HW-50F columns from BW2001/pUAEHB cells, in which human APEX nuclease was expressed, essentially as described (Seki et al., 1992). The recombinant APEX nuclease was used as purified APEX nuclease for the following priming activity assay and reconstitution experiments of repair of Fe^{3+}-NTA-H_2O_2-damaged DNA.

Purification of a 45-kDa protein

The APEX nuclease preparation partially purified by sequential chromatographies with DEAE-cellulose and phosphocellulose columns contained factors for supporting the repair of Fe^{3+}-NTA-H_2O_2-damaged DNA. The preparation was electrophoresed on a large sodium dodecyl sulfate (SDS)-polyacrylamide gel (17 × 15 × 0.1 cm) as described by Laemmli (1970). The gel was stained with Coomassie brilliant blue R-250 (CBB) for 40 min and destained for 3 h. The 45-kDa protein band was cut out from the gel in which the band was well resolved from its surroundings. The protein was extracted, and electrophoresed again on a gel having the similar size. The 45-kDa protein extracted from the gel was denatured, renatured, and then used for the repair experiments essentially as described previously (Hager and Burgess, 1980; Seki et al., 1993).

Assay of Fe^{3+}-NTA-H_2O_2-induced DNA damage

The reaction mixture (15 μl final volume, adjusted with distilled water) for assaying Fe^{3+}-NTA-H_2O_2-induced DNA damage contained 0.25 μg superhelical pUC18 DNA, 100 μM Fe^{3+}-NTA and 100 μM H_2O_2 at the standard condition. In some experiments, a radical scavenger (ethanol, D-mannitol or DMSO) was added to the reaction mixture. The reaction was performed at 37° C for 30 min, and stopped by chilling to 0° C and adding 10 mM deferoxamine. After addition of 3.0 μl 6-fold concentrated gel loading buffer to the reaction mixture, the sample was applied to a slot of an agarose gel, and electrophoresed for conformational analysis of DNA as described previously (Seki et al., 1989,1990).

Repair of Fe^{3+}-NTA-H_2O_2-damaged DNA

Repair of Fe^{3+}-NTA-H_2O_2-induced single-strand DNA breaks was analyzed essentially as described previously (Seki et al., 1990). The reaction mixture (adjusted to a final volume of 15 μl with Triton buffer B consisting of 0.0175% Triton X-100, 0.25 M sucrose, 10 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM EDTA and 6 mM 2-mercaptoethanol, pH 8.0) for DNA repair consisted of 0.50 μg of Fe^{3+}-NTA-H_2O_2-treated pUC18 DNA, 3.0 μl of 5-fold diluted, partially purified APEX nuclease stock solution, 2.5 μl of DNA polymerase β, 2.5 mM ATP, 40 units T4 DNA ligase and 1.5 μl of 10-fold concentrated substrate mixture for DNA synthesis (0.5 M Tris-HCl, pH 8.0 at 25° C, 50 mM $MgCl_2$, 500 μM each of dATP, dGTP, dCTP and dTTP). In some experiments, 400 ng of purified recombinant APEX nuclease and 300 ng of the 45-kDa protein were added to the reaction mixture in place of the partially purified APEX nuclease. The repair reaction was performed at 37° C for 180 min. After the incubation, 100 μg/ml proteinase K was added to the incubation mixture, and the mixture was incubated at 37° C for 90 min to digest proteins in the reaction mixture. The repaired DNA was analyzed by agarose gel electrophoresis.

Assay of priming activity of APEX nuclease for DNA polymerase on Fe^{3+}-NTA-H_2O_2-damaged DNA

Priming activity of APEX nuclease for DNA polymerase on Fe^{3+}-NTA-H_2O_2-damaged DNA was measured at 37° C for 30 min in a reaction mixture (60 μl in final volume) consisting of 40 μl of Triton buffer B supplemented with 3 μg Fe^{3+}-NTA-H_2O_2-treated pUC18 DNA and APEX nuclease, and 20 μl of a substrate mixture for DNA synthesis (100 mM Tris-HCl, 7.5 mM $MgCl_2$, 240 mM NaCl, 150 μM dATP, 150 μM dCTP, 150 μM dGTP and 7.5 μM [^{3}H]dTTP at 15 Ci/mmol, pH 8.0 at 25° C) supplemented with 0.04 units Klenow polymerase (or DNA polymerase β with an equivalent activity) (Seki and Oda, 1988; Seki et al., 1990). The radioactivity incorporated into acid-insoluble materials was measured by the disk

method. The sample was pipetted onto a glass fiber disk (Whatman GF/C) numbered with India ink, and the disks were batch-washed in 5% trichloroacetic acid, 95% ethanol and ether. The radioactivity was measured as described (Seki and Oda, 1988; Seki et al., 1990).

Detection of a priming enzyme for repair of Fe^{3+}-NTA-H_2O_2-induced single-strand DNA breaks on SDS-polyacrylamide gel

DNA repair enzymes possibly involved in initiation of DNA repair for DNA polymerase on Fe^{3+}-NTA-H_2O_2-treated DNA were examined by the activity blotting method as described previously (Ikeda et al., 1991; Seki et al., 1993). Briefly, Fe^{3+}-NTA-H_2O_2-treated DNA-fixed membranes were prepared by treating native DNA-fixed nylon membranes at 37° C for 30 min with a solution containing 500 μM Fe^{3+}-NTA and 500 μM H_2O_2. Proteins fractionated on a SDS-polyacrylamide gel were renatured and blotted at 30° C for 24 h onto a Fe^{3+}-NTA-H_2O_2-treated DNA-fixed membrane, during which process incision and/or excision were introduced to the damaged DNA by DNA repair enzymes. The blotted membrane was incubated with Klenow polymerase in the presence of digoxigenin-dUTP and other deoxynucleoside triphosphates. The site of the digoxigenin-labeled DNA on the membrane, reflecting molecular weight of the repair enzyme, was finally visualized by non-radioactive detection procedure (Seki et al., 1993).

Other methods

Preparation of DNA polymerase β (Seki and Oda, 1988) and agarose gel electrophoresis (Zhang et al., 1991) were conducted as described previously. Protein concentrations were determined by the BCA protein assay (Pierce, IL, USA), using bovine serum albumin as the standard.

3. Results

Effects of Fe^{3+}-NTA and H_2O_2 on DNA

Untreated pUC18 DNA was composed of abundant superhelical DNA and small amount of nicked, open circular DNA (Fig. 1). The treatment of pUC18 DNA with 100 μM Fe^{3+}-NTA alone or 100 μM H_2O_2 alone did not cause single-strand breaks (Fig. 1A,B). When the superhelical pUC18 DNA was treated with a mixture of 100 μM Fe^{3+}-NTA and varying concentrations (over 10 μM) of H_2O_2, nicked open circular DNA increased in a dose-dependent manner (Fig. 1A). When the DNA was treated with a mixture of 100 μM H_2O_2 and varying concentrations (over 10 μM) of Fe^{3+}-NTA, nicked open circular DNA also increased in a dose-dependent manner (Fig. 1B). By treating pUC18 DNA with 100 μM Fe^{3+}-NTA plus 100 μM H_2O_2 at the conditions described in Materials and methods, closed superhelical pUC18 DNA was mostly converted to nicked open circular DNA. By further increasing the concentration of H_2O_2, linear and fragmented DNAs appeared and increased (Fig. 1A).

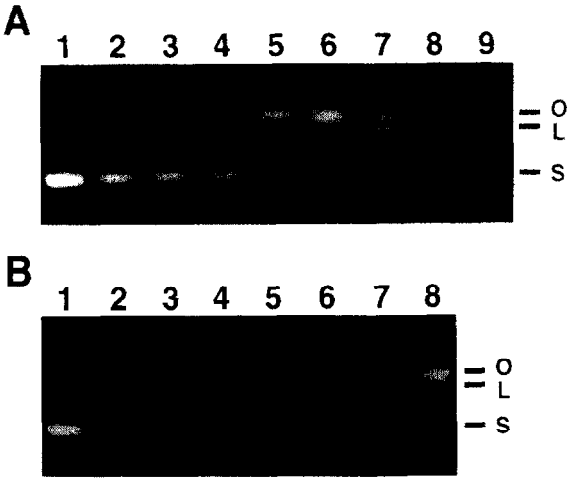

Fig. 1. Effects of H_2O_2 and Fe^{3+}-NTA at various concentrations on DNA damage. The amount of DNA loaded into each slot was 0.25 μg. The concentrations of H_2O_2 in A and Fe^{3+}-NTA in B were as follows: lane 1, untreated pUC18 DNA; lanes 2–9 in A, pUC18 DNA treated with 100 μM Fe^{3+}-NTA and 0, 10, 20, 50, 100, 200, 500 and 700 μM H_2O_2, and lanes 2–8 in B, 100 μM H_2O_2 and 0, 10, 20, 50, 100, 200 and 500 μM Fe^{3+}-NTA. Abbreviations: O, open circular DNA; L, linear DNA; S, closed circular superhelical DNA.

Kinetics of DNA damage by Fe^{3+}-NTA and H_2O_2

When superhelical pUC18 DNA was incubated at 37° C with 100 μM each of Fe^{3+}-NTA

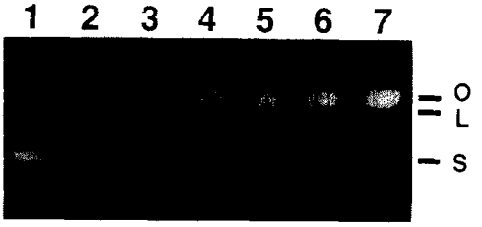

Fig. 2. Kinetics of Fe^{3+}-NTA and H_2O_2-induced DNA damage. The reaction mixture was the same as described in Materials and methods except that the incubation time varied as follows: lane 1, untreated pUC18 DNA; lanes 2–7, pUC18 DNA treated at 37°C with 100 μM Fe^{3+}-NTA and 100 μM H_2O_2 for 0, 5, 10, 20, 30 and 45 min. Abbreviations are the same as in Fig. 1.

and H_2O_2, the closed superhelical form of DNA was converted into the nicked open circular form, and the conversion was completed in 30 min (Fig. 2).

Effects of radical scavengers and deferoxamine on Fe^{3+}-NTA-H_2O_2-induced DNA damage

Effects of hydroxyl radical scavengers on Fe^{3+}-NTA-H_2O_2-induced DNA damage were tested in order to determine whether hydroxyl radical was

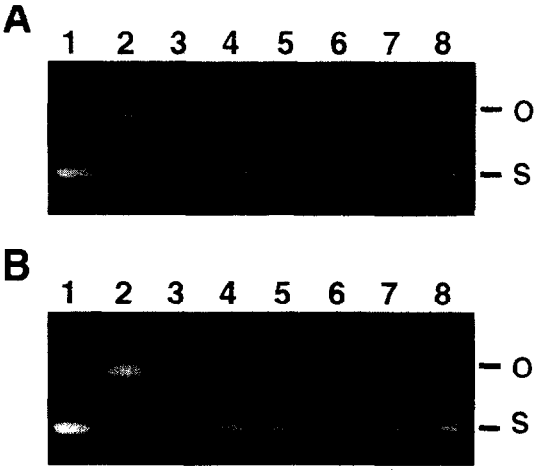

Fig. 3. Effects of hydroxyl radical scavengers and deferoxamine mesylate on DNA damage induced by Fe^{3+}-NTA and H_2O_2. The following scavenger or deferoxamine mesylate was added to the reaction mixture prior to the addition of H_2O_2, and the concentrations of H_2O_2 and Fe^{3+}-NTA were 100 μM each. Lane 1, untreated pUC18 DNA; lane 2, 100 μM Fe^{3+}-NTA and 100 μM H_2O_2-treated pUC18 DNA; lanes 3, 5 and 7 in A, DNA damaged in the presence of 20 mM D-mannitol, DMSO and ethanol; lanes 4, 6 and 8 in A, DNA damaged in the presence of 50 mM D-mannitol, DMSO and ethanol; lanes 3–8 in B, 0.01, 0.1, 1, 5, 7 and 10 mM deferoxamine mesylate.

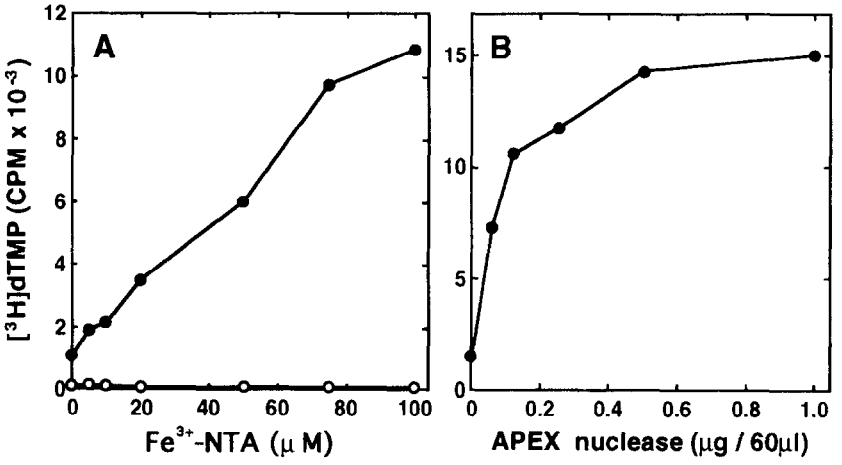

Fig. 4. Effects of Fe^{3+}-NTA (A) and APEX nuclease (B) at various concentrations on the template-primer activity of Fe^{3+}-NTA-100 μM H_2O_2-damaged pUC18 DNA. The template-primer activity for Klenow polymerase of the damaged DNA was measured by the single step method as described in Materials and methods. (A) Concentration of Fe^{3+}-NTA varied as indicated. The template-primer activity was assayed in the presence of 1 μg recombinant APEX nuclease (●) or in the absence of APEX nuclease (○). (B) DNA was treated with 100 μM Fe^{3+}-NTA and 100 μM H_2O_2 at the standard condition. Recombinant APEX nuclease was added at the indicated concentration to the reaction mixture. Data are expressed as cpm of [^{3}H]dTMP incorporated into 3 μg DNA in a reaction tube as described in Materials and methods.

actually responsible for the Fe^{3+}-NTA-H_2O_2-induced DNA damage. All hydroxyl radical scavengers tested significantly inhibited production of the Fe^{3+}-NTA-H_2O_2-induced DNA damage, and the inhibitory effect of the scavengers at 50 mM was higher in the order of ethanol < D-mannitol < DMSO (Fig. 3A). 50 mM DMSO inhibited 100 μM Fe^{3+}-NTA-100 μM H_2O_2-induced single-strand breaks of pUC18 DNA almost completely. In order to determine whether ferrous ions were essential for the single-strand breaks by Fe^{3+}-NTA and H_2O_2, the effect of deferoxamine, a ferrous ion-specific chelator, on single-strand breaks was tested. Single-strand DNA breaks induced by 100 μM Fe^{3+}-NTA plus 100 μM H_2O_2 were almost completely inhibited by 10 mM deferoxamine (Fig. 3B).

Priming activity of purified APEX nuclease for DNA polymerase on Fe^{3+}-NTA-H_2O_2-damaged DNA

The DNA damage-dependent (A) and APEX nuclease-dependent (B) DNA syntheses shown in Fig. 4 indicate that purified recombinant APEX nuclease clearly has priming activity for DNA polymerase on Fe^{3+}-NTA-H_2O_2-damaged DNA. Considering the enzymatic property of APEX nuclease, 3′-blocked termini on the damaged DNA should be removed by DNA 3′-5′ repair diesterase activity and AP sites on the damaged DNA should be nicked at the 5′ side by 5′-AP endonuclease activity of APEX nuclease.

Further evidence for the involvement of APEX nuclease in repair of Fe^{3+}-NTA-H_2O_2-induced, single-strand DNA breaks

The activity blotting method (Ikeda et al., 1991; Seki et al., 1993) was used to detect the enzyme(s) involved in the initial step of repair of Fe^{3+}-NTA-H_2O_2-induced, single-strand DNA breaks. The result of SDS-PAGE of the partially purified APEX nuclease fraction indicates that the fraction contained various proteins besides APEX nuclease (Fig. 5, lane 2). APEX nuclease was gel-purified to become a 35-kDa single protein band on SDS-polyacrylamide gels (Fig. 5, lane 3). The proteins fractionated on the SDS-polyacrylamide gels were activity-blotted on the nylon membrane coated with Fe^{3+}-NTA-H_2O_2-treated DNA, as described previously (Seki et al., 1993). The positive signal was detected only at the position of APEX nuclease of both the partially purified APEX nuclease-loaded lane and the purified APEX nuclease-loaded lane on the Fe^{3+}-NTA-H_2O_2-treated DNA-fixed membrane, but not on the native DNA-fixed membrane (Fig. 5). The result indicates that the priming factor for repair on Fe^{3+}-NTA-H_2O_2-induced single-strand DNA breaks in the partially purified APEX nuclease preparation is APEX nuclease itself.

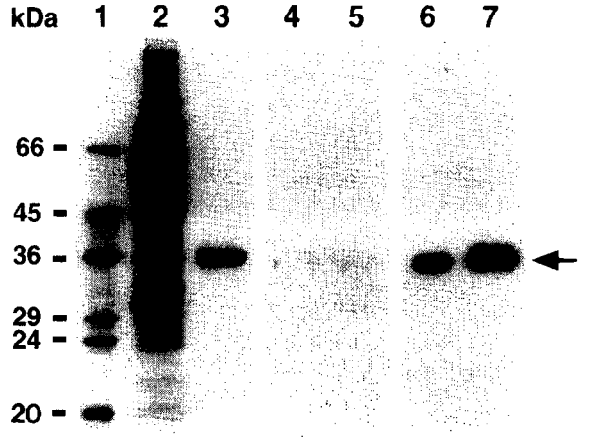

Fig. 5. Activity blotting for detection of a priming factor(s) in the APEX nuclease fraction partially purified from mouse ascites sarcoma cells for repair of Fe^{3+}-NTA and H_2O_2-damaged DNA. The molecular weight standard was electrophoresed in lane 1. The partially purified APEX nuclease (lanes 2, 4 and 6) and APEX nuclease highly purified by SDS-PAGE (lanes 3, 5 and 7), 10 μl each, were electrophoresed. Proteins electrophoresed in lanes 1, 2 and 3 were stained with CBB. The electrophoresed, renatured enzymes were activity-blotted onto a native DNA-fixed membrane (lanes 4 and 5) or a Fe^{3+}-NTA plus H_2O_2-damaged DNA-fixed membrane (lanes 6 and 7). Klenow polymerase was used for reparative DNA synthesis. The arrow indicates the position of APEX nuclease.

Repair of Fe^{3+}-NTA and H_2O_2-induced single-strand DNA breaks

Superhelical pUC18 DNA was converted largely into the open circular form with a single-strand break(s) and slightly into the linear form

with a double-strand break by treating the DNA with 100 μM Fe^{3+}-NTA plus 100 μM H_2O_2 (Fig. 6A,B, lane 2). When the nicked open circular DNA was incubated for DNA repair with the partially purified APEX nuclease fraction and analyzed by agarose gel electrophoresis in the presence of ethidium bromide (EtdBr) at 0.5 μg/ml, the band of the open circular DNA was markedly reduced and a new thick band ('R', repaired DNA band) showing almost the same electrophoretic mobility as that of superhelical pUC18 DNA appeared (Fig. 6A, lane 3; Fig. 7A, lane 3). The 'R' band was shown to be the band of repaired DNA by two-dimensional electrophoresis. When the DNA incubated for DNA repair was electrophoresed on an agarose gel without EtdBr (first dimension) (Fig. 7B, lane 3) and then under the presence of EtdBr (second dimension), repaired closed circular DNA was clearly distinguishable from the open circular and linear DNAs by its mobility (Fig. 7C) (Seki et al., 1989,1990). When the nicked DNA was incubated for DNA repair with the purified recombinant APEX nuclease in place of the partially purified APEX nuclease, only a little repaired DNA was observed (Fig. 6B, lane 3). The result suggests that a factor(s) in addition to APEX nuclease in the partially purified APEX nuclease fraction is required for repair of the Fe^{3+}-NTA-H_2O_2-induced single-strand DNA breaks. To identify the factor, the partially purified APEX nuclease sample was fractionated by electrophoresis on a SDS-polyacrylamide gel (Fig. 8). Protein bands were cut out separately, and protein was eluted from the each gel band and renatured (Hager and Burgess, 1980; Seki et al., 1993). The repair supporting activity of these eluted proteins was tested with the above mentioned DNA repair system. DNA repair equivalent to that with the partially purified APEX nuclease was obtained using a 45-kDa protein and the purified recombinant APEX nuclease in place of the partially purified APEX nuclease (Fig. 6B, lane 4). The 45-kDa protein (Fig. 8) did not have detectable AP endonuclease activity, non-specific endonuclease activity and priming activity for DNA polymerase on Fe^{3+}-NTA-H_2O_2-induced single-strand DNA breaks and acid-depurinated DNA. The protein was inactivated at 60° C for 10 min, and the repair supporting activity was inhibited by 100 mM NaCl. Repair of acid-depurinated DNA was also initiated by APEX nuclease and supported by the 45-kDa protein (data not shown).

Densitometric analysis of the electrophoretic pattern showed that more than 50% of Fe^{3+}-

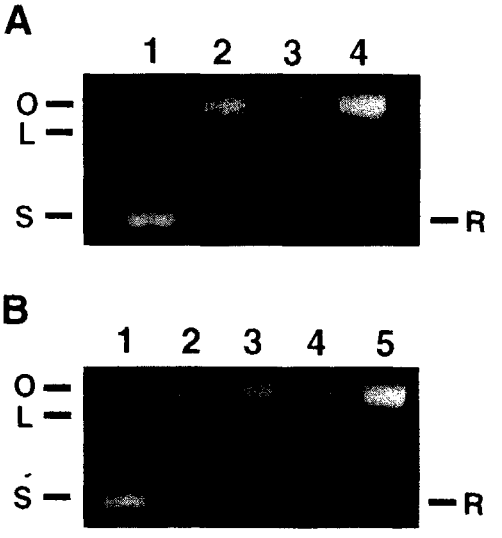

Fig. 6. Gel electrophoretic analysis of repair of Fe^{3+}-NTA and H_2O_2-damaged DNA. The complete assay mixture (15 μl) contained 0.5 μg damaged pUC18 DNA, the partially purified APEX nuclease fraction (A) or purified APEX nuclease plus 45-kDa protein (B), four dNTPs, ATP, DNA polymerase β and T4 DNA ligase. Incubation for DNA repair was conducted at 37° C for 180 min, and then the mixture was incubated at 37° C for 90 min after addition of 100 μg/ml proteinase K. The amount of DNA loaded in each slot was 0.30 μg. (A) Lane 1, untreated pUC18 DNA; lane 2, 100 μM Fe^{3+}-NTA and 100 μM H_2O_2-damaged DNA; lane 3, Fe^{3+}-NTA and H_2O_2-damaged DNAs incubated for DNA repair with the complete assay mixture containing the partially purified APEX nuclease; lane 4, without the APEX nuclease fraction. (B) Lane 1, untreated pUC18 DNA; lane 2, 100 μM Fe^{3+}-NTA and 100 μM H_2O_2-damaged DNA; lane 3, Fe^{3+}-NTA and H_2O_2-damaged DNA incubated for DNA repair with the complete assay mixture with the exception of the 45-kDa protein; lane 4, with the complete assay mixture containing 400 ng purified APEX nuclease and 300 ng 45-kDa protein; lane 5, with the complete assay mixture with the exception of the 45-kDa protein and APEX nuclease. Abbreviations: O, open circular DNA; L, linear DNA; S, closed circular superhelical DNA; R, repaired closed circular DNA.

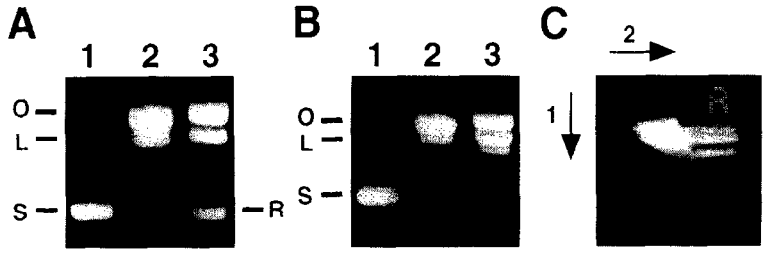

Fig. 7. Detection of repaired DNA on agarose gel electrophoresis. (A and B) Lane 1, untreated pUC18 DNA; lane 2, 100 μM Fe^{3+}-NTA and 100 μM H_2O_2-damaged DNA; lane 3, Fe^{3+}-NTA and H_2O_2-damaged DNA incubated for DNA repair in the complete assay mixture described in legend to Fig. 6A. Agarose gels were prepared and the samples were electrophoresed in TBE buffer supplemented with 0.5 μg/ml EtdBr in A or without EtdBr in B. After electrophoresis, DNA in B was stained with EtdBr at a concentration of 0.5 μg/ml. (C) Two-dimensional gel electrophoresis. Fe^{3+}-NTA and H_2O_2-damaged DNA was incubated for DNA repair with the complete assay mixture. The sample was electrophoresed firstly at 50 V for 90 min without EtdBr in the direction of arrow 1. The gel was shaken gently for 90 min in TBE buffer supplemented with EtdBr at 0.02 μg/ml and then electrophoresed at 50 V for 90 min in the direction of arrow 2 in TBE buffer supplemented with EtdBr at 0.02 μg/ml. After the electrophoresis, DNA was stained with EtdBr at 0.5 μg/ml.

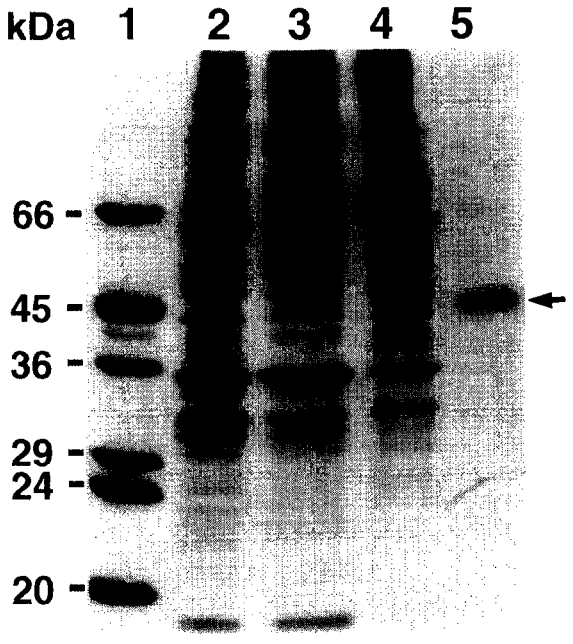

Fig. 8. SDS-PAGE of fractions obtained in the purification steps of the 45-kDa protein. The 45-kDa protein was extracted from permeable SR-C3H/He cells and purified as described in Materials and methods. Fractions in the purification steps were electrophoresed on SDS-polyacrylamide gels. Samples and the amount of protein loaded per lane were as follows: lane 1, molecular weight standard; lane 2, 33 μg of the crude cell extract; lane 3, 43 μg of the DEAE-cellulose flow-through fraction; lane 4, 43 μg of the phosphocellulose fraction; lane 5, 1 μg of the gel-purified 45-kDa protein (arrow).

NTA-H_2O_2-induced single-strand DNA breaks were repaired and converted to closed circular DNA in the present repair system, but double-strand DNA breaks were not repaired.

4. Discussion

Oxygen free radicals generate various types of DNA damage, such as base damages, AP sites and single- and double-strand breaks. The lesions are generally repaired by cellular DNA repair systems, but these repair mechanisms sometimes fail, resulting in genetic changes or cell death. In the present work, oxygen radical-induced (Fe^{3+}-NTA plus H_2O_2-induced) single-strand DNA breaks and their repair were investigated.

Fe^{3+}-NTA-H_2O_2-induced DNA breaks were inhibited by deferoxamine mesylate and by hydroxyl radical scavengers such as DMSO, D-mannitol and ethanol indicating that the DNA damage was caused by hydroxyl radicals which were generated by reaction of Fe^{3+}-NTA with H_2O_2. The results agree well with those reported previously (Inoue and Kawanishi, 1987; Aruoma et al., 1989; Toyokuni and Sagripanti, 1993). It is known that some free radical-mediated single-strand breaks contain 3′-phosphoglycolate termini and 3′-phosphate termini (Imlay and Linn, 1988; Tee-

bor et al., 1988). Both types of strand breaks were reported to contain 5′-phosphate termini (Teebor et al., 1988). Abasic (AP) sites are also known to be produced by oxidative free radical-mediated DNA damage (Teebor et al., 1988). Ionizing radiation is also known to produce AP sites and single-strand DNA breaks with 5′-phosphate and 3′-phosphoglycolate or 3′-phosphate termini by essentially the same mechanism as oxidative free radical-mediated DNA damage (Henner et al., 1983; Breimer and Lindahl, 1984).

In *E. coli*, repair of 3′-blocked termini and AP sites is known to be initiated mostly by exonuclease III and endonuclease IV (Breimer, 1988; Imlay and Linn, 1988; Teebor et al., 1988). 5′-Phosphodeoxyribose tags (a kind of 5′-blocked terminus) produced by the action of exonuclease III or endonuclease IV on AP sites are suggested to be removed by DNA deoxyribophosphodiesterase (dRpase) (Franklin and Lindahl, 1988; Price and Lindahl, 1991). Sandigursky and Franklin (1992) reported that dRpase of *E. coli* was associated with exonuclease I. Recently, Dianov et al. (1994) suggested that the dRpase was not exonuclease I but recJ product.

In mammalian cells, a few enzymes showing priming activity for DNA polymerase on DNAs having single-strand breaks with 3′-blocked termini and AP sites have been reported (Seki and Oda, 1988; Seki et al., 1991a; Chen et al., 1991; Demple et al., 1991; Robson and Hickson, 1991; Winters et al., 1992). Among them, the mammalian major AP endonuclease was highly purified and shown to have 5′-AP endonuclease, DNA 3′ repair diesterase, 3′-5′ exonuclease and DNA 3′ phosphatase activities (Seki et al., 1991a,b; Demple et al., 1991). cDNA for the enzyme was cloned independently by three groups: HAP1 and BAP1 by Robson and Hickson (1991) and Robson et al. (1991), APEX by Seki et al. (1991a,1992) and APE by Demple et al. (1991).

The highly purified APEX nuclease contributes to only a small fraction of repair of Fe^{3+}-NTA plus H_2O_2-induced single-strand DNA breaks as well as X-ray-induced single-strand DNA breaks, although more than 50% of X-ray-induced single-strand DNA breaks was repaired by the partially purified preparation of APEX nuclease (Seki et al., 1990). In the present experiment, therefore, we searched for other repair factors in addition to APEX nuclease in the partially purified APEX nuclease preparation. We identified a 45-kDa protein as the repair supporting factor. DNA repair equivalent to that with the partially purified APEX nuclease was obtained by using the 45-kDa protein and purified APEX nuclease in place of the partially purified APEX nuclease. The reconstituted repair system suggested that more than 50% of Fe^{3+}-NTA and H_2O_2-induced single-strand DNA breaks as well as X-ray-induced single-strand DNA breaks is repaired in the presence of APEX nuclease, the 45-kDa protein, DNA polymerase, four dNTPs, Mg^{2+}, DNA ligase and ATP. The tentative partial amino acid sequence analysis of the 45-kDa protein suggested that a protein with the identical sequence has not been reported (Seki, in preparation).

The repair supporting property of the 45-kDa protein suggested that the protein is involved in the repair of oxidative DNA damage by removing 5′-blocked termini similar to dRpase (Franklin and Lindahl, 1988; Price and Lindahl, 1991) and DNase IV (Lindahl et al., 1969; Murray et al., 1994; Robin et al., 1994). Further characterization of the 45-kDa protein is necessary to distinguish it from dRpase or DNase IV, and we are trying to determine the partial amino acid sequence of the 45-kDa protein and its cDNA cloning to isolate an enzymatically active recombinant protein. The enzyme is labile and difficult to purify from mammalian cells in its intact form for enzymatic characterization.

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Beneficial effects of hypertonic mannitol in acute ischemia — reperfusion injuries in humans

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Acute ischemia–reperfusion of extremities is characterized by edema, compartment syndrome and neuromuscular dysfunction. Intravenous hypertonic mannitol has been shown to be of benefit in several experimental models. The authors' 5-year experience with the use of hypertonic mannitol and the treatment of acute ischemia reperfusion injuries in humans has been reviewed. Some 186 patients with acute arterial occlusion following thromboembolism (149) and trauma (37) were treated. Hypertonic mannitol (25 g intravenous bolus followed by 5–10 g intravenous/h) was given perioperatively. Length of preoperative ischemia varied from 1 to 24 h. Some 57.5% of patients had preoperative neuromuscular dysfunction. Following revascularization, limb salvage was obtained in 97.7% of surviving patients and neuromuscular dysfunction improved in 89%. Overall, 15% required fasciotomy. The mortality rate was 3.2%. These data suggest that hypertonic mannitol may have some protective effect in acute ischemia–reperfusion injuries of human extremities. It may decrease the need for fasciotomy and minimize neuromuscular dysfunction.

Keywords: arterial thrombosis, embolism, fasciotomy, mannitol

Following a period of ischemia and subsequent reperfusion a series of metabolic events occurs leading to cellular injury. Microvascular permeability is increased[1–3]. Edema raises pressure within the fascial compartments, which impairs venous return and muscle perfusion, leading to necrosis. Irreversible changes can occur after as few as 4 h of ischemia. It is now suggested that partial ischemia can be more damaging than complete vascular occlusion. In addition, the rate of reperfusion may be an important element in regulating free radical injury[4,5]. Features of the developing compartment syndrome include pain, parasthesias and paralysis. These deficits in neuromuscular function can be permanent if a decompressing fasciotomy is not performed. Limb loss and mortality rates in acute ischemia have been significant[6,7].

Hypertonic mannitol has been postulated to be beneficial in ischemia–reperfusion injury, both as an osmotic agent and as a scavenger of free radical hydroxyl molecules. Previous animal studies in this institution have demonstrated the local and systemic physiologic effects of mannitol in ischemia–reperfusion models[8–10]. This review is undertaken to examine the outcome of human limbs managed with hypertonic mannitol after acute arterial ischemia.

Patients and methods

The records of all patients presenting with acute arterial ischemia in the past 5 years were reviewed. This included limb-threatening ischemia secondary to thrombotic, embolic and traumatic injuries, limited to a period no greater than 24 h. These patients underwent prompt revascularization, delayed only by management of life-threatening complications or transfer from another institution. Arteriography was performed depending on the cause of ischemia and clinical condition of the extremity. Hypertonic mannitol (20%) 25 g was given to all patients as an intravenous bolus before the operative procedure. A 5–10 g continuous infusion was simultaneously started and maintained through the postoperative period (12 h to 3 days). The addition of

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fasciotomy, either concurrently with the revascularization or at a second operation, was based on clinical signs of compartment syndrome. These included a swollen, tender muscle compartment, pain on passive muscle stretch, parasthesias and/or measured intracompartmental pressure > 30 mmHg.

Data obtained in this review include demographics, etiology and duration of ischemia, pre- and postoperative incidence of neuromuscular dysfunction, type of operative procedure, and rates of fasciotomy, limb salvage and death.

Results

One hundred and eighty-six people presented with acute lower-extremity ischemia during the period from 1988 to 1993. Of these, 108 were males and 78 females. The mean age was 61.4 years (range 5–94 years). Some 53% had a history of coronary artery disease.

The etiology of ischemia was thromboembolism in 149 cases (80.1%) and trauma in 37 cases (19.9%). Embolism was noted in 51 patients and acute thrombosis in 98 (*Table 1*). Vascular bypass occlusion comprised 45 of the 98 thrombosis patients. The trauma was blunt in 25 patients (68%), penetrating in six (16%) and iatrogenic catheter injury in six (16%). The average duration of ischemia was 10.9 h (s.d. 7.9 h, s.e. 0.58 h).

On examination, 107 of the patients (57.5%) exhibited preoperative signs of neuromuscular dysfunction. Of these, 68 were found to have both motor and sensory deficits, whereas 39 had sensory loss alone. The remaining 79 patients complained of pain in their lower extremities or were noted to have signs of ischemia on clinical examination.

Some 75 (40%) of the 186 patients underwent thromboembolectomy, with or without patch graft, to restore perfusion. Vascular bypass, with or without thromboembolectomy, was performed in 104 (55.9%) cases. Seven revascularizations were achieved by direct repair. A total of 28 patients underwent lower-extremity open fasciotomy (15.1%). A majority of these fasciotomies (82.1%) were done simultaneously with the revascularization. Five patients had a staged fasciotomy after development of signs of compartment syndrome.

Table 1 Etiology of acute lower-extremity ischaemia

Etiology	No. of ischaemic episodes	Fasciotomy rates
Trauma	37	10/37 (27.0)
Blunt	25	8?25 (32.0)
Penetrating	6	2/6 (33.3)
Iatrogenic	6	0/6 (0)
Thromboembolism	149	18/149 (12.1)
Thombosis	98	16?98 (16.3)
Embolism	51	2/51 (3.9)
Total	186	28/186 (15.1)

Values in parentheses are percentages

Ninety of those 101 patients (89.1%) with preoperative neuromuscular dysfunction who survived without amputation had improvement in their symptoms after revascularization. Complete resolution was noted in 60 patients (59.4%). Partial resolution was seen in 30 patients (29.7%), most often recorded as residual parasthesias. Nine patients were lost to follow-up. One child, who was hit by a car, suffered a traction injury to the peroneal nerve; she had a persistent foot drop. Two patients with spinal cord ischemia had no improvement in their preoperative condition. Besides these patients, all surviving limbs were functional.

The mortality rate was 3.2% (six of 186 patients). Four of these patients developed acute lower-extremity thrombosis after emergent aortic replacement and died from multisystem organ failure. Two of the patients had primarily cardiac-related deaths, succumbing to acute myocardial infarction and arrhythmias. None of the deaths was caused directly by limb ischemia.

Limb salvage was achieved in 176 of the 180 surviving patients (97.8%). The four amputations followed acute thrombotic episodes, two having thrombosis of previously placed vascular bypasses. One patient with acute aortic occlusion was transferred from another institution after 10 h of lower-extremity paralysis. She underwent simultaneous inflow procedure and amputation of one of her extremities as the muscle was non-viable. The second patient presented with recurrent occlusion of a distal bypass. He was transferred to the authors' institution after an attempt at revision also failed. An inflow procedure, distal revascularization and fasciotomy were performed. The bypass again occluded due to lack of adequate outflow and conduit. Amputation was performed on postoperative day 5. The third patient had thrombosis of his *in situ* bypass on completion of an abdominal peroneal resection at another institution. After transfer an inflow procedure, graft thrombectomy and fasciotomy were performed. The calf muscle showed progressive necrosis and amputation was performed on the fifth postoperative day. The fourth patient had a history of diabetes, dialysis-dependent renal failure and contralateral amputation. She developed acute ischemia 24 h after iliac angioplasty. Iliofemoral and femoral tibial bypass were performed with the distal portion subsequently occluding. The patient declined further attempts at revascularization.

Discussion

Within 6 h of ischemia, skeletal muscle energy stores in the form of creatine phosphate and glycogen are depleted. Adenosine triphosphate (ATP) is catabolized to adenosine, inosine, hypoxanthine and xanthine. Also

during the ischemic period the enzyme xanthine dehydrogenase is converted to xanthine oxidase. During reperfusion, xanthine oxidase catalyzes the production of superoxide free radicals from oxygen and hypoxanthine or xanthine. The superoxide anion reacts with hydrogen peroxide to generate the hydroxyl free radical. In the ischemic extremity, the endothelial cell is a source of xanthine oxidase, as xanthine dehydrogenase is not converted in skeletal muscle. No xanthine oxidase activity was found in isolated canine gracilis muscle subjected to ischemia and reperfusion[11]. In addition, inhibitors of xanthine oxidase (allopurinol and oxypurinol) did not limit ischemic muscle necrosis following reperfusion[12]. After adherence to the endothelium, activated neutrophils can amplify the generation of free radicals by a cell surface enzyme NADPH oxidase[13–15]. Neutrophil adherence to the endothelium after ischemia–reperfusion is associated with an increase in microvascular permeability in skeletal muscle; this has been blocked with monocolonal antibody to the CD11/CD18 receptor[3].

Free radicals damage lipid cellular membranes by lipid peroxidation. This causes cell edema, changes in electrolyte concentration, uncoupling of oxidative phosphorylation, cell dysfunction and cell death. Microvascular permeability increases[1–3]. The effects of free radical injury can become systemic with activation of neutrophils and the complement cascade, release of cellular toxins and rhabdomyolysis[16,17].

Mannitol is one of many free radical scavengers and is primarily active against the hydroxyl radical. This occurs in the extracellular space. Mannitol has been used experimentally to limit injury following ischemia and reperfusion. Walker *et al.*[5] used a gracilis muscle preparation subjected to 5 h of ischemia. Reperfusion was performed in a 'controlled fashion', i.e. oxygen was gradually introduced over a 1-h period. One group also received reperfusate containing mannitol. Muscle necrosis was measured at 48 h by histologic staining. The greatest preservation of muscle structure was seen in the group with controlled oxygen reperfusion and infusion of free radical scavengers. Those with controlled reperfusion alone showed less necrosis than the control group. Ricci and colleagues[18] used an *in vivo* canine model of compartment syndrome to compare the effects of prophylactic fasciotomy, superoxide dismutase and mannitol on muscle necrosis and muscle function. Using technetium pyrophosphate scanning, the mannitol group was noted to have significantly increased muscle preservation compared with fasciotomy or superoxide dismutase. Prophylactic fasciotomy showed superior results in preserving muscle function compared with any other group. In this protocol, however, only a single dose of mannitol (25 g bolus) was given rather than a continuous infusion. This dose could have been cleared early in the 15-h reperfusion period. Hoch *et al.*[19] noted a significant reduction in muscle damage and improved neuromuscular function in a canine *in vivo* model in those animals reperfused with a continuous infusion of mannitol compared with control or superoxide dismutase groups.

Previously reported by this author is a canine hindlimb model of ischemia–reperfusion in which a bolus of hypertonic mannitol followed by a continuous infusion was employed[9]. One hindlimb underwent hypoperfusion at a blood pressure of 50 mmHg for 90 min. During reperfusion, blood flow, oxygen delivery, oxygen consumption and oxygen diffusing capacity all dropped in untreated limbs, but equaled control values in those limbs treated with mannitol. In a separate experiment, canine hindlimbs underwent hypoperfusion (90 min) followed by reperfusion with a bypass procedure. Untreated dogs showed a decrease in femoral artery flow accompanied by an increase in peripheral vascular resistance at 5, 60 and 120 min of reperfusion. A significant increase in wet-to-dry ratio was also noted. These changes were not seen in the mannitol-treated animals[8–10].

Encouraged by previous results, hypertonic mannitol has been used as adjunctive therapy in nearly all patients presenting to the authors' institution with acute limb-threatening ischemia. The mortality rate in the present review of 3.2% compared favorably with other studies reporting rates of 8–22%[6,7]. Aortoiliac thrombosis carries an even higher risk of death, approximately 50%[20]. Unlike results reported by Cambria and Abbott[6] in which mortality was higher after embolic (20%) rather than thrombotic (8%) events, five of the present six deaths came after acute thrombosis. None of these patients had amputations before death. In four cases acute ischemia occurred following other major surgery (mesenteric ischemia in two and ruptured aortic aneurysm in two). These patients succumbed to multisystem organ failure in the postoperative period. Two patients died of myocardial infarction or arrhythmia. Only four amputations were required in this series, achieving a limb salvage rate of 97.7%. These occurred after acute thrombotic episodes. One patient underwent immediate amputation after aortic occlusion due to extensive muscle necrosis. Three patients had failure of revascularization. This limb salvage rate is higher than reported for acute ischemia in the literature, ranging from 53 to 95%[6,7,21,22]. No amputations were required in this group of 37 trauma patients, which included 11 with popliteal fossa injuries. It should be stressed that functional limb salvage was achieved. Some 89% had improvement of their preoperative neuromuscular dysfunction; the majority have complete resolution.

Further comment on those patients that required fasciotomy is warranted. Ten of these patients had a traumatic injury, eight of which were traumas with a crush component. This comprises a 27% fasciotomy rate for all trauma patients in this series. A similar figure was reported by Patman[23]. This supports the premise that mechanical crush forces cause damage beyond free radical injury. Therefore, it is unlikely that free radical

scavengers would be as protective in crush injuries.

Sixteen of those patients undergoing fasciotomy had acute arterial thrombosis, which is 16.3% of all thrombosis patients. The majority were noted to have lower-extremity ischemia after an initial major operative procedure with accompanying hemodynamic and metabolic instability (ruptured aortic aneurysm, thoracic aortic dissection, mesenteric ischemia, radical nephrectomy and abdominal perineal resection). Two patients had thrombosis of popliteal aneurysms. Two of the 51 embolic injuries (3.9%) underwent fasciotomy. Interestingly, the duration of ischemia for those patients requiring fasciotomy was not significantly different from those without fasciotomy.

In conclusion this retrospective review describes the clinical course of patients presenting with limb-threatening ischemia treated with hemodynamic stabilization, prompt revascularization, fasciotomy for those with signs of compartment syndrome, and hypertonic mannitol infusion through the reperfusion period. Mannitol is an osmotic diuretic with free radical scavenging properties. Results with this protocol are encouraging, showing significant recovery of neuromuscular function, and low rates of amputation and mortality. The need for fasciotomy was also low in localized thromboembolic events. This study supports the use of hypertonic mannitol in the management of acute arterial ischemia.

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Glycyrrhizin ameliorates metabolic syndrome-induced liver damage in experimental rat model

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Abstract Glycyrrhizin, a major constituent of licorice (*Glycyrrhiza glabra*) root, has been reported to ameliorate insulin resistance, hyperglycemia, dyslipidemia, and obesity in rats with metabolic syndrome. Liver dysfunction is associated with this syndrome. The objective of this study is to investigate the effect of glycyrrhizin treatment on metabolic syndrome-induced liver damage. After induction of metabolic syndrome in rats by high fructose (60 %) diet for 6 weeks, the rats were treated with glycyrrhizin (50 mg/kg body weight, single intra-peritoneal injection). After 2 weeks of treatment, rats were sacrificed to collect blood samples and liver tissues. Compared to normal, elevated activities of serum alanine transaminase, alkaline phosphatase and aspartate transaminase, increased levels of liver advanced glycation end products, reactive oxygen species, lipid peroxidation, protein carbonyl, protein kinase Cα, NADPH oxidase-2, and decreased glutathione cycle components established liver damage and oxidative stress in fructose-fed rats. Activation of nuclear factor κB, mitogen-activated protein kinase pathways as well as signals from mitochondria were found to be involved in liver cell apoptosis. Increased levels of cyclooxygenase-2, tumor necrosis factor, and interleukin-12 proteins suggested hepatic inflammation. Metabolic syndrome caused hepatic DNA damage and poly-ADP ribose polymerase cleavage. Fluorescence-activated cell sorting using annexin V/propidium iodide staining confirmed the apoptotic hepatic cell death. Histology of liver tissue also supported the experimental findings. Treatment with glycyrrhizin reduced oxidative stress, hepatic inflammation, and apoptotic cell death in fructose-fed rats. The results suggest that glycyrrhizin possesses therapeutic potential against hepatocellular damage in metabolic syndrome.

Keywords Metabolic syndrome · Glycyrrhizin · Liver damage · Oxidative stress · Apoptosis · Inflammation

Introduction

Metabolic syndrome or syndrome-X is characterized by insulin resistance, hyperglycemia, dyslipidemia, hypertension, and obesity. The syndrome increases the risk of type 2 diabetes and cardiovascular diseases [1]. Liver damage and non-alcoholic fatty liver disease are common in patients with metabolic syndrome [2]. Increased fructose consumption through soft drinks, juice beverages, and prepackaged food increases the risk of this syndrome [3]. Fructose is a highly lipogenic nutrient increasing the triglyceride level in body. Fructose-fed animals are widely used as the experimental model of metabolic syndrome [4]. Fructose is primarily metabolized in liver through an insulin-independent and uncontrolled pathway. Due to its strategic position, between the intestinal bed and the systemic circulation, the liver is regarded as the buffer organ for the regulation of metabolic fluxes [5]. In pathological

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conditions, the biomarkers of oxidative stress are elevated rapidly in the liver. For example, in hyperglycemic condition, the liver is subjected to reactive oxygen species (ROS)-mediated injury [6]. In fructose-induced metabolic syndrome, the burden of high levels of fructose metabolism generates oxidative stress and inflammation in liver [7].

In recent years, scientists are in search of easily available, inexpensive therapeutics for the effective treatment of diseases. For these, phytochemicals from different herbal sources are being increasingly used [8, 9]. Glycyrrhizin, a triterpene saponin, is the major water soluble component of licorice (*Glycyrrhiza glabra*) root extract. Glycyrrhizin has two major active metabolites, 18β-glycyrrhetinic acid and 3-monoglucuronyl-glycyrrhetinic acid [10, 11]. According to Glavac and Kreft [12], after oral ingestion of 600 mg of glycyrrhizin in human subjects, the metabolites appear in urine ranging from 1.5 to 14 h and can be detected in the urine even after 4 days. Protection of liver cells from experimentally induced metabolic disorders and hepatocellular injury by glycyrrhizin has been reported in different publications [13–19]. Antidiabetic effect of glycyrrhizin and its metabolite 18β-glycyrrhetinic acid has been reported in streptozotocin-induced type 1 diabetes mellitus in rat model [20, 21]. Eu et al. [22] and Chandramouli et al. [23] have shown that glycyrrhizin exhibits ameliorative effect in high-fat diet and high sucrose diet-induced obese rats, respectively. In the previous study, we have reported that glycyrrhizin treatment increases peroxisome proliferator-activated receptor γ (PPARγ) and glucose transporter 4 (GLUT 4) protein levels in fructose-fed rats and improves lipid profile, insulin sensitivity, and glucose homeostasis in metabolic syndrome [24]. As metabolic syndrome is associated with inflammation and hepatocellular injury, we have undertaken a study to investigate the effect of glycyrrhizin treatment against metabolic syndrome-induced liver damage in fructose-fed rats. For this, we have examined the status of the enzymatic indices related to hepatic damage, oxidative stress markers, glutathione cycle components, histological changes in liver and expression of nuclear factor κB (NF-κB), mitogen-activated protein kinases (MAPKs), and other inflammatory proteins as well as mitochondria-dependent cell death pathway.

Materials and methods

Chemicals

Collagenase type IV, 1-chloro-2, 4-dinitrobenzene (CDNB), 2, 4-dinitrophenylhydrazine (DNPH), dithiothreitol (DTT), glycyrrhizin, reduced glutathione (GSH), 2′, 7′-dichlorodihydrofluorescein diacetate acetyl ester (H_2DCF-DA), 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethyl benzimidazolyl carbocyanine iodide dye (JC-1), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), thiobarbituric acid (TBA), annexin V- fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit, genomic DNA isolation kit, protease inhibitor cocktail, and Oil Red O (ORO) dye were purchased from Sigma-Aldrich Company (St Louis, USA). Glucose, cholesterol, and triglyceride kits were purchased from Span Diagnostics Ltd. (Mumbai, India). Rat insulin ELISA kit was purchased from DRG Diagnostic (Frauenbergstr, Germany). Primary and secondary antibodies and Western blot Luminol reagent were purchased from Santa Cruz Biotechnology (Texas, USA). Other chemicals used in this study were of analytical grade and obtained from Sisco Research Laboratory Pvt. Ltd. (Mumbai, India).

Animals

Animals were maintained in accordance with the regulations specified and monitored by the institutional animal ethics committee of the University of Calcutta, Kolkata, India (Registration No. 935/c/06/CPCSEA, 30.06.2009). Male Wistar rats, body weight ranging from 100 to 110 g, were obtained from the university approved animal supplier. The rats were housed in an animal room under controlled conditions on a 12 h light/dark cycle at 26–28 °C, 60–80 % relative humidity.

Toxicity study of glycyrrhizin

To find the toxic effect of glycyrrhizin (dissolved in 50 mM phosphate buffer, pH 7.4) on normal rats, several markers were studied at different time intervals. Male Wistar rats were divided into four groups having five rats in each group as follows:

NC group: normal control rats treated with only buffer,
NT_{50} group: rats treated with glycyrrhizin (50 mg/kg body weight),
NT_{100} group: rats treated with glycyrrhizin (100 mg/kg body weight),
NT_{200} group: rats treated with glycyrrhizin (200 mg/kg body weight).

In each case, single dose of glycyrrhizin solution was administered intraperitoneally (i.p). All the rats were supplied with normal diet and water ad libitum.

Blood samples (fasting) were collected from tail vein. Red blood cells were isolated from heparinized blood for scanning electron microscopy (SEM). Serum was separated from the clotted blood by centrifugation at $3500 \times g$ for 10 min at 4 °C to estimate biochemical parameters. Fasting blood glucose, cholesterol, and triglycerides were measured in the serum using commercial assay kits. Different

biochemical parameters (indices of hepatic damage) namely serum alanine transaminase (ALT), alkaline phosphatase (ALP), and aspartate transaminase (AST) activities were assayed according to the methods of Reitman and Frankel [25], Thambidorai and Bachawat [26], and Amador and Wacker [27], respectively. For total GSH in liver, the tissue was homogenized in 20 mM Tris–HCl, pH 7.4 containing 250 mM sucrose and centrifuged at 20,000×*g* for 30 min at 4 °C to collect the supernatant. GSH content was measured in the supernatant following the method of Ellman [28]. Protein contents in different samples were measured following the method of Lowry et al. [29]. Histological studies of liver and kidney tissues were done using hematoxylin and eosin staining.

The effect of glycyrrhizin on fructose-induced metabolic syndrome in rats

Experimental design

Rats were divided into two groups and were maintained as follows:

Normal control group (NC)—the animals ($n = 6$ rats) received control diet (containing 60 % corn starch) and water.

High fructose diet-fed group (HFD)—the animals ($n = 12$ rats) received high fructose diet (containing 60 % fructose) and water.

Both normal and high fructose diets contained 20 % casein, 0.7 % methionine, 5 % groundnut oil, 10.6 % wheat bran, 3.5 % salt, and 0.2 % vitamins. The diets were prepared freshly following the published report of Nandhini et al. [30]. During the experimental period, same amount of diet (10 g per day) was supplied to each rat. As reported in a published study [24], after 6 weeks of high fructose diet feeding in rats (HFD group), induction of metabolic syndrome was evident from high levels of fasting blood glucose, insulin, and triglyceride and low level of high-density lipoprotein (HDL) cholesterol.

HFD group of rats was then treated with glycyrrhizin solution or buffer as follows:

HFD rats treated with glycyrrhizin (HFDT)—HFD rats ($n = 6$ rats) were treated with single intra-peritoneal (i.p) injection of glycyrrhizin solution in 50 mM potassium phosphate buffer, pH 7.4, at a dose of 50 mg/kg body weight.

HFD rats (untreated group)—HFD rats ($n = 6$ rats) were treated (i.p) with only buffer.

Both HFDT and HFD groups were continued feeding with the high fructose diet for 2 weeks after administration of glycyrrhizin solution/buffer. Blood glucose, insulin, and serum lipids were checked once a week during this period. The rats were then sacrificed by cervical dislocation and liver tissues were collected for subsequent experiments.

Measurement of blood glucose, insulin, serum lipids, and enzymes

Fasting blood glucose, insulin, triglyceride, and HDL cholesterol in serum samples were measured using commercial assay kits according to the manufacturer's protocols. Serum ALP, ALT, and AST enzyme activities were estimated according to the methods, as stated before.

Estimation of lipid peroxidation product and protein carbonyl content in liver

For lipid peroxidation product, liver tissue was homogenized in 0.1 M Tris–HCl buffer, pH 7.4. TBA reactive substance present in this homogenate was measured following the method of Buege and Aust [31]. For protein carbonyl content, liver tissue was homogenized in 10 mM HEPES buffer, pH 7.4 containing 137 mM NaCl, 4.6 mM KCl, 1.0 mM KH_2PO_4, and 0.6 mM $MgSO_4$, and was centrifuged at 20,000×*g* for 30 min at 4 °C. Carbonyl content in the supernatant was estimated using DNPH according to Levine et al. [32].

Determination of advanced glycation end product (AGE) content in liver

Minced liver tissue was delipidated with chloroform and methanol (2:1, v/v) and homogenized in 0.1 N NaOH. After stirring for 3 h, the homogenate was centrifuged at 8000×*g* for 15 min at 4 °C. The amount of alkali-soluble AGEs in the supernatant was estimated by measuring the AGE fluorescence at 440 nm (excitation wavelength 370 nm) [33].

Estimation of glutathione cycle components in liver

For total GSH, glutathione reductase (GR), and glutathione peroxidase (GPx), the tissue was homogenized in 20 mM Tris–HCl, pH 7.4 containing 250 mM sucrose and centrifuged at 20,000×*g* for 30 min at 4 °C to collect supernatant. For glutathione *S*-transferase (GST), the liver extract was prepared similarly, except 1 mM DTT was included in homogenizing buffer. GSH content and activities of GR, GPx, and GST were measured following the methods of Ellman [28], Carlberg and Mannervik [34], Rotruck et al. [35], and Habig et al. [36], respectively.

Measurement of protein levels in liver by Western blotting

Total cell lysate, nuclear extract, cytosol, and mitochondrial fraction were prepared from liver tissue following the method of Kim et al. [37]. Western blotting experiments

for different molecular markers except for cytochrome c were done with total cell lysate. Cytochrome c was measured in mitochondrial and cytosolic fraction. NF-κB p65 level was also measured in nuclear extract. Proteins (60 μg) were separated by SDS-PAGE and immunoblotting was performed with primary antibodies: mouse monoclonal antibody for β-actin, lamin B1, NF-κB p65, protein kinase Cα (PKCα), poly-ADP ribose polymerase (PARP), interleukin-4 (IL-4), B cell lymphoma 2 (Bcl-2), and Bcl-2 associated X protein (Bax) (dilution 1:500 in each case); goat polyclonal antibody for inducible nitric oxide synthase (iNOS), inhibitor of NF-κB (IκBα), interleukin-12 (IL-12), cyclooxygenase-2 (COX-2), NADPH oxidase-2 (NOX-2 or $gp91^{phox}$), receptor of AGE (RAGE), tumor necrosis factor α (TNFα), apoptotic protease activating factor-1 (Apaf-1), caspase-3, complex IV and cytochrome c (dilution 1:500 in each case); rabbit polyclonal antibody for phosphorylated NF-κB p65 (p-NF-κB p65), MAPK p38, phosphorylated MAPK p38 (p-MAPK p38), extracellular signal-regulated protein kinase (ERK) 1/2, and phosphorylated ERK1/2 (p-ERK1/2) (dilution 1:500 in each case). Secondary antibodies donkey anti-mouse IgG-HRP or donkey anti-goat IgG-HRP or donkey anti-rabbit IgG-HRP with dilution 1:5000 were used as appropriate. Band intensities were quantified by densitometry using Bio-Rad GelDoc apparatus with ImageJ software.

Estimation of MTT reduction and ROS generation in liver mitochondria

For mitochondria isolation [38], liver tissue was homogenized in a buffer containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mg/mL BSA, and pH 7.4. The homogenate was centrifuged at $2000\times g$ for 10 min at 4 °C to remove nuclei and cell debris. The supernatant was centrifuged at $12{,}000\times g$ for 10 min at 4 °C. The mitochondrial pellet was washed twice with wash buffer (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, pH 7.4) and resuspended in isotonic buffer containing 80 mM KCl, 50 mM sucrose, 5 mM NaCl, 1 mM EGTA and 1 mM $MgCl_2$ in 10 mM phosphate buffer, pH 7.4. Mitochondrial MTT reduction was estimated following the method of Cohen et al. [39]. ROS generation in mitochondria was measured spectrofluorimetrically (excitation wavelength 507 nm, emission wavelength 530 nm) using H_2DCF-DA [40].

Assessment of genomic DNA damage

DNA damage was assayed by electrophoresis of genomic DNA samples, isolated from rat liver using commercial kit, on 0.8 % agarose gel and staining by ethidium bromide [41]. Gel image was captured using Bio-Rad GelDoc apparatus.

Fluorescence-activated cell sorting (FACS) analysis of liver cells for studying apoptosis and mitochondrial membrane potential

For isolation of liver cells [42], the tissue was irrigated in 10 mM HEPES buffer, pH 7.4 containing 3 mM KCl, 130 mM NaCl, 1 mM NaH_2PO_4, and 10 mM glucose and then incubated in 5 mM $CaCl_2$, containing 0.05 % collagenase type IV for 1 h at 37 °C. After passing through 80 μm Dacron mesh, the cells were centrifuged at $500\times g$ for 10 min at 4 °C and the pellet was suspended in PBS, pH 7.4. For apoptosis detection, the cells were stained with annexin V-FITC and PI using commercial kit according to the manufacturer's protocol. Stained cells were analyzed in a FACS analyzer (BD Biosciences, USA) equipped with FACSDiva Version 6.1.3 software. Mitochondrial transmembrane potential was assessed by labeling liver cells with cationic JC-1 dye and measuring red and green fluorescence of the dye in the FACS analyzer [43].

Histology of liver tissue

For hematoxylin and eosin staining, liver tissue was fixed in 5 % paraformaldehyde solution in 50 mM phosphate buffer, pH 7.4, and processed for paraffin sectioning. Sections of about 5 μm thickness were cut using microtome and stained with hematoxylin and eosin. For lipid staining, frozen liver tissues were cut into sections (5 μm) using a cryostat and stained with 0.3 % ORO in isopropanol, followed by counterstaining with hematoxylin [44]. The pathophysiological changes are visualized under Motic BA 400 light microscope.

Statistical analysis

Results were expressed as mean ± standard deviation (SD) obtained for n number of rats. Statistical significance was determined by AcaStat 6 software using unpaired Student's t test, and $P < 0.05$ was considered to be significant.

Results

Results of toxicity study

Compared to normal rats, no considerable hair loss or differences in hair color, food intake, water intake, and behavior were observed for next 3 weeks of the administration of glycyrrhizin (50, 100, and 200 mg/kg body weight) in normal rats. No change was found in the

morphology of red blood cells isolated from different groups of rat (Supplementary Fig. 1). There were no significant differences between the groups regarding body weight gain, fasting blood glucose, cholesterol, and triglyceride levels (Supplementary Fig. 2). No significant changes were found in the indices of liver damage between normal and treated groups of rat (Supplementary Fig. 3). There were no considerable abnormalities in the hepatic lobular morphology in different groups of rat (Supplementary Fig. 4). Glomerular structure was also normal with regular capsular spaces in kidney tissue of all the groups (Supplementary Fig. 4). These results indicate that glycyrrhizin has no toxic effect on the rats up to the dose of 200 mg/kg body weight. According to Isbrucker and Burdock [45], lethal dose 50 (LD_{50}) of glycyrrhizin by i.p administration is 840 mg/kg body weight in rats. The existing reports including a previous study from our laboratory also suggest that i.p administration of glycyrrhizin in rats at a dose of 100 mg/kg body weight causes no toxicity [20, 46, 47]. In the present study, considerably lower dose of glycyrrhizin (50 mg/kg body weight) was administered in rats having fructose-induced metabolic syndrome.

Effect of glycyrrhizin on primary complications and serum enzymes related to liver damage in metabolic syndrome

During the experimental period (8 weeks), weight gain by high fructose diet-fed rats was more than two folds of the weight gain by control diet-fed animals (Table 1). Similar weight gains (two to four fold) by feeding high fructose diet have also been reported by other groups using restricted supply (16 g per day per rat) [37] or ad libitum [30, 48–50]. Excess weight gain and increased levels of fasting blood glucose, insulin and triglyceride and decreased levels of HDL cholesterol suggested induction of metabolic syndrome in HFD group (Table 1). Glycyrrhizin treatment reduced the weight gain and levels of blood glucose, insulin and triglyceride in HFDT group of rats. The treatment improved HDL cholesterol level in HFDT rats. Improvement of PPARγ and GLUT4 protein levels in metabolic syndrome-induced rats by glycyrrhizin [24] possibly associated with the reduction of primary complications. ALT, ALP, and AST are liver-specific enzymes. Damage of liver cells releases these enzymes in blood. A significant increase in activities of these enzymes in serum of HFD rats compared to normal rats indicated hepatocellular damage in metabolic syndrome (Table 2). Treatment with glycyrrhizin lowered the enzymes activities in HFDT group suggesting improvement of liver condition.

Effect of glycyrrhizin on lipid peroxidation, protein carbonyl content, ROS, and free radical-generating protein levels in liver

Lipid peroxidation and protein carbonyl content are widely used as markers of cell membrane damage and oxidative modifications of proteins, respectively. In this study, lipid peroxidation was measured by estimating its end product as TBA reactive substance. Metabolic syndrome increased the levels of TBA reactive substance and protein carbonylation in HFD group of rats (Fig. 1a, b). Increased ROS is responsible for complications in different pathophysiological conditions. Mitochondrial electron transport chain is the major source of intracellular ROS [51]. ROS generation increased in mitochondria isolated from liver tissue of fructose-fed rats compared to normal (Fig. 1c). Glycyrrhizin treatment effectively decreased these oxidative stress markers in HFDT group. PKC and NOX family proteins stimulate ROS production in various pathological conditions [52, 53]. We checked the levels of two important members of PKC and NOX family namely PKCα and NOX-2 (also known as $gp91^{phox}$), respectively, by immunoblotting. The hepatic levels of PKCα and NOX-2 proteins were found to increase in HFD rats, compared to those in NC rats (Fig. 1d). Treatment with glycyrrhizin reduced the protein levels in HFDT group. These findings suggested antioxidative role of the herbal agent in metabolic syndrome.

Effect of glycyrrhizin on hepatic AGE and RAGE level

Increased protein glycation in hyperglycemic condition increases AGE formation. AGEs are important source of

Table 1 Metabolic syndrome parameters

Groups	Weight gain (g)	Blood glucose (mg/dL)	Insulin (μg/L)	Triglyceride (mg/dL)	HDL cholesterol (mg/dL)
NC	23.3 ± 3.0	84.9 ± 6.0	0.96 ± 0.08	68.2 ± 8.8	37.9 ± 5.4
HFD	53.0 ± 9.2*	168.5 ± 8.7*	1.89 ± 0.60*	167.6 ± 12.4*	15.3 ± 3.8*
HFDT	37.5 ± 6.4$^{\#}$	121.0 ± 5.9$^{\#}$	1.10 ± 0.16$^{\#\#}$	118.3 ± 8.6$^{\#}$	37.3 ± 3.3$^{\#}$

The parameters were measured after 2 weeks of glycyrrhizin treatment

The results are mean ± SD, $n = 5$ for insulin and $n = 6$ for other parameters

* $P < 0.01$ versus NC; $^{\#}$ $P < 0.01$ versus HFD; $^{\#\#}$ $P < 0.05$ versus HFD

Table 2 Serum enzymes related to hepatocellular damage

Groups	ALT activity (μmole of pyruvate liberated/min/mg protein)	ALP activity (μmole of para-nitro phenol formed/min/mg protein)	AST activity (μmole of NADH oxidized/min/mg protein)
NC	3.68 ± 0.94	55.8 ± 11.4	0.784 ± 0.165
HFD	10.19 ± 2.21*	97.5 ± 11.6*	1.322 ± 0.097*
HFDT	5.51 ± 0.90#	71.8 ± 6.6#	0.957 ± 0.122#

The enzymes were assayed after 2 weeks of glycyrrhizin treatment

The results are mean ± SD, $n = 5$

* $P < 0.01$ versus NC; # $P < 0.01$ versus HFD

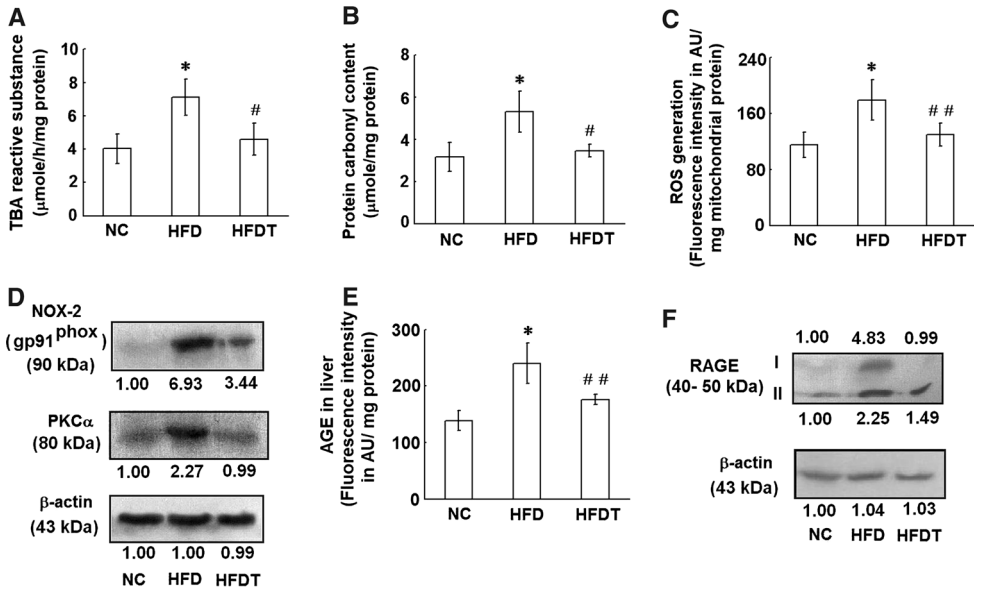

Fig. 1 Oxidative stress markers in liver. **a** TBA reactive substance, **b** protein carbonyl content, and **c** mitochondrial ROS generation. For TBA reactive substance and protein carbonyl content, liver homogenates ($n = 6$) and for ROS, liver mitochondrial preparations ($n = 5$) were used. The results are mean ± SD. (D) representative Western blots of PKCα and NOX-2 ($gp91^{phox}$) in liver cell lysate. Fold change in protein levels compared to normal (NC) is mentioned in the bottom of the blots. **e** Liver AGE content. AGE levels were measured spectrofluorimetrically in arbitrary units (AU) (excitation wavelength 370 nm and emission wavelength 440 nm). The results are mean ± SD, $n = 4$. *$P < 0.01$ versus NC; #$P < 0.01$ versus HFD; ##$P < 0.05$ versus HFD. **f** Representative Western blot of RAGE in liver cell lysate. Two bands (I and II) were appeared for RAGE within molecular weight range 40 kDa to 50 kDa. Fold change in protein levels compared to normal (NC) are mentioned in the above (for band I) and bottom (for band II) of the blot

free radicals [54]. AGE content in liver tissue of HFD rats was significantly higher than in NC rats (Fig. 1e), indicating higher extent of protein glycation and free radical generation in metabolic syndrome. RAGE, the transmembrane receptor of AGEs, mediates the effect of AGEs [55]. We assessed the hepatic level of RAGE by Western blotting. RAGE expression increased in HFD group compared to normal as indicated by increased protein level in band I and II (Fig. 1f). AGE content and RAGE level were decreased by glycyrrhizin treatment in HFDT group.

Effect of glycyrrhizin on glutathione cycle in liver

Glutathione cycle maintains the intracellular redox balance by scavenging free radicals [56]. High amount of GSH is consumed to accomplish this task. Whenever the GSH level decreases below the threshold level, the concentration of reactive radicals becomes elevated causing oxidative stress. The non-enzymatic antioxidant total GSH and enzymatic antioxidants GPx, GR, and GST activities were found to be significantly lower in fructose-fed rats than in normal rats (Table 3). All the components of glutathione cycle increased significantly by glycyrrhizin treatment in HFDT group suggesting reduction of free radical level by the herbal agent.

Effect of glycyrrhizin treatment on hepatic NF-κB

NF-κB is an important transcription factor regulating oxidative stress-responsive cell signaling [57]. It can be activated by a variety of stimulating factors relevant to pathophysiology. Transcriptional activity of NF-κB can be

Table 3 Glutathione cycle components in liver

Groups	GSH content (μg/mg protein)	GR activity (μmole of NADPH oxidized/min/mg protein)	GPx activity (μmole of GSH oxidized/min/mg protein)	GST activity (mmole of CDNB-GSH conjugate formed/min/mg protein)
NC	19.5 ± 2.0	113.2 ± 7.7	261.7 ± 34.0	17.6 ± 1.7
HFD	8.4 ± 3.0*	63.1 ± 7.7*	78.6 ± 19.7*	6.9 ± 1.2*
HFDT	17.5 ± 2.1#	107.6 ± 8.0#	214.7 ± 23.2#	12.1 ± 1.1#

The results are mean ± SD, $n = 5$

* $P < 0.01$ versus NC; # $P < 0.01$ versus HFD

regulated by phosphorylation and degradation of its inhibitor IκBα as well as phosphorylation of its p65 subunit. In this study, we used Western immunoblotting to determine the involvement of NF-κB signaling in metabolic syndrome-associated hepatic damage and its prevention by glycyrrhizin. We observed that in cell lysate, IκBα level decreased in HFD group with elevated expression of p-NF-κB p65 (Fig. 2a). Total NF-κB p65 level was unchanged in cell lysate among these groups. NF-κB p65 level was higher in nuclear extract isolated from fructose-fed rat liver compared to normal suggesting increased nuclear translocation of NF-κB from cytoplasm. Glycyrrhizin treatment was effective in preventing degradation of IκBα and therefore inhibiting nuclear translocation and phosphorylation of NF-κB p65 in HFDT group.

Effect of glycyrrhizin treatment on MAPKs in liver

MAPKs are the critical upstream signaling proteins regulating various cellular networks including apoptosis and inflammation [58]. To assess the effect of metabolic syndrome on the activation of the MAPKs and the effect of herbal treatment, the total cell lysate from different groups of rat was analyzed for both total and phosphorylated forms of two important MAPKs namely MAPK p38 and ERK1/2 by Western blotting. In HFD group, p-MAPK p38 and p-ERK1/2 levels were increased leaving their total levels almost unchanged compared to normal (Fig. 2b). Treatment with glycyrrhizin was found to inhibit the phosphorylation of MAPK p38 and ERK1/2 in HFDT group.

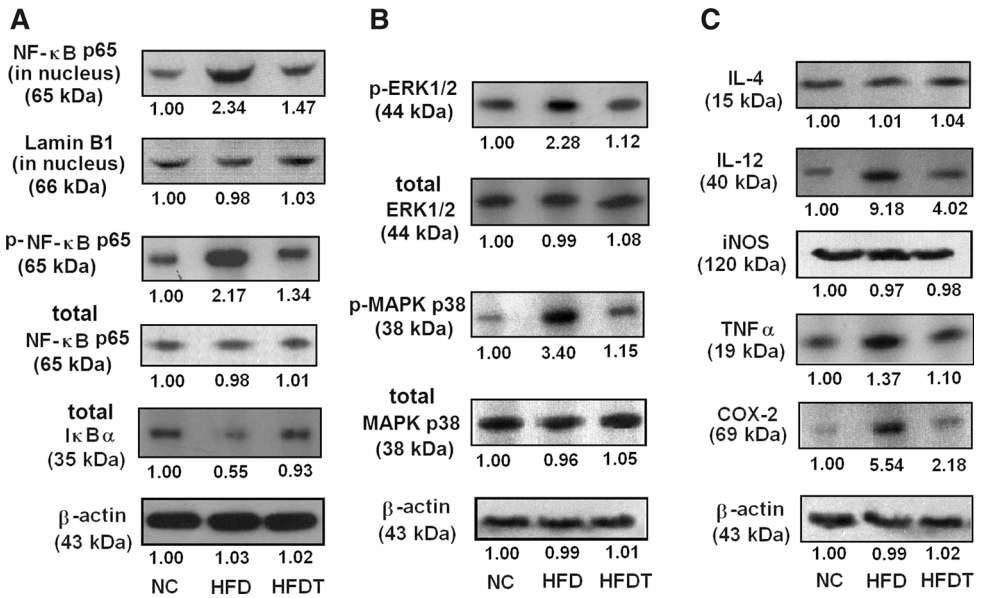

Fig. 2 Representative Western blots of proteins involved in oxidative stress-responsive cell signaling and inflammation in liver. **a** Immunoblots showing total IκBα, total NF-κB p65, and phosphorylated NF-κB p65 (p-NF-κB p65) in liver cell lysate. NF-κB p65 was also measured using nuclear fraction. **b** Immunoblots of two important members and their phosphorylated forms of MAPK family. Total MAPK p38, phosphorylated MAPK p38 (p-MAPK p38), total ERK1/2, and phosphorylated ERK1/2 (p-ERK1/2) were measured using liver cell lysate. **c** Immunoblots of several proteins involved in inflammation. COX-2, TNFα, iNOS, IL-12, and IL-4 were measured using liver cell lysate. Fold change in protein levels compared to normal (NC) is mentioned in the bottom of the blots

Effect of glycyrrhizin on hepatic levels of various inflammatory proteins

NF-κB and MAPK signaling result in inflammation. Hepatic levels of different inflammatory proteins like COX-2, TNFα, iNOS, IL-12, and IL-4 were checked by immunoblotting. Figure 2c shows that the levels of COX-2, TNFα, and IL-12 increased in fructose-fed rat than the normal indicating liver inflammation. Glycyrrhizin treatment effectively reduced these protein levels in HFDT group compared to HFD group. However, there were no considerable differences between the groups regarding the hepatic iNOS and IL-4 protein levels.

Effect of glycyrrhizin against mitochondria-dependent apoptotic pathway

Bcl-2 family proteins are upstream regulators of the mitochondria-mediated apoptosis pathway [59]. Loss of mitochondrial membrane potential, dehydrogenase activity and subsequent release of cytochrome c into cytosol are the biomarkers of cell death via mitochondria-dependent pathway. Release of cytochrome c causes the activation of caspases via the formation of apoptosome with Apaf-1. To check an antiapoptotic role of glycyrrhizin, we determined mitochondrial membrane potential, dehydrogenase activity and studied the expression of Bcl-2 family proteins, cytochrome c, Apaf-1 and caspase-3 by Western blotting. It was observed that metabolic syndrome upregulated proapoptotic Bax and downregulated antiapoptotic Bcl-2 protein in liver of fructose-fed rats (Fig. 3a). Glycyrrhizin treatment could repress the syndrome-induced alterations in Bcl-2 family proteins. Elevated levels of Apaf-1, caspase-3 (Fig. 3a), and cytochrome c (in cytosol) (Fig. 3b1) were observed in liver tissue isolated from HFD group, and the levels appear to decrease by glycyrrhizin treatment. For caspase-3, only the intact form (32–35 kDa), not the cleaved form (activated, 17–19 kDa), was found in our experiment.

The tetrazolium dye MTT accepts electron from mitochondrial electron transport chain. Therefore, mitochondrial MTT reduction ability gives an indirect assessment of its electron transport chain activity and associated metabolic function [39]. The MTT reduction ability of liver mitochondria in HFD rats was found to be decreased in comparison with that in NC rats (Fig. 3b2), indicating loss of mitochondrial function. Figure 3b3 shows the changes in mitochondrial transmembrane potential. The negative charge established by mitochondrial membrane potential allows the lipophilic cationic JC-1 dye to enter into mitochondrial matrix, where it accumulates to form oligomer that has red fluorescence [60]. In apoptotic cells, the mitochondrial membrane potential collapses and JC-1 cannot accumulate within the mitochondria. In these cells, JC-1 remains in the cytoplasm as a monomer having green fluorescence. Mitochondrial transmembrane potential appeared to be lower in HFD rats than in normal rats, as indicated by reduced red fluorescence of the JC-1 as measured by FACS analyzer. These findings suggested mitochondria-dependent apoptosis of liver cells in this pathological condition. Treatment with glycyrrhizin effectively inhibited metabolic syndrome-induced mitochondria-dependent apoptotic cell death.

Effect of glycyrrhizin treatment against hepatic DNA damage and PARP expression

DNA fragmentation and cleavage of PARP are two hallmarks of apoptosis [61]. Metabolic syndrome caused DNA damage as indicated by trailing of DNA samples from HFD group in agarose gel electrophoresis in comparison with intact DNA band in samples from normal rats (Fig. 3c). Figure 3d shows that cleaved PARP (84 kDa) level increased in fructose-fed rats compared to normal. However, the expression of cleaved PARP in HFD group was not quite strong to make a considerable difference in intact PARP (120 kDa) levels between the different groups. Glycyrrhizin treatment reduced DNA fragmentation and PARP cleavage in HFDT group.

Results of FACS analysis

FACS analysis of liver cells using annexin V and PI double staining supported apoptotic cell death in metabolic syndrome. Annexin V specifically binds with the externalized phosphatidylserine residues in cell membrane of apoptotic cells [62]. FACS data revealed that there was increase of annexin V-stained cells (30.4 %) in liver of HFD rats in comparison with only 3.5 % such cells in normal rats (Fig. 3e), indicating increased liver cell apoptosis in induced metabolic syndrome. Late apoptotic cells, stained with both annexin V and PI, appeared to be much higher in liver of HFD rats (20.2 %) than in normal rats (0.4 %). Necrotic cells, stained with only PI, were also found in HFD rat liver (1.1 %). Treatment with glycyrrhizin showed fewer early apoptotic (16.5 %) and late apoptotic (6.4 %) cells as well as necrotic cells (0.6 %) suggesting its effectiveness in lowering liver cell apoptosis in the syndrome.

Histological assessment

Histology of liver sections is presented in Fig. 4. Hematoxylin and eosin staining showed that hepatic architecture was clear and intact without abnormalities in the liver tissue of normal rats. Granuloma, an indicator of

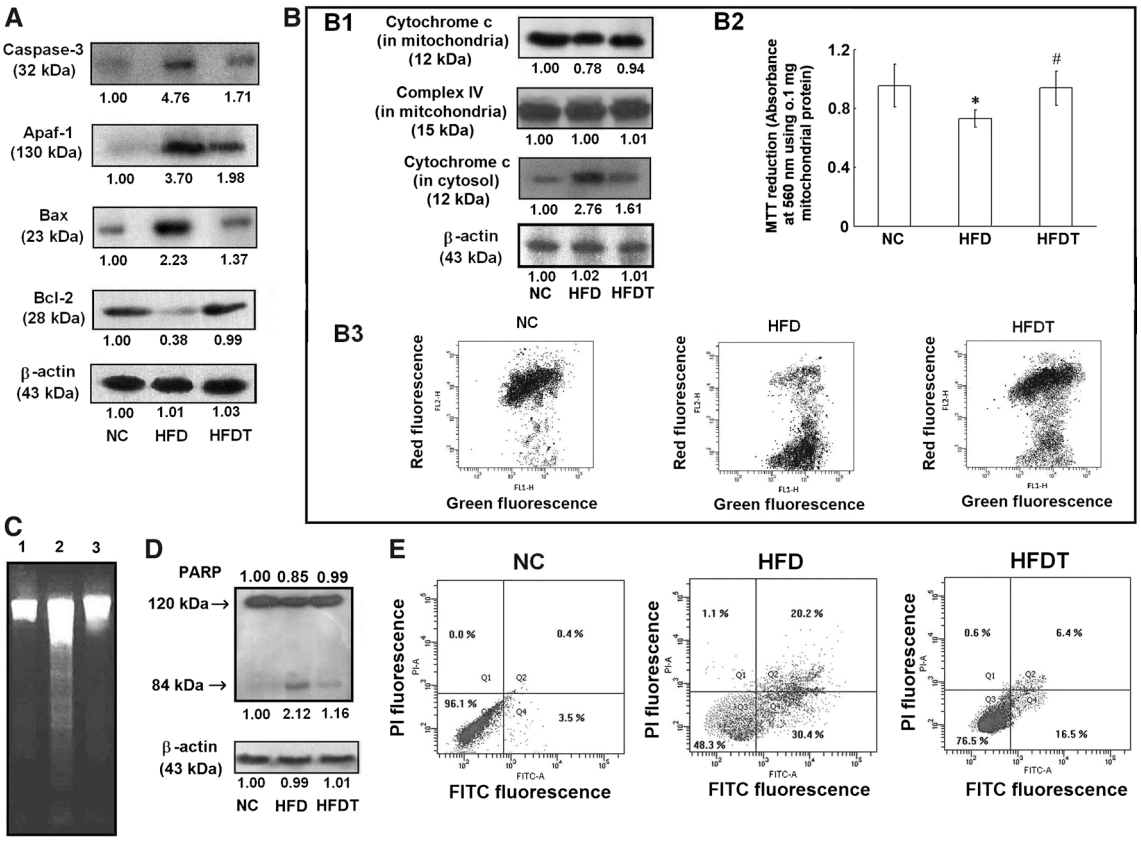

Fig. 3 Apoptotic changes in liver. **a** Representative Western blots of proteins involved in the apoptosis. Bcl-2, Bax, Apaf-1, and caspase-3 were measured using liver cell lysate. Fold change in protein levels compared to normal (NC) is mentioned in the bottom of the blots. **b** Mitochondria-dependent apoptosis signaling (*b1*) representative Western blot of cytochrome c in liver mitochondrial and cytosolic fractions (*b2*) Mitochondrial dehydrogenase activity as indicated by MTT reduction assay. The results are mean ± SD, $n = 5$. $^{*}P < 0.05$ versus NC; $^{\#}P < 0.05$ versus HFD (*b3*) Mitochondrial transmembrane potential as indicated by JC-1 fluorescence using FACS. Results are shown as a representative dot plot of red fluorescence (FL2-H) versus green fluorescence (FL1-H). **c** A representative experiment of agarose gel (0.8 %, w/v) electrophoresis followed by ethidium bromide staining of genomic DNA samples isolated from liver tissues. *Lane 1* DNA from NC rat, *Lane 2* DNA from HFD rat, *Lane 3* DNA from HFDT rat. **d** Representative Western blot of PARP expression in liver showing 120 kDa intact PARP and 84 kDa cleaved PARP level in cell lysate. Fold change in protein level of cleaved PARP (84 kDa) and intact PARP (120 kDa) compared to normal (NC) are mentioned in the bottom and above of the blot, respectively. **e** Flow cytometric analysis of cell distribution using annexin V-FITC binding and PI uptake. Representative dot plot of PI fluorescence (PI-A) versus FITC fluorescence (FITC-A) showing percentage distribution of viable ($FITC^{-}/PI^{-}$), early apoptotic ($FITC^{+}/PI^{-}$), late apoptotic ($FITC^{+}/PI^{+}$), and necrotic cells ($FITC^{-}/PI^{+}$) in liver tissue

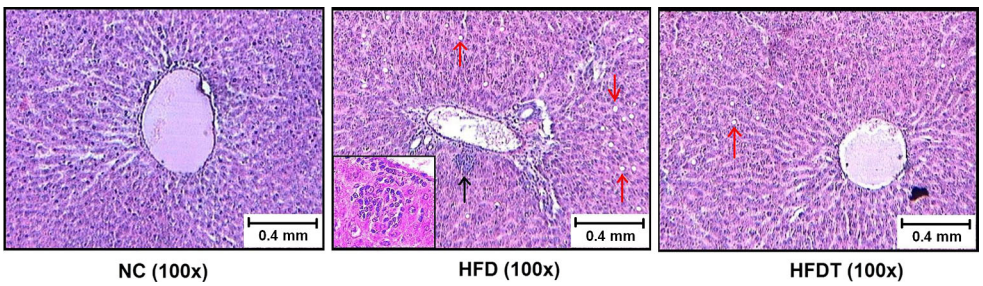

Fig. 4 Histological assessment of liver tissues. Representative picture (magnification ×100) of the hematoxylin/eosin stained liver tissue. Granuloma formation in HFD tissue is shown by *black arrowhead*, *Inset* Higher-magnification (×200) image of the granuloma. Small, unstained vacuole (as shown by *red arrowhead*) indicates lipid deposition. (Color figure online)

inflammation [63], was observed in liver sections of fructose-fed rats (indicated by black arrow). Such granuloma structure was not found in the liver section of HFDT group of rat indicating lowering of hepatic inflammation by glycyrrhizin. Small, unstained vacuoles (indicated by red arrow) suggested lipid deposition in fructose-fed rat liver.

The herbal treatment reduced the number of lipid vacuoles in HFDT group. Histological sections of ORO staining are shown in Supplementary Fig. 5. ORO, a fat soluble dye, was used for identification of lipid droplets in tissue sections. The staining indicated lipid deposition in fructose-fed rat livers, while glycyrrhizin treatment lowered the deposition of the droplets.

Discussion

Excess weight gain and high levels of blood glucose, insulin and triglyceride and low level of HDL cholesterol characterize metabolic syndrome [1]. Previous reports [3, 24] as well as the present study are consistent with these features of metabolic syndrome in fructose-fed rats (Table 1). Increased activities of liver-specific enzymes in blood serum suggest liver damage in this syndrome (Table 2). Oxidative stress is suggested to play a central role in the pathogenesis and progression of metabolic syndrome and its associated complications [64]. In this study, we observe that metabolic syndrome increases lipid peroxidation, protein carbonylation, and mitochondrial ROS generation resulting oxidative stress in liver (Fig. 1a, b, c). The levels of PKC and NOX proteins are associated with oxidative stress in various pathophysiological conditions [52, 53]. PKC activates several downstream kinases resulting increased free radical generation. NOX transfers reducing equivalents from NADPH or NADH to oxygen resulting superoxide (O_2^-) generation. Elevated levels of these proteins have been reported in hyperglycemic condition [64]. Metabolic syndrome causes elevation of hepatic PKCα and NOX-2 protein levels (Fig. 1d) which may be related to the syndrome-induced oxidative stress. Hyperglycemia in diabetes induces protein glycation and AGE formation, leading to different complications [55]. High blood glucose in metabolic syndrome also increases AGE accumulation and RAGE level in liver (Fig. 1e, f). AGE-RAGE interaction may activate PKC and other protein kinases resulting increased free radical generation and activation of NF-κB signaling [65]. Glycyrrhizin treatment reduces AGE-mediated signaling in liver by lowering blood glucose level in metabolic syndrome.

Oxidative stress, developed in this syndrome, alters both non-enzymatic and enzymatic antioxidant defenses [66, 67]. GSH maintains intracellular redox balance by detoxifying various xenobiotics as well as scavenging free radicals [56]. Enzymes of glutathione cycle namely GST and GPx utilize GSH during their reactions. Decrease in the GSH content due to oxidative stress simultaneously decreases the activities of GST and GPx, with a concomitant decrease in the activity of the GSH-regenerating enzyme, GR. All the components of glutathione cycle decreases in fructose-induced metabolic syndrome (Table 3). The findings suggest impaired antioxidant defense and elevated oxidative damage of liver tissue under this pathological condition. Treatment with glycyrrhizin decreases oxidative stress as well as augments the intracellular antioxidant defense and liver health.

NF-κB is a transcription factor that plays a central role in inflammatory reactions and cellular apoptosis [57]. It is composed of p50 and p65 subunits. In normal condition, NF-κB exists in an inactive form in the cytosol by associating with its inhibitory protein IκBα through the p65 subunit. In pathological condition, various inducers (including ROS, AGE-RAGE interaction, etc.) cause activation of different protein kinases resulting phosphorylation and degradation of IκBα. Free NF-κB complex then translocates to nucleus and regulates target gene transcription including COX-2, TNFα, iNOS, IL-12, and Bcl-2 family proteins [68]. Phosphorylation at multiple serine residues of the p65 subunit increases the transcriptional activity of NF-κB in nucleus [69]. In this study, we observe increased NF-κB translocation and phosphorylation of the p65 subunit together with decreased IκBα level in liver of rats with metabolic syndrome (Fig. 2a). These findings suggest activation of NF-κB signaling in metabolic syndrome. Treatment with glycyrrhizin, however, reduces the syndrome-induced disorders in IκBα/NF-κB level.

MAPKs are a family of serine/threonine kinases that are activated by dual phosphorylation on threonine and tyrosine residues. MAPKs are found to be activated by osmotic perturbations derived from glucose itself or by glucose-induced oxidative stress and by AGEs via its receptor RAGE [58]. MAPK signaling has been implicated in the activation of NF-κB via the phosphorylation of its inhibitor, IκBα. Again in response to TNFα, MAPKs increase the transcriptional activity of NF-κB via phosphorylation of its p65 subunit at serine 276 residue [69]. Our findings show increased phosphorylation of two important members of MAPK family namely MAPK p38 and ERK1/2 in metabolic syndrome indicating involvement of MAPKs in hepatic damage (Fig. 2b). Treatment with glycyrrhizin prevents phosphorylation of MAPKs and thus cellular damage via the MAPK signaling pathway.

COX-2 is an inducible enzyme, becoming abundant at the sites of inflammation, and generates free radicals during its reaction [70]. TNFα, a cytokine involved in systemic inflammation, can activate NF-κB through binding with TNF receptor (TNF-R) in cell membrane [57]. Elevation of these protein levels suggests hepatic inflammation in metabolic syndrome-induced rats (Fig. 2c). IL-12 is the modulator of T helper type 1 cells (Th1)-mediated immune response [71]. High IL-12 level in fructose-fed rat liver suggests Th1 inflammatory response in this syndrome. Lowering of these inflammatory protein levels in liver by

glycyrrhizin treatment suggests the antiinflammatory role of the phytochemical. Inhibition of high-mobility group box 1 protein activity by glycyrrhizin may also be responsible for reduction of inflammation [72–74]. However, iNOS and IL-4 protein levels remain almost same in different groups (NC, HFD, and HFDT) of rat.

Apoptosis is a precisely controlled programmed cell death pathway. Mitochondrial signal has been reported to take a critical part in apoptosis in most pathological conditions [75]. Bcl-2 family proteins act on the mitochondria to regulate mitochondria-dependent cell death [59]. There are two classes of regulatory proteins in the Bcl-2 family that confer opposite effects on apoptosis: the antiapoptotic member (e.g., Bcl-2) protects cells against apoptosis, whereas the proapoptotic member (e.g., Bax) promotes programmed cell death. The proapoptotic member of the Bcl-2 family physically interacts to form oligomer that can move onto the mitochondrial membrane and release cytochrome c from mitochondria to cytosol. In the cytosol, cytochrome c interacts with Apaf-1 to form the apoptosome that triggers the activation of caspase-3. Caspase-3 degrades DNA in the nucleus resulting cell death. Change in Bcl-2 and Bax proportion has been reported in the liver tissue of fructose-fed rats [37]. Our study also suggests that metabolic syndrome upregulates proapoptotic Bax and downregulates antiapoptotic Bcl-2 proteins, causing a reduction in the mitochondrial membrane potential and functionality (Fig. 3a, b). Release of cytochrome c in the cytosol, increased Apaf-1 and caspase-3 level indicate apoptotic changes which may be related to the pathological consequences in metabolic syndrome. Treatment with glycyrrhizin attenuates apoptotic cell death by regulating the Bcl-2 family proteins and their effects on the mitochondria-dependent cell death pathway.

DNA damage and increased level of cleaved PARP (84 kDa) supports liver cell apoptosis in fructose-induced metabolic syndrome (Fig. 3c, d). Once caspase-3 is activated, it leads to DNA breakdown and cleavage of several proteins including PARP [60]. This cleavage leads to the inactivation of PARP that ultimately prevents the futile DNA repair. According to another theory, PARP depletes the ATP level of a cell in an attempt to repair the damaged DNA and promotes apoptosis [76]. Glycyrrhizin treatment effectively decreases metabolic syndrome-induced DNA damage and PARP cleavage and thereby reduces liver cell apoptosis. FACS analysis using annexin V/PI double staining (Fig. 3e) supports the antiapoptotic effect of the herbal agent in metabolic syndrome-induced liver damage. Liver damage in high fructose diet-fed rats is evident from the appearance of granulomas, which are focal collection of epithelial cells, including macrophages, mononuclear, and other inflammatory cells that may fuse together to form multinucleated giant cells, usually in response to variety of infections and immunologic disorders [77]. Glycyrrhizin treatment inhibits granuloma formation and lipid deposition in fructose-fed rat liver (Fig. 4 and Supplementary Fig. 5).

In conclusion, the results of our study suggest that metabolic syndrome causes liver dysfunction and hepatic cell death via the activation of oxidative stress-responsive cell signaling pathways including signals from mitochondria. Treatment with glycyrrhizin effectively ameliorates liver damage in this pathological condition. However, it is not yet clear if the herbal agent controls glucose and glycation level leading to the observed ameliorative effects by lowering AGE, ROS, etc. or it also acts at different downstream sites of the cell signaling.

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Conflict of interest There are no conflicts of interest.

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Glycyrrhizin ameliorates insulin resistance, hyperglycemia, dyslipidemia and oxidative stress in fructose-induced metabolic syndrome-X in rat model

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This study investigates if glycyrrhizin, a constituent of licorice (*Glycyrrhiza glabra*) root, is able to treat the complications (insulin resistance, hyperglycemia, dyslipidemia and oxidative stress) of metabolic syndrome. Metabolic syndrome was induced in rats by feeding a fructose-enriched (60%) diet for six weeks, after which single dose of glycyrrhizin (50 mg/kg body weight) was administered intraperitoneally. Different biochemical parameters from blood were estimated during three weeks after treatment. Then the rats were sacrificed to collect skeletal muscle tissue. Glycyrrhizin reduced the enhanced levels of blood glucose, insulin and lipids in metabolic syndrome group. Increased advanced glycation end products of hemoglobin, glycohemoglobin, hemoglobin-mediated iron release and iron-mediated free radical reactions (arachidonic acid and deoxyribose degradation) in metabolic syndrome were inhibited by glycyrrhizin treatment. Reduced activities of enzymatic antioxidants (superoxide dismutase and catalase) and elevated oxidative stress markers (malonaldehyde, fructosamine, hemoglobin carbonyl content and DNA damage) in metabolic syndrome were reversed to almost normal levels by glycyrrhizin. The decreased levels of peroxisome proliferator activated receptor γ (PPARγ) and glucose transporter 4 (GLUT4) proteins in skeletal muscle of metabolic syndrome group were elevated by glycyrrhizin, indicating improved fatty acid oxidation and glucose homeostasis.

Keywords: Metabolic syndrome, High fructose diet, Glycation, Insulin resistance, Peroxisome proliferator activated receptor γ, Glucose transporter 4

Metabolic syndrome-X is one of the important health problems in the present world. It is a combination of clinical abnormalities including insulin resistance, hyperglycemia, dyslipidemia, hypertension and obesity. The syndrome increases the risk of cardiovascular diseases and type 2 diabetes[1]. It also leads to non-alcoholic fatty liver disease interfering with normal liver function[2]. Development of oxidative stress, an imbalance in prooxidant-antioxidant status, is the final mediator by which metabolic syndrome contributes to different pathological complications[3].

Fructose, a highly lipogenic nutrient, is a contributor to nearly all of the classic manifestations of metabolic syndrome. Nowadays increased fructose consumption through high fructose corn syrup present in soft drinks, juice beverages and pre-packaged food increases the risk of this syndrome[4]. Long-term intake of a fructose-enriched diet increases the risk of insulin resistance. Insulin resistance, in which the insulin level is not reduced but its function is affected, plays a key role in the development of metabolic syndrome[5]. Insulin resistance causes reduced glucose uptake and utilization by skeletal muscle and adipose tissues, due to reduced level of glucose transporter 4 (GLUT4), which is associated with insulin-regulated glucose transport[6]. Increased cellular lipid content due to reduced mitochondrial and peroxisomal β-oxidation also reduces insulin sensitivity in metabolic syndrome[7]. Cellular β-oxidation depends on peroxisome proliferator activated receptor γ (PPARγ), which is a ligand activated transcription factor belonging to the nuclear receptor superfamily. It regulates fatty acid oxidation in peroxisome and mitochondria. PPARγ expression level decreases in metabolic syndrome[8]. Therefore, improvement of GLUT4 and PPARγ levels may be a potential target in the treatment of this syndrome.

In recent years, scientists are in search of easily available, inexpensive therapeutics having minimum side effects for the better treatment of diseases. For these, phytochemicals from different herbal sources

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are being increasingly used. Licorice (*Glycyrrhiza glabra*) is a widely used medicinal plant. Glycyrrhizin, a triterpene saponin, is the major constituent of licorice root. It has long been studied for its medicinal activity. Takii *et al.*[9] have shown the antidiabetic effect of glycyrrhizin in genetically diabetic KK-A^y mice. Recent studies have reported the curative effect of glycyrrhizin[10] or its metabolite, 18β-glycyrrhetinic acid[11-13] in streptozotocin-induced type 1 diabetes mellitus in rat model. Glycyrrhizin has also been shown to upregulate total PPARγ expression in different tissues of normal rats[14]. Eu *et al.*[15] have shown that glycyrrhizin increases insulin sensitivity in high fat diet-induced obese rats. These findings have prompted us to undertake the present study to find the effect of glycyrrhizin on fructose-induced metabolic syndrome with respect to insulin resistance, hyperglycemia, dyslipidemia and oxidative stress as well as the expression of GLUT4 and PPARγ.

Materials and Methods

Materials—Arachidonic acid, deoxyribose, dinitrophenyl hydrazine (DNPH), dithiothreitol (DTT), ferrozine, glycyrrhizin, histopaque 1077, malonaldehyde (MDA), nitroblue tetrazoloium (NBT), phenylmethylsulfonylfluoride (PMSF), pyrogallol, sephadex G100, thiobarbituric acid (TBA) and protease inhibitor cocktail were purchased from Sigma-Aldrich, USA. Rat insulin ELISA kit and glycohemoglobin (GHb) estimation kit were purchased from DRG Diagnostic, Germany, and Transasia Biomedicals, India, respectively. Kits for estimating blood glucose, total cholesterol (TC), triglyceride (TG) and high-density lipoprotein-cholesterol (HDL-C) were purchased from Span Diagnostics, India. Primary and secondary antibodies and western blot Luminol reagent were purchased from Santa Cruz Biotechnology, USA. Other chemicals used in this study were of analytical grade and obtained from SRL and E. Merck, India.

Animals—Animals were maintained in accordance with the regulations specified and monitored by the Institutional Animal Ethics Committee of the Department of Biophysics, Molecular Biology and Bioinformatics, University of Calcutta, Kolkata (Registration number: 935/c/06/CPCSEA, 30.06.2009). Male Wistar rats, weighing 90-100 g body weight were obtained from university approved animal supplier. They were housed in an animal room under controlled conditions of 12 h light/dark cycle at 26-28 °C and 60-80% RH.

Toxicity study of glycyrrhizin—To find the toxic effect of glycyrrhizin on normal rats, several toxicity markers were checked. Rats were treated intraperitoneally (ip) with different single doses of glycyrrhizin (50, 100, and 200 mg/kg body weight in 50 m*M* potassium phosphate buffer, *p*H 7.4) with five rats in each group. Rats were supplied normal diet and water *ad libitum*. No significant hair loss or differences in hair colour, food intake, water intake, body weight gain, and behaviour were observed for next 4 weeks. Different biochemical parameters namely, serum alanine transaminase, alkaline phosphatase, xanthine oxidase, aspartate transaminase activities and reduced glutathione content of liver were estimated according to the standard methods[16-20]. No considerable change was found in the parameters between normal and treated rats, indicating no toxicity up to the dose of 200 mg/kg body weight (unpublished data). In the present experiments, glycyrrhizin was administered at a dose of 50 mg/kg body weight to find its effect.

Experimental design—The experimental design is presented in Scheme 1. Total 24 rats were divided primarily into two groups and were maintained as follows:

Normal control group (NC): The animals (n = 8 rats) received a control diet and water *ad libitum*.

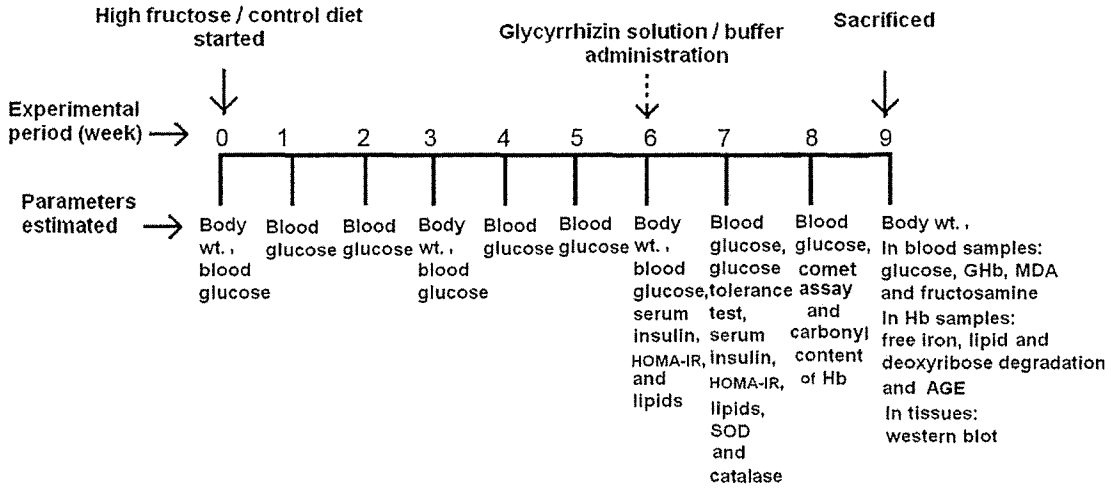
High fructose diet group (HFD): The animals (n = 16 rats) received a fructose-enriched diet and water *ad libitum*.

The diet composition is given in Table 1[21]. Each rat was supplied with 10 g freshly-prepared diet during daytime. After six weeks, the levels of fasting blood glucose, insulin, TC and TG appeared to be significantly higher ($P < 0.01$) in HFD group of rats in comparison with those of NC group of rats, indicating induction of metabolic syndrome in HFD rats. On day 43, HFD rats were treated with glycyrrhizin solution or buffer as follows:

HFDT group: HFD rats (n = 8 rats) were treated with single injection (ip) of glycyrrhizin solution in 50 m*M* potassium phosphate buffer, *p*H 7.4, at a dose of 50 mg/kg body weight.

HFD group: HFD rats (n = 8 rats) were similarly treated with only buffer.

Both HFD and HFDT groups of rat were still on the fructose-enriched diet until sacrificed after three weeks of glycyrrhizin treatment.



Scheme 1—Schematic representation of the experimental design. It shows the parameters tested at different time intervals in different groups of rat. Control diet was supplied to NC group and fructose-enriched diet was supplied to HFD and HFDT groups. Buffer was administered in HFD group and glycyrrhizin solution was administered in HFDT group of rats.

Collection of serum, hemoglobin and tissue— Blood samples (fasting) were collected from tail vein without and with heparin for estimation of enzymes and other parameters, respectively. For estimation of serum insulin level, blood was drawn from the retro-orbital plexus. Serum was separated by centrifugation at 3,500 *g* at 4 °C for 10 min. Hemolysates were prepared from erythrocytes after washing with normal saline (0.9% NaCl) and hypotonic lysis with distilled water. Hemoglobin (Hb) was isolated from hemolysate by centrifugation at 17,500 *g* at 4 °C for 15 min and purified by sephadex G100 column pre-equilibrated with 50 m*M* potassium phosphate buffer, *p*H 7.4. The concentration of Hb was measured from the soret absorbance with molar extinction coefficient 125 mM^{-1} cm^{-1} (monomer basis)[22]. Rats were sacrificed by cervical dislocation. Quadricep muscle tissue was dissected out immediately, washed with ice-cold phosphate buffer saline, *p*H 7.4 and preserved at -80 °C for Western blot experiments done within next three weeks.

Blood glucose, GHb, insulin, homeostasis model of assessment-insulin resistance (HOMA-IR) index, lipids and intraperitoneal glucose tolerance test (IPGTT)—Blood glucose was estimated following the conventional glucose oxidase/peroxidase method by using the assay kit[23]. Serum insulin, TC, TG, HDL-C and GHb were measured using commercial assay kits according to the manufacturer's protocols. Low-density lipoprotein-cholesterol (LDL-C) was estimated according to the Friedewald equation[24]. Very low-density lipoprotein-cholesterol (VLDL-C) was obtained by subtracting both HDL-C and LDL-C from TC. HOMA-IR index was calculated according to the formulae of Matthews *et al*[25]. For glucose tolerance test, a sterile solution of 20% glucose was injected (ip) at a dose of 2 g/kg body weight to overnight fasting animals. Blood was collected to estimate glucose from tail vein before (0 min) and 30, 60, 90 and 120 min after injection.

Table 1— Composition of diets (g/100g)

Ingredients	Control diet	High-fructose diet
Corn starch	60	--------
Fructose	--------	60
Casein	20	20
Methionine	0.7	0.7
Groundnut oil	5	5
Wheat bran	10.6	10.6
Salt	3.5	3.5
Vitamin	0.2	0.2

Superoxide dismutase (SOD), catalase, MDA and fructosamine in serum— SOD and catalase activities were assayed according to the methods of Murklund and Murklund[26] and Beers and Sizer[27] using pyrogallol and hydrogen peroxide, respectively. Protein content was measured following the method of Lowry *et al.*[28] using bovine serum albumin as the standard. MDA level was measured as TBA reactive substance[29]. For fructosamine (Amadori product), NBT was used to detect the formazan formed[30].

Free iron level in Hb, Hb-mediated lipid peroxidation and deoxyribose degradation— Free iron in Hb was measured by reaction with ferrozine according to the method of Panter[31]. Hb-mediated lipid (arachidonic acid) peroxidation was estimated

according to the method of Sadrzadeh *et al.*[32] with modifications as described before[33]. The values were corrected for endogenous TBA reactive substance present in arachidonic acid. Hb-mediated deoxyribose degradation was assayed spectrofluorimetrically following the method of Gutteridge[34].

Carbonyl content and advanced glycation end products (AGEs) in Hb— Carbonyl content in Hb was measured using DNPH according to the method of Levine *et al*[35]. AGEs in Hb were estimated spectrofluorimetrically from fluorescence emission at 440 nm after exciting Hb samples at 370 nm[36].

Single cell gel electrophoresis (comet assay) of lymphocytes— Lymphocytes were isolated from blood using histopaque 1077 and were resuspended in phosphate buffer saline, *p*H 7.4. Comet assay and scoring were performed according to the method of Singh *et al.*[37] and Collins *et al.*[38], respectively. Photomicrograph of ethidium bromide-stained cells was taken in Olympus BX51 fluorescence microscope with Evolution VF cool CCD camera at 200 magnifications.

Western blotting experiment with quadricep muscle tissue— Quadricep muscle tissue was homogenized with ice-cold lysis buffer containing 25 m*M* Tris HCl, *p*H 7.5, 250 m*M* NaCl, 1% Triton X-100 (v/v), 1 m*M* DTT, 1 m*M* PMSF and protease inhibitor cocktail and the supernatant was collected by centrifugation at 20,000 *g* at 4 °C for 30 min[39]. Proteins were separated by SDS-PAGE (10%) and western blot experiments were performed using mouse monoclonal antibody for β-actin (1:500 dilution) and GLUT4 (1:250 dilution) and goat polyclonal antibody for PPARγ (1:250 dilution). Band intensities were quantified by densitometry using Biorad Geldoc apparatus with Quantity One software.

Statistical analysis— Results were expressed as mean±SD obtained for *n* experiments; *n* represents the number of rats. Statistical significance was determined by AcaStat 6 software using unpaired Student's *t*-test and $P < 0.05$ was considered to be significant.

Results and Discussion

Metabolic syndrome parameters: effect of glycyrrhizin—Several metabolic syndrome parameters namely, fasting blood glucose, serum insulin, lipid profile and body weight were measured in NC and HFD group of rats. Six weeks of fructose-enriched diet led to significant increase in fasting blood glucose, serum insulin, and HOMA-IR index in HFD rats in comparison with those in NC rats (Table 2). At week six, serum levels of TC, TG, LDL-C and VLDL-C were significantly higher and HDL-C was significantly lower in HFD group than the respective levels in normal rats. High fructose diet induced significant increase in body weight in HFD group in comparison with weight gain by NC group having normal diet (Table 3). The weight gain, abnormal lipid profile together with high blood glucose, serum insulin and HOMA-IR index of HFD group of rats demonstrated the onset of metabolic syndrome induced by fructose-enriched diet. The results are consistent with previous reports of other groups[4,8].

The metabolic syndrome parameters were measured at different intervals after glycyrrhizin treatment of HFD rats (HFDT) and compared them with respective levels of HFD and NC group of rats.

Table 2— Metabolic syndrome parameters of different groups of rat after six weeks of high fructose diet feeding

[Values are mean ± SD from each group]

Groups	Blood glucose (mg/dL)	Insulin (μg/L)	HOMA -IR	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	VLDL-C (mg/dL)
NC	80.6 ± 5.8	0.82 ± 0.09	5.54 ±0.81	64.7 ± 7.5	72.5 ± 9.8	35.8 ± 4.2	14.4 ± 3.4	14.6 ± 2.0
HFD	160.5 ±7.8 [a]	2.14 ± 0.22 [a]	21.17 ± 3.33 [a]	131.8 ± 10.4 [a]	163.0 ± 11.3 [a]	13.3 ± 3.2 [a]	76.4 ± 13.5 [a]	32.7 ± 1.5 [a]

For insulin and HOMA-IR, $n = 6$ rats and for other parameters, $n = 8$ rats.
[a] $P < 0.01$ *versus* NC.

Table 3— Body weight of different groups of rat

[Values are mean ± SD from each group]

Groups	Initial body weight (g)	Body weight (g) after 6 weeks of control/fructose diet feeding	Body weight (g) after 3 weeks of buffer/ glycyrrhizin administration (total 9 weeks)		Body weight gain (g) in 9 weeks
NC ($n = 8$)	91.7 ± 6.5	103.7 ± 8.3	118.6 ± 5.0		26.4 ± 4.7
HFD ($n = 16$)	92.2 ± 8.7	122.6 ± 6.7	HFD ($n = 8$)	151.4 ± 7.3	58.9 ± 7.4 [a]
			HFDT ($n = 8$)	134.3 ± 5.0	40.3 ± 7.5 [b]

[a] $P < 0.01$ *versus* NC; [b] $P < 0.05$ *versus* HFD.

The dose of glycyrrhizin used in the present study (50 mg/kg body weight) was much less than the LD_{50} dose (0.84 g/kg body weight, ip)[40]. The existing reports suggest that ip administration of glycyrrhizin in rats at a dose of 100 mg/kg body weight caused no toxicity[41,42]. No toxic effect of glycyrrhizin was found up to administration of 200 mg/kg body weight (unpublished data). After one week of treatment with glycyrrhizin, fasting blood glucose level decreased significantly in the treated group, as compared with HFD group (Fig. 1a). For next two weeks, HFDT group exhibited slow increase in blood glucose level, which might be due to continuous feeding of high fructose diet. However, still there was a significant difference in blood glucose levels between HFD and HFDT groups after three weeks of treatment. After one week of glycyrrhizin treatment, insulin level and HOMA-IR index decreased significantly in HFDT group, as compared with HFD group (Fig. 1b and 1c), indicating increased insulin sensitivity in the treated group. Glucose tolerance behaviour, as indicated by IPGTT done after one week of glycyrrhizin treatment, appeared almost normal in HFDT group of rats in comparison with the normal and abnormal tolerant profiles in NC and HFD groups, respectively (Fig. 1d). Increased serum levels of TC, TG, LDL-C and VLDL-C and decreased level of HDL-C in HFD rats reverted significantly towards respective normal levels after one week of treatment with glycyrrhizin, (Fig. 2). Glycyrrhizin treatment was also effective in lowering body weight gained by fructose-enriched diet (Table 3), probably by increasing fatty acid

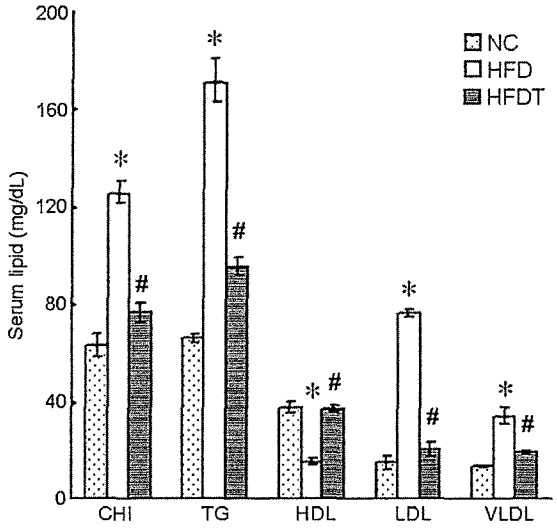


Fig. 2— Serum lipid profiles of different groups of rat. The levels of TC, TG, HDL-C, LDL-C and VLDL-C were estimated after one week of glycyrrhizin treatment. The results are mean ± SD, n = 8 in each group. * $P < 0.01$ *versus* NC; # $P < 0.01$ *versus* HFD.

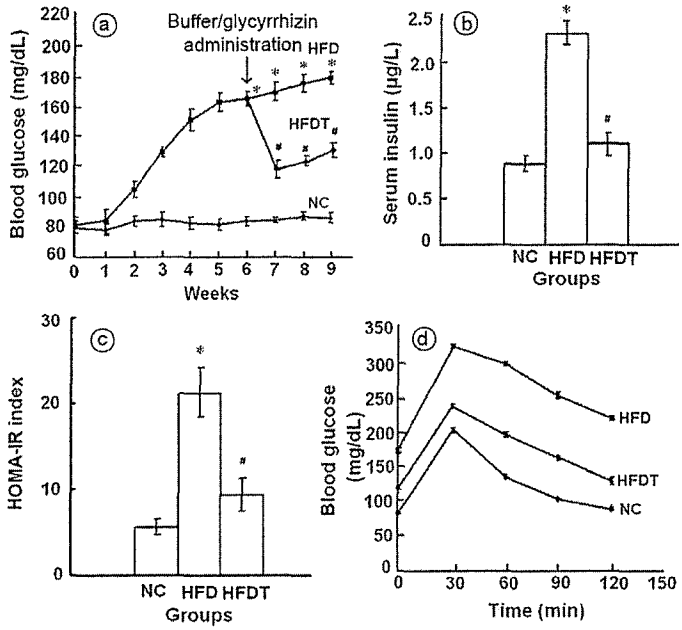


Fig. 1— Blood glucose and insulin level during experimental period. (a) Change of blood glucose with time before and after glycyrrhizin treatment. n = 8 in each group. (b) Serum insulin level of different groups of rat after one week of glycyrrhizin treatment. n = 6 in each group. (c) HOMA-IR index of different groups of rat after one week of glycyrrhizin treatment. n = 6 in each group. (d) Glucose tolerance curves of NC, HFD and HFDT rats after one week of glycyrrhizin treatment. n = 5 in each group. The results are mean ± SD. * $P < 0.01$ *versus* NC; # $P < 0.01$ *versus* HFD.

oxidation and thereby decreasing total lipid storage in body. Increased insulin sensitivity and reduction in body weight in glycyrrhizin-treated high fat diet-induced obese rats have been reported by Eu *et al*[15].

Oxidative stress markers in blood samples of metabolic syndrome group of rats: effect of glycyrrhizin— Free radical scavenging enzyme (SOD and catalase) activities in serum were reduced and serum MDA and fructosamine levels were increased significantly in HFD rats, compared to those in normal rats (Table 4). Glycyrrhizin treatment significantly enhanced the levels of antioxidative enzymes and reduced MDA and fructosamine levels in HFDT group of rats, thereby indicating antioxidant function of the herbal agent. SOD and catalase activities were assayed after one week and MDA and fructosamine levels were determined after three weeks of glycyrrhizin treatment.

Since metabolic syndrome exhibits increased blood glucose level, GHb level was measured in blood samples of different groups of rat. The level increased significantly in HFD group, in comparison with the NC group, and reverted to the near normal level after three weeks of treatment with glycyrrhizin (Table 5). The lowering of blood glucose level due to treatment with the herbal agent may have reduced the GHb level.

Both *in vitro*[33,44,45] and *in vivo*[10,46] studies suggest that GHb is a source of catalytic iron causing oxidative stress in hyperglycemic condition. Ferrozine-detected free iron level was estimated in Hb samples of different groups of rat and was found to increase significantly in HFD rats in comparison with the normal level (Table 5). H_2O_2 is known to react with ferrous iron to form hydroxyl radical[47], which, in turn, causes free radical reactions. Oxidative reactions namely, arachidonic acid and deoxyribose breakdown were assayed in presence of H_2O_2 and Hb samples of different groups of rat. Both these reactions were significantly increased with Hb samples of HFD group than with those of normal group, indicating enhanced Hb-catalyzed oxidative reactions in metabolic syndrome (Table 5). However, glycyrrhizin treatment effectively lowered free iron level and both arachidonic acid and deoxyribose breakdown reactions, as demonstrated with Hb samples of HFDT group of rats. Sen *et al.*[10] have also reported similar effect of glycyrrhizin on streptozotocin-induced diabetes in rats. Thus both in type 1 diabetes and high fructose-induced metabolic syndrome, glycyrrhizin treatment interrupts the glycation cascade, preventing the potential pathological consequences.

Metal-catalyzed oxidation may cause covalent modification of proteins by introducing carbonyl

Table 4— Effect of glycyrrhizin on free radical scavenging enzymes (SOD and catalase) and oxidative stress markers (MDA and fructosamine) in serum of different groups of rat

[Values are mean ± SD from each group]

Groups	SOD (Units/min/mg protein)	Catalase (Units/min/mg protein)	MDA (Fluorescence intensity in arbitrary unit/mg protein)	Fructosamine (μmol/mg protein)
NC	0.956 ± 0.117	0.255 ± 0.032	11.5 ± 2.2	142.2 ± 21.5
HFD	0.378 ± 0.118 [a]	0.137 ± 0.015 [a]	48.8 ± 7.6 [a]	464.9 ± 37.2 [a]
HFDT	0.658 ± 0.044 [b]	0.217 ± 0.011 [b]	28.5 ± 4.8 [b]	257.7 ± 23.3 [b]

Serum enzymes were assayed after one week and serum MDA and fructosamine levels were assayed after three weeks of glycyrrhizin treatment.
For each parameter, $n = 7$ rats.
[a] $P < 0.01$ *versus* NC; [b] $P < 0.01$ *versus* HFD.

Table 5— GHb, free iron in Hb and Hb-mediated oxidative reactions in different groups of rat

[Values are mean ± SD from each group]

Groups	GHb (%)	Free iron in Hb (μg/g Hb)	Arachidonic acid peroxidation (μmol of TBA reactive substance formed/h/mg Hb)	Deoxyribose degradation (Fluorescence intensity in arbitrary unit/mg Hb)
NC	5.90 ± 0.63	96.7 ± 14.9	15.6 ± 6.8	235.3 ± 38.3
HFD	10.07 ± 1.04 [a]	244.1 ± 26.1 [a]	60.3 ± 10.0 [a]	778.2 ± 62.7 [a]
HFDT	6.77 ± 0.34 [c]	147.3 ± 9.0 [b]	25.3 ± 4.7 [b]	416.6 ± 54.6 [b]

The experiments were done after three weeks of glycyrrhizin treatment.
For GHb, $n = 6$ rats and for other parameters $n = 7$ rats.
[a] $P < 0.01$ *versus* NC; [b] $P < 0.01$ *versus* HFD; [c] $P < 0.05$ *versus* HFD.

groups into amino acid residues of proteins. Such oxidative modification is an index of oxidative stress. The extent of carbonyl formation was estimated in Hb samples isolated from different groups of rat. Carbonyl content of Hb samples of HFD rats were significantly higher in comparison with those of NC rats (Fig. 3a). Glycyrrhizin efficiently improved this stress condition by decreasing carbonyl content of Hb samples of HFDT rats. The results obtained with Hb after two weeks of treatment have been presented.

AGEs, formed by long-term glycation of proteins with reducing sugars, lead to free radical generation and oxidative stress. Compared to NC rats, AGE formation was higher in Hb of HFD rats (Fig. 3b). Glycyrrhizin treatment effectively decreased AGE content of Hb by maintaining reduced blood glucose level for three weeks in HFDT rats compared to HFD rats. The treatment thus helps to decrease oxidative stress in metabolic syndrome.

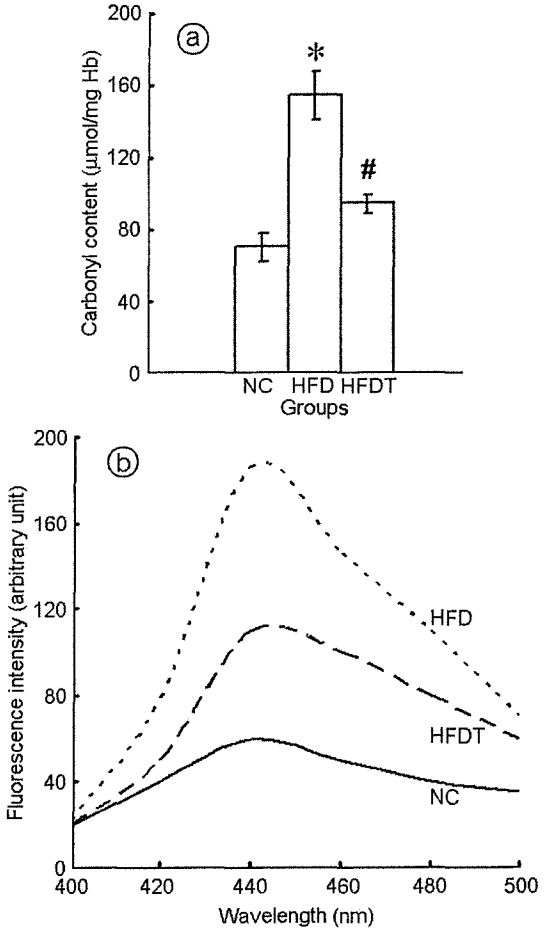


Fig. 3— Oxidative stress markers in Hb of different groups of rat. (a) Levels of carbonyl content in Hb samples from different groups of rat. The results are mean ± SD, $n = 7$ in each group. * $P < 0.01$ *versus* NC; # $P < 0.01$ *versus* HFD. Experiment was done after two weeks of glycyrrhizin treatment. (b) Representative fluorescence emission spectra (excitation 370 nm) showing extent of AGE formation in Hb samples (20 µM) from NC, HFD and HFDT groups of rat. This is the representative spectra of six independent experiments. Experiment was done after three weeks of glycyrrhizin treatment.

To study oxidative stress-mediated DNA damage, single cell gel electrophoresis (comet assay) of lymphocytes was done. Comet formation in the lymphocytes of HFD rats increased significantly in comparison with those of NC rats. However, glycyrrhizin treatment decreased comet formation, as shown in the lymphocytes of HFDT rats (Fig. 4), indicating reduced oxidative damage of DNA in the treated group. The experiment was done after two weeks of glycyrrhizin treatment.

Expression levels of GLUT4 and PPARγ in quadricep muscle extracts of metabolic syndrome group of rats: effect of glycyrrhizin— Western blot experiment (Fig. 5) revealed that the levels of GLUT4 and PPARγ decreased in quadricep muscle extracts of HFD rats, as compared with the levels of NC rats. However, glycyrrhizin treatment of HFD rats caused improvement and increased the proteins toward normal levels of expression. Shih *et al.*[8] have also reported that PPARγ and GLUT4 expressions decrease in skeletal muscle of fructose-fed rats having metabolic syndrome. On the other hand, the finding of Yin *et al.*[14] showing enhanced level of PPARγ in glycyrrhizin-treated normal rats is also in agreement with our observation in HFDT rats. Increased PPARγ expression enhances peroxisomal and mitochondrial β-oxidation, reducing cellular lipid content and insulin resistance[8]. Again, up regulation of PPARγ has been shown to increase GLUT4 expression in skeletal muscle[43], and is consistent with our result. Increased GLUT4 in HFDT rats, in turn, helps to improve cellular insulin sensitivity and glucose homeostasis in metabolic syndrome.

In conclusion, the present findings suggest the beneficial effect of glycyrrhizin against several complications of metabolic syndrome namely, insulin resistance, hyperglycemia, dyslipidemia and oxidative stress. Regulation of GLUT4 and PPARγ expression in skeletal muscle may be one of the mechanisms of glycyrrhizin action. However, it is not yet clear if glycyrrhizin administered intraperitoneally acts as such or through a hydrolyzed/metabolized product. Further studies are also necessary to elucidate the

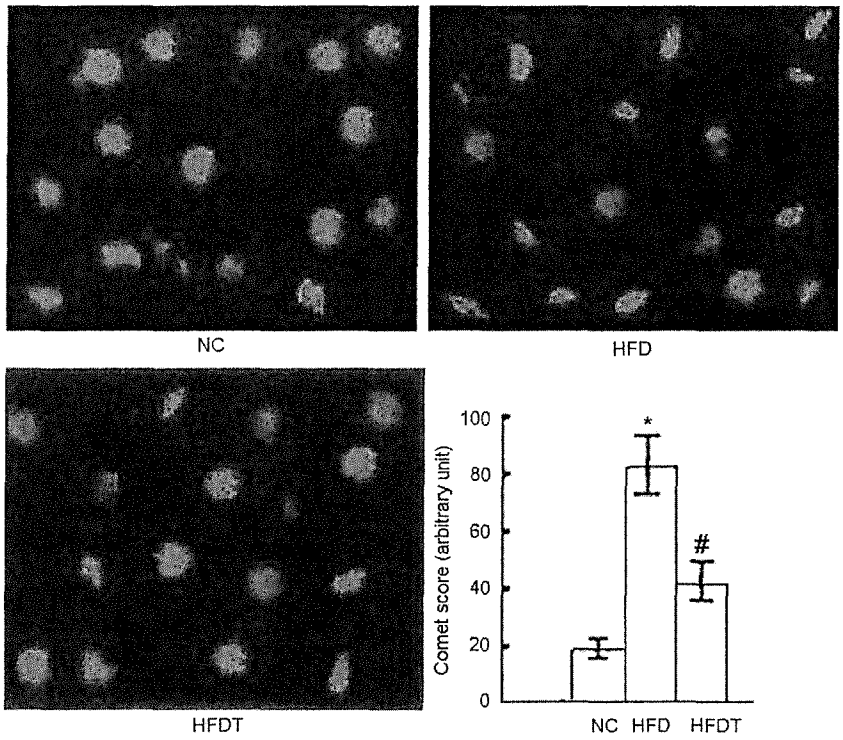


Fig. 4— Effect of glycyrrhizin treatment on DNA damage in lymphocytes. Single cell gel electrophoresis in lymphocytes showing comets (200 magnifications). Comet scores are shown on different groups of rat. The results are mean ± SD of six experiments in each case. * $P < 0.05$ *versus* NC; # $P < 0.05$ *versus* HFD. Experiment was done after two weeks of glycyrrhizin treatment.

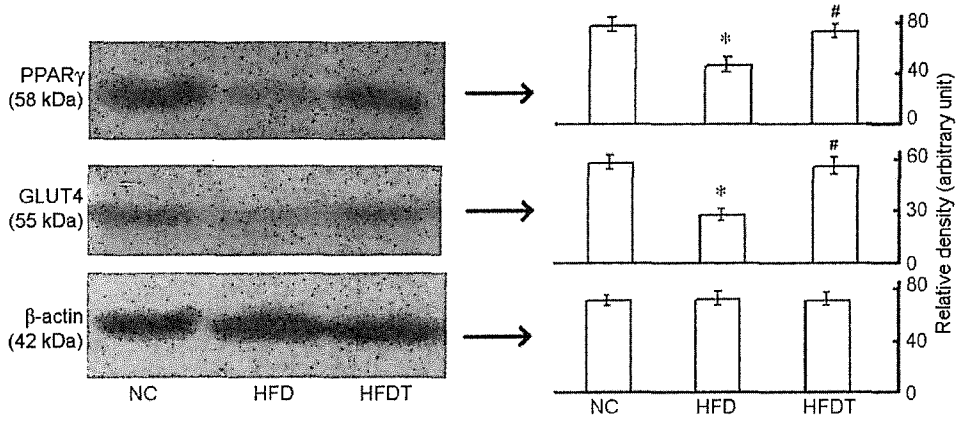


Fig. 5— Expression of PPARγ and GLUT4 in skeletal muscle of different groups of rat. Representative western blot and densitometry data depicting PPARγ and GLUT4 protein in the quadricep muscle of different groups of rat. The densitometry results are mean ± SD of six independent experiments. * $P < 0.05$ *versus* NC; # $P < 0.05$ *versus* HFD.

effect of glycyrrhizin on other underlying mechanisms of the syndrome affecting normal metabolism.

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The efficiency of Magnesium isoglycyrrhizinate in treatment of autoimmune hepatitis cirrhosis with decompensated liver inflammatory activity

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Objective To investigate the therapeutic effect of magnesium isoglycyrrhizinat on the discompensation stage cirrhosis of autoimmune he patitis.Methods Eighty-eight patients were randomly allocated to a treatment group(n=44) and a control group(n=44).Patients in the treatmen t group received magnesium isoglycyrrhizinate 200 mg once daily.Patients in the control group received compound glycyrrhizin 200 mg once daily.The Course takes 3 weeks,and was observed in terms of the change of clinical manifestations,biochemical and immunological indexes. Results Patients in both groups showed therapeutic effect with statistical significance(P0.01).In both groups,after treatment,the degree of bio chemical indicators declined significantly in the experimental group than the control group(P0.05 or P0.01).Conclusion The result shows that glycyrrhizic acid is an effective drug for controlling inflammation activity of the discompensation stage cirrhosis of autoimmune hepatitis and magnesium isoglycyrrhizinate is a more effective and safer agent than Compound Glycyrrhizin.It may be used extensively.

【Key Words】： magnesium isoglycyrrhizinate autoimmune hepatitis cirrhosis

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STATE OF THE SCIENCE REPORT ON THE EFFECTS OF MODERATE DRINKING

NATIONAL INSTITUTE ON ALCOHOL ABUSE AND ALCOHOLISM

NATIONAL INSTITUTES OF HEALTH

DEPARTMENT OF HEALTH AND HUMAN SERVICES

DECEMBER 19, 2003

PURPOSE/CHARGE

In support of the planned 2005 update of the Dietary Guidelines, NIAAA has been asked to assess the strength of the evidence related to health risks and potential benefits of moderate alcohol consumption, with particular focus on the areas of cardiovascular disease, breast cancer, obesity, birth defects, breastfeeding, and aging.

The following report was prepared by NIAAA scientific staff experts in areas of basic research (e.g., metabolism, toxicity, neuroscience), nutrition, and epidemiology, and was reviewed by external researchers with extensive research backgrounds on the consequences and benefits of alcohol consumption. (Participating staff and external reviewers are listed in the Appendix).

The report consists of several sections:

I. BACKGROUND INFORMATION

About 35% of the adult US population abstains from alcohol use, about 60% are occasional to moderate drinkers, and about 5 to 7 % are diagnosable with alcohol abuse or dependence (NIAAA, 1997). Of the some 16 million Americans who meet the diagnostic criteria for abuse or dependence, only about 1.5 million seek and receive treatment (SAMHSA, 2003).

Alcohol consumption causes some 100,000 deaths annually in the US, including more than 16,000 alcohol related traffic fatalities (Meister et al., 2000; NIAAA, 2000). Compared with abstainers, drinkers – particularly heavy or excessive drinkers – have higher death rates from injuries, violence, suicide, poisoning, cirrhosis, certain cancers, and possibly hemorrhagic strokes (Gutjahr et al., 2001; Thun et al., 1997).

However, because of alcohol's apparent protective effect against coronary heart disease (CHD) and other atherosclerotic diseases, which are the most common causes of death in the US, the consequences of alcohol use must be evaluated in conjunction with its potential benefits. For example, at least one estimate predicts that if all current consumers of alcohol abstained from drinking, another approximately 80,000 CHD deaths would occur each year (Pearson & Terry, 1994).

Over the past 50 years, numerous studies have investigated the relationship of alcohol consumption and the development of many medical conditions including cancers, cardiovascular disease, diabetes and dementia. Studies have also investigated the relationship of maternal alcohol consumption during pregnancy and breast feeding to the health and development of infants and children. Many of these studies have evaluated dose response relationships and therefore may provide comparative information about zero, low, moderate and heavy levels of ethanol consumption and the various outcomes of interest. Both epidemiologic and basic science studies have addressed the relationship of moderate alcohol consumption and medical consequences and both must be considered in evaluating the relationship of moderate drinking and health. However, certain complications are inherent in interpreting this literature. "Moderate" drinking is the only level of drinking that has been shown to have potential health benefits, and the levels of drinking that are classified as "moderate" and "heavy" have not been defined consistently across studies (Gaziano et al., 2000; Klatsky, 2002; NIAAA, 1992). Further, they are not always consistent with the definition of moderate drinking in the USDA/DHHS Dietary Guidelines (2000; i.e., no more than one drink per day for women and no more than two drinks per day for men). Furthermore, the amount considered moderate in some situations may be excessive under other circumstances (e.g., pregnancy; intent to drive). Also, it is important to note that many "moderate drinkers" have occasions of high-risk drinking, including heavy episodic drinking and acute intoxication leading to injuries and violence (Gutjahr et al., 2001).

The difficulty in defining moderate drinking is to some extent a result of individual differences. The amount a person can drink without

intoxication may vary according to drinking experience and tolerance (Bondy et al., 1999). Individual metabolic differences can lead to a wide range of blood alcohol content (BAC) levels for the same consumption (Ramchandani et al., 2001a). Also important is the time over which the alcohol is consumed: 3 drinks in one hour will produce a much higher BAC than 3 drinks over the course of 3 hours, and therefore different effects. Thus, definitions solely based on the number of drinks are not the best approach.

Another complicating factor in the interpretation of this complex literature is the interaction of genetic vulnerability to a particular medical condition with the effects of alcohol consumption: risk and protection from alcohol's effects may vary considerably across groups or individuals in the population. Confounding and modification by lifestyle variables also could be a factor in the observed health differences between drinkers and nondrinkers. Various studies have found that nondrinkers are less likely to exercise regularly and have a higher body mass index than their drinking counterparts; they also report lower vegetable intakes and higher fat consumption (Barefoot et al., 2002). Moderate drinkers are found to monitor their health (e.g., blood pressure and preventive dental care) more often than abstainers and heavy drinkers, and female drinkers over age 50 report significantly higher mammography rates than nondrinkers (Green & Polen, 2001). Studies suggest that life-long abstainers tend to be older, poorer, religious, disabled or in poor health, less physically and socially active, and to have more symptoms of depression (Ashley et al., 1994); while some of these traits (e.g. health status) may stem from their abstention, others obviously do not.

Research on basic mechanisms of alcohol effects may explain observed epidemiological phenomena associated with moderate drinking. In this report, we summarize both the epidemiological and selected basic research studies that may contribute to the understanding of the consequences and benefits of moderate drinking. However, it is important to note that there is a difference between epidemiological data (e.g., population-based averages) and experimental/clinical data (e.g. looking at specific individuals in specific confounding or co-occurring environmental, physiological, and genetic contexts). Moreover, the interpretation of and conclusions drawn from all of these studies must be tempered by the following considerations:

Pharmacokinetics and Pharmacodynamics

The variation in host responses to alcohol is exemplified in the variability in the rate and extent of absorption, distribution and the metabolism of ethanol, (i.e. the pharmacokinetics), and in the effects (i.e., the pharmacodynamics) of alcohol. To date, researchers have found as much as 3- to 4-fold differences in metabolic and 2- to 3-fold differences in behavioral responses to alcohol between different individuals (Ramchandani et al., 2001a). In other words, whereas alcoholic drinks may be standardized, drinkers are not. There are genetic as well as environmental contributions to this variation. For example, alcohol elimination rates have shown as much as an average 45% increase following the environmental factor of food consumption, compared with that following fasting (Ramchandani et al., 2001b).

Demographics

Alcohol's influence on mortality risk is influenced by age, because the leading causes of death differ by age group (Ashley et al., 1997; Gronbaek, 2001; Meister et al., 2000; Thun et al., 1997). For example, among US men aged 15-29, deaths from injuries and other external causes predominate, accounting for 75% of all deaths; only 4 % are from cardiovascular conditions. On the other hand, for men over 60, only 3% of deaths are from external causes, and over 45% are from cardiovascular conditions (Thun et al., 1997). This affects the balance of risks and benefits.

The effects of moderate drinking, as well as the level deemed moderate, may also vary by age and by gender due to the metabolic and pharmacokinetic effects described above. Likewise, the risks and benefits of alcohol use may fluctuate according to genetic-based susceptibility to various diseases (including alcoholism itself), which in turn may be associated with ethnic background or gender.

Definition of "moderate"

Moderate drinking can mean drinking in moderation, where the term "moderation" is defined by Webster's Dictionary (1984) as "within limits; reasonable; of average or medium quantity or extent" that is, drinking such that there is no ensuing harm. Alternatively, the term moderate drinking can be used as a descriptor of quantity/frequency of intake, particularly in comparison to the "extremes" of total abstinence and heavy drinking. Both of these definitions share the problem of not accounting for the pattern of intake over time, which can be a major determinant of whether drinking is harmful or beneficial. Other conceptions of moderate include nonintoxicating; noninjurious; or statistically "normal" (Eckardt et al., 1998), definitions which can vary by individual or by socio-cultural context. Studies cited throughout this report use a wide range of consumption levels to represent moderate drinking: some consider it to be 1 drink per week or less (e.g., Berger et al., 1999) while others use as many as 4 drinks per day (e.g., Nicolas et al., 2002) making comparisons and generalizations across studies and across areas of harm difficult.

Drinking Patterns

Drinking patterns are as important as total consumption, not only in terms of alcohol's benefits, but also its harmful consequences. Risks for alcohol abuse and/or dependence jump dramatically for men who exceed 4 drinks per occasion and for women who exceed 3 drinks per occasion (NIAAA/NLAES 1992). Some of the studies addressed in this report have specifically looked at the differences between low per-occasion consumption occurring regularly (e.g., 1or 2 drink per day, 4 days per week) and the same total weekly consumption occurring all at once (e.g., Mukamal et al., 2003a). However, the pattern of drinking was not assessed by many of the studies, and so the consumption level of "an average of 1 drink per day" could reflect either a true "daily" drinker; a 3-times-per-week, 2-drinks-per-occasion pattern; or a weekend heavy drinker, making comparison across studies and the determination of clear conclusions difficult.

Drink size

Because some researchers present results in terms of number of drinks and others in terms of "grams of alcohol" (which differs across alcoholic beverage types and according to portion sizes), this report will use the approximation of 1 drink = 15 grams of alcohol in presenting all data in order to facilitate evaluations. However, drink sizes vary by country, alcohol content varies by type of beverage, and recall/reporting by the study participants may be inaccurate (intentionally or not). Thus, while this report "standardizes" drink size for ease of readability, a true comparison of actual alcohol ingestion level across studies is unreliable.

In summary, discrepancies in findings across studies can arise from pattern of alcohol consumption and differences in modes of administration, differences in definition of drink size or in the number of drinks that constitutes "moderate" use, differences between in vivo and in vitro reactivity, and the use of different animal models and the validity of their extrapolation to humans. In human studies, gene-environment interactions, co-morbidity, medications, age, self-reporting and alcohol use assessment, gender and lifestyle effects further complicate interpretation.

II. AREAS OF SPECIFIC FOCUS

A. Cardiovascular Disease

Cardiovascular disease, in particular coronary heart disease (CHD) and associated myocardial infarction (MI), is the leading cause of death among adults in the United States (CDC, 2002). Cardiovascular causes account for about 45% of all deaths among men over 35 years old and 37% of all deaths among women over 35 (Thun et al., 1997). In numerous studies – cross-sectional, longitudinal, cohort, case-control, individual, meta-analysis – differing considerably in their adjustments for confounding risk factors, the data on CHD-related death are remarkably consistent: the relationship between alcohol consumption and mortality follows a J-shaped or U-shaped curve, with one to four drinks daily significantly reducing risk and five or more drinks daily significantly increasing risk (Booyse & Parks, 2001; Corrao et al., 2000; Hines & Rimm, 2001; Murray et al., 2002; Perret et al., 2002; Rehm et al., 2001; Rehm et al., 2003; Rimm, 2000; Rotondo et al., 2001). This

inverse association between light-to-moderate alcohol consumption and CHD morbidity and mortality had been demonstrated independent of age, sex, smoking habits, and body mass index.

Most recent studies have found that the trend for beneficial CHD effects first appears when daily drinking exceeds 1 and 1.5 drinks per day for women and men, respectively (Baer et al.; 2002; Hines & Rimm, 2001; Murray et al., 2002; Sillanaukee et al., 2000). The relative risk of MI is reduced by 25% in men consuming up to 2 drinks per day and by 50% in those consuming more than 2 drinks. The association holds even for men with a prior history of MI (Shaper & Wannamethee, 2000). Lower levels of consumption were not significantly associated with CHD (Murray et al., 2002). However, recently Mukamal et al. (2003a) found that the protective effect was more a function of frequency of consumption than of volume; small amounts consumed several times a week reduced risk to a greater extent than the same amount consumed over fewer occasions. In pre-menopausal women, for whom overall CHD risk is lower, the effects of alcohol are less likely to reach significance, although there have been studies showing significant HDL cholesterol-increasing and LDL cholesterol-decreasing effects (Baer et al., 2002). However, in one of the few studies large enough to offset the rarity of CHD in younger women (age 34-59 at start), a 20-40% lower CHD risk was found for moderate drinkers as compared with nondrinkers (Stampfer et al., 1988). In post-menopausal women, for whom CHD risk is higher than for their younger counterparts, similar lipid profile effects, as well as reduced CHD risk, have been found to correspond with moderate (1-2 drinks/day) alcohol consumption (Baer et al., 2002). However, for both men and women, any report of heavy episodic drinking was associated with a significantly increased risk of CHD, at 2.26 for men and 1.10 for women (Murray et al., 2002; Rehm et al., 2001).

There are also cardiovascular risks associated with alcohol consumption, at least at heavier drinking levels. Consistent heavy consumption of alcohol often leads to impairment of left ventricular function, which can result in cardiomyopathy (Walsh et al., 2002). Although most likely pluri-causal with at least some genetic component, alcoholic cardiomyopathy is often a complication of longstanding alcohol abuse, related to a person's lifetime dose of ethanol; it can eventually lead to congestive heart failure (Meister et al., 2000). Alcoholism is one of the most important factors in dilative cardiomyopathy, associated with up to 30% of the cases and typically occurring in men between age 30 and 55 who have regularly consumed more than 5 drinks per day for more than 10 years (Flesch et al., 2001; Walsh et al., 2002). Total abstinence has been the standard treatment for alcoholic cardiomyopathy, based on the assumption that any further alcohol consumption is deleterious. However, an evaluation of the effect of reduced drinking in patients with cardiomyopathy found that cardiac contractility improved in all patients who reduced their daily intake to 1-4 drinks/day (n = 15). Drinking at a level of 4-5 drinks/day had mixed results, and functional deterioration continued in most of those who continued to exceed 5 drinks/day (Nicolas et al., 2002). An exception within the last (i.e., > 5 drinks) group were those who, although exceeding 5 drinks/day, nonetheless decreased their previous intake by 50% or more; these 4 patients actually demonstrated a functional improvement.

The observed cardioprotective effect of moderate alcohol consumption may be related to alcohol-induced changes in lipids, lipoproteins, fibrinogen and insulin resistance, as well as to other unknown mechanisms or combinations of mechanisms. Approximately 50% of the reduction in risk has been attributed to moderate alcohol-induced increases in high-density lipoprotein cholesterol (HDL-C) (e.g., De Oliveira e Silva et al., 2000; Gronbaek, 2002; Sillanaukee et al., 2000; van der Gaag et al., 2001). Several other biological mechanisms proposed as alcohol-related contributors to CHD risk reduction include decreased low-density lipoprotein (LDL) oxidation (Durrington et al., 2001; Griffin, 1999; Serafini et al., 2000; Sierksma et al., 2002) and reduced blood clotting and platelet aggregation (Dimmitt et al., 1998; Grenett et al., 1998; Lacoste et al., 2001; Mukamal et al., 2001b; Pellegrini et al., 1996; Ruf, 1999; Sierksma et al., 2001; Tabengwa et al., 2002).

Summary -- CHD

The J-shaped curve has accumulated considerable evidence in cohorts of individuals 40 and over, and it persists after the empirical testing of major alternative explanations such as lifestyle and dietary factors, or composition of abstainer group (Corrao et al., 2000; Rehm et al., 2001). The largest potential benefits of alcohol use in terms of CHD mortality and morbidity apply to older individuals and those otherwise at risk for heart disease (Mukamal, 2003; Mukamal & Rimm, 2001); insufficient research has been done on the lifetime accumulation of CHD benefits – or risks – that may accompany moderate drinking begun in young adulthood.

B. Breast Cancer

The effect of alcohol on the risk for breast cancer remains controversial. Methodological problems are common, including the lack of reporting information about other breast cancer risk factors such as family history and estrogen replacement therapy (ERT). Even in well done case control and cohort studies, researchers use a variety of somewhat arbitrary cutoffs in assessing levels, doses, or amounts of alcohol consumed (Ginsburg, 1999). Thus, when comparing the outcomes of various studies, results for pre- and post-menopausal women are inconclusive; there is no clear evidence of a dose-response relationship; there is a large range of threshold values (between <1/2 and 4 or 5 drinks per day); and, as the strength of the association seems to decrease with an increase in follow-up time, results from 5 year versus 15 year follow-ups are often in conflict (Mannisto et al., 2000).

Although some studies have found a positive correlation between alcohol and breast cancer, others have not (Clavel-Chapelon et al., 2002; Colditz & Rosner, 2000; Ellison et al., 2001; Feigelson et al., 2003; Garland et al.; 1999; Gronbaek, 2001; Horn-Ross et al., 2002; Lash & Aschengrau, 2000; Lenz et al., 2002; Rohan et al., 2000; Smith-Warner et al., 1998; Tjonneland et al., 2003; Zhang et al., 1999); there have even been a few findings of lowered relative risk among light-to-moderate drinkers as compared with abstainers (Baumgartner et al., 2002; Kropp et al., 2001). A substantial number of the "positive" findings have failed to reach standard levels of statistical significance; the researchers generally attribute this to their study's sample size and subsequent limited power to detect associations of the low magnitude observed for alcohol and breast cancer. Other studies report a "significant trend for increasing risk with increasing consumption", although none of the individual levels of consumption actually demonstrate a statistically significant risk. Even when results are statistically significant, in some studies the magnitude of the change in risk for an individual woman is quite small, making the clinical importance of such findings debatable; however, the public health implications, when the change in risk level is applied across 150 million US women, may be substantial.

In a large collaborative re-analysis of 53 studies, one of the larger analyses with statistically significant findings, compared with women who reported drinking no alcohol, the relative risk of breast cancer was increased by a third for an intake of 2 ½ to 3 drinks per day and by nearly half for more than 3 drinks per day. Specifically, the relative risk of breast cancer increased by 7% for each additional two-thirds drink per day (Hamajima et al., 2002). This means that the cumulative incidence of breast cancer by 80 years is estimated to increase from 8.8 per 100 women in non-drinkers to 9.4, 10.1, 10.8, 11.6, 12.4, and 13.3 per 100 women consuming an average of 1, 2, 3, 4, 5, and 6 drinks per day (Hamajima et al., 2002). The risk at higher doses (e.g., 7-8 drinks per day) is difficult to determine, because in most studies the vast majority of participants report less than 4 drinks per day.

One group that does seem to be at substantially increased risk even at low doses is women with a family history of breast cancer. Vachon et al. (2001) found a risk ratio of 2.45 in daily drinkers who were first-degree relatives of breast cancer probands, as compared with never-drinkers. The risk for second degree relatives was not significant, and there was no association for women who had married into the families (i.e., were not biologically related).

A number of pooled studies and meta-analyses have been undertaken to provide the level of statistical power needed to resolve the issue of nonsignificant findings. One pooled analysis of 6 cohort studies found a significant dose response effect with 1 or more drinks per day increasing breast cancer risk by 9%, and 2-5 drinks per day increasing it by 41%. (Smith-Warner et al., 1998). A meta-analysis of 38 studies indicates a steady but modest increase in risk of breast cancer with increasing daily alcohol consumption (Ashley et al., 1997). However, the association is a relatively weak one, and researchers have suggested that, not only can associations of this magnitude be due to bias or measurement error, but that investigation of other factors that may differ by alcohol use (e.g., age, obesity, smoking, reproductive factors, etc.) is necessary before any conclusions can be drawn (Meister et al., 2000). There is some evidence of a monotonic increase in the relative

risk of breast cancer with alcohol consumption; however, the magnitude of the risk was modest – in comparison with non-drinkers, there is a 10% increase in risk for women averaging 1 drink/day (Ellison et al., 2001).

The picture for older women is slightly different. Although there is no consensus on the comparative risks for premenopausal versus postmenopausal women overall, findings for a subset of postmenopausal women have been consistent. Epidemiological evidence indicates that estrogen replacement therapy (ERT) after menopause increases breast cancer risk, and there are data suggesting that ERT combined with alcohol use magnifies that risk (Ginsburg, 1999). In particular, a significant risk is associated with intake of more than 2 drinks/day over a period of years (Stoll, 1999). However, in some studies, even lower levels of alcohol consumption add risk. The Iowa Women's Health Study found that women who consumed an average of one-half drink per day or more manifested increased risk of breast cancer with estrogen administration; lesser consumption or none at all showed no increased risk (Zumoff, 1997). A prospective cohort of 44,187 post menopausal women found that, while there was no significant increase in risk for women who drank at least 1 ½ drinks per day but did not use ERT, the women consuming that amount of alcohol and also using ERT for 5 or more years had a relative risk twice that of non-drinking, non ERT users, i.e., a woman whose lifetime risk for breast cancer is 4% would increase her risk to 8% with 5 or more years of current ERT use and the consumption of > 1 ½ drinks daily (Chen et al., 2002).

The underlying mechanisms of association between alcohol consumption and breast cancer risk are not clear. The role of estrogen and its metabolism is one candidate for causality. Several studies have reported acute and chronic effects of alcohol in raising levels of circulating estrogen (Ginsburg et al., 1996; Ginsburg, 1999), but other studies have failed to observe this effect (Purohit, 1998). Zumoff (1997) proposed that estrogen levels modulate breast cancer risk in individuals with particular genotypes or dietary and exposure history, which may account for conflicting findings across studies.

Several studies have suggested a role for genetic polymorphisms in the alcohol/breast cancer association. Premenopausal (but not postmenopausal) women with the ADH1C 1-1 genotype with even quite low alcohol intake (> 1 ½ drinks per week) regularly over a period of 20 years had a breast cancer odds ratio of 3.6 in relation to women with the ADH1C 1-2 or 2-2 genotypes (Freudenheim et al., 1999). A recent case-control study on glutathione S-transferase (GST) M1 and T1 polymorphisms (Zheng et al., 2003) found that ever-drinking women with the GST M1A genotype had a 2.5 fold increased risk of breast cancer compared to never-drinking GST M1A women, a risk that increased with daily amount and/or duration of alcohol consumption. Postmenopausal women with the GST T1-null genotype and a lifetime consumption of more than 1500 drinks (e.g., 3 drinks per week for a duration of 10 years, or 1 drink per week for 30 years) had an almost seven-fold increase in breast cancer risk. Women at that consumption level with both the GST M1A and the GST T1-null had an even greater odds ratio, 8.2. These findings are consistent with mechanisms that may not necessarily involve estrogen. For example, Rundle et al. (2003) reported that for nontumor tissue of breast cancer cases, current drinkers possessing the GST M1-null genotype exhibited significantly higher levels of DNA damage from polycyclic aromatic hydrocarbons, compared to nondrinkers.

Summary – Breast Cancer

In summary, overall evidence from epidemiologic data seems to indicate that alcohol may be associated with an increase in the risk of breast cancer in the population overall, but that the relative effect of moderate consumption is small at the individual level but can be substantial at the population level; the increase in risk is most clearly evident for women with a family history of breast cancer, and for those using ERT. A degree of uncertainty remains about the effect of a given amount of alcohol on the risk of developing breast cancer in the absence of confounding risk factors, as well as whether there may be a threshold dose below which alcohol has no effect. Although not well-investigated other than via consideration as confounds, individual genetic variations in metabolism and their interaction with carcinogens and dietary factors may play a role. Individual women, with the help of their physicians, must weigh their potential increased risk for breast cancer against their potential reduced risk for CHD in determining whether alcohol consumption should be reduced.

C. Obesity

Obesity results from an imbalance between energy intake and energy expenditure over a prolonged period of time. Given the energy content of alcohol (7.1 kcal/g, as compared to 4.5 kcal/g for protein, 5 kcal/g for carbohydrate and 9 kcal/g for fat), weight gain attributable to drinking could arise if corresponding food intake was not adjusted sufficiently to maintain energy balance. DeCastro and Orozco (1990) found that alcohol supplements rather than displaces food-supplied calories. However, a recent animal model study designed to evaluate the effects of chronic moderate alcohol intake (5% ethanol in drinking water) on energy balance using male rats that are maintained on either a low-fat or a high-fat diet suggests that rats fully compensate for the excess calories associated with alcohol and maintain energy balance regardless of the fat content of the diet (Cornier et al., 2002). Looking at actual changes in weight or body mass index (BMI) rather than calorie-source replacement, a prospective study by Wannamethee and Shaper (2003) found that, over a five year follow-up period, mean body mass index and the prevalence of men with a BMI of 28 or greater (i.e., top quintile of the BMI distribution) increased significantly from the light-moderate to the very heavy alcohol (defined in this study as 2 or more drinks per day) intake group even after adjustment for potential confounding factors. However, a prospective study with a ten year follow-up (Koh-Banerjee et al., 2003) found that changes in levels of alcohol consumption were not associated with changes in waist circumference. Over a shorter timeframe, Cordain et al. (2000) found that the addition of two glasses of red wine to the evening meals for 6 weeks did not adversely affect body weight. Thus far, the evidence on the relationship between moderate alcohol consumption and obesity remains inconclusive.

Metabolic Syndrome:

Metabolic syndrome, which predisposes people to CHD and diabetes and is frequently associated with obesity, has been defined as reaching (or exceeding) threshold levels for any three of five conditions: abdominal obesity, elevated fasting blood triglycerides, low levels of HDL or "good" cholesterol, high fasting blood sugar (glucose) and high blood pressure (National Cholesterol Education Program, cited in Sattar et al, 2003.). An earlier World Health Organization definition also included evidence of insulin resistance in people with normal glucose tolerance as a required factor for diagnosis.

A recent study that examined the association between the quantity and type of alcohol intake with clinical and biochemical markers of metabolic syndrome (e.g. lipid profile, fasting blood glucose, hemoglobin A1c, and fasting serum insulin) in severely obese individuals, revealed that light alcohol consumers (< 7 drinks/week) showed a marked reduction in relative risk of developing Type II diabetes (a frequent complication of obesity) compared with rare or non-consumers, and the frequency of consumption did not influence metabolic syndrome measures (Dixon et al., 2002). Kroenke et al. (2003) found an inverse association of alcohol intake and insulin, but only for women with a BMI >= 25. Insulin levels were lowest for episodic drinkers consuming 2 or more drinks per day, up to 3 days per week, suggesting that moderate alcohol consumption of 1-2 drinks per day on a few to several days per week may have a beneficial glycemic effect, particularly among overweight women.

Diabetes:

The relationship between alcohol intake and the relative risk of developing Type II diabetes is U- or J-shaped. Several studies have demonstrated that moderate drinking is associated with a reduced incidence of Type 2 diabetes in both men and women (Ajani et al., 2000; Rimm et al., 1995). The risk is lower by about 1/3 in moderate drinkers as compared to abstainers, and the association is even stronger for those who drink at levels somewhat beyond the limits of moderation, with the risk decreasing progressively up to 6 drinks/day (Meister et al., 2000; Wannamethee et al., 2002) in some populations. In a 10 year follow-up study, Wannamethee et al. (2003) found a progressively decreasing risk for those consuming ½ (20% reduction) through 2 drinks (nearly 60% reduction) per day, but 3 or more drinks per day conferred the same level of risk as total abstention. Looking at Native American Indian populations, Lu et al. (2003) found a similar pattern, but at lower consumption levels: light (3 drinks/week) and moderate (4-12 drinks/week) drinkers had a lower relative risk of developing Type 2 diabetes while heavier drinkers had an increased risk.

The diabetes-related benefits seem to derive from alcohol's effects on insulin secretion, resistance and sensitivity (Davies et al, 2002; Rimm, 2000). Regular moderate alcohol consumption (4.5 to 11.5 drinks/week) is associated with decreased insulin resistance (Flanagan et al., 2000). Alcohol consumption of 1-2 drinks per day by both men and women was associated with enhanced insulin-mediated glucose uptake, lower plasma glucose and insulin concentrations in response to oral glucose (Facchini et al., 1994). The exact mechanism underlying the insulin sensitizing action of alcohol remains unresolved.

Summary – Obesity & Related Conditions

The relationship between moderate alcohol consumption and weight gain, BMI, or obesity remains inconclusive. However, there appears to be some protective effect of moderate consumption on two of the major sequelae of obesity, i.e., metabolic syndrome and diabetes.

D. Birth Defects

Research over three decades in both human epidemiological studies and animal models has clearly established that alcohol at high consumption levels can cause both physical and neurobehavioral birth defects (Institute of Medicine report -- Stratton et al., 1996). These findings have led to the issuance of a Health Advisory from the Surgeon General of the United States (U.S. Public Health Service, 1981). A specific dysmorphic syndrome, named "fetal alcohol syndrome" (FAS) (Jones and Smith, 1973) was identified and confirmed through research (Stratton et al., 1996). As research has clearly identified three domains of deficits in FAS -- in growth, physical malformations, and neurological/cognitive effects -- it is principally in these domains that potential effects of moderate alcohol exposure could be looked for. However, to date few studies have been undertaken on the effects of low-or-moderate alcohol exposure levels and therefore findings are more limited.

Effects on Growth:

A longitudinal study of alcohol exposure in pregnancy reported a 4 pound decrease in weight at ages 10 and 14 resulting from first trimester exposure to an average daily volume of one drink compared with zero exposure (Day et al., 1999 and 2002). Among women who drank one or more drinks per day during the third trimester, Day et al. (1991) observed continuing smaller size of offspring, including a 1.6 pound decrease in weight at age 3, compared to the offspring of abstainers. In a longitudinal study Sampson et al. (1994) found that effects on size were observable at birth and at 8 months, but not thereafter. A recent case-control study by Yang et al. (2001), provided no evidence of an independent association between moderate maternal alcohol consumption (<14 drinks per week) and risk for intrauterine growth retardation (IUGR). However, unlike the previous studies cited, Yang et al. (2001) collected the maternal alcohol data retrospectively rather than during pregnancy, an approach that appears to be less effective in detecting subtle alcohol effects (S. Jacobson et al., 2002). Additionally, the outcome measure used was a major growth deficit rather than continuous measures of growth, another difficulty in determining subtle effects. Therefore, studies on the risks of moderate prenatal alcohol exposure associated with effects on growth have not yet been definitive.

Morphological Effects:

In general, studies have not found dysmorphology or physical malformation at low to moderate prenatal alcohol exposure levels. In a longitudinal study Sampson et al. (1994) found that dysmorphology of facial features occurred only at the highest levels of consumption. Moreover, the results of a meta-analysis combining seven case-control and cohort studies suggested that moderate alcohol consumption in the first trimester of pregnancy does not increase the risk of major fetal malformations (Polygenis et al. 1998).

Although oral cleft defects are not typically associated with FAS, a case-control study undertaken by Lorente et al. (2000) found a greater than two-fold increase in cleft palate associated with more than 1 drink per day during the first trimester. However, a case-control study by Natsume et al. (2000) found significantly more defects when mothers had less than 1 drink per week than in comparison to the offspring of mothers with higher levels of consumption.

Neurological/Cognitive Effects:

It is the behavioral teratogenic effects of prenatal alcohol exposure (i.e., Alcohol-Related Neurodevelopmental Disorder, or ARND -- the low end of the Fetal Alcohol Spectrum Disorders continuum) that appear to be more sensitive on a dose response basis than the physical teratogenic effects of alcohol, and therefore, are more important in the context of assessing lower dose injury from alcohol. A study examining exposure during the first trimester to an average daily volume of one drink found there were significant effects in verbal learning and memory as measured by the Wide Range Assessment of Memory and Learning (Richardson et al., 2002).

Several other studies have also found deficits in neurodevelopmental parameters at levels averaging 1 to 2 drinks per day. Sampson et al. (1994) found that neurobehavioral functioning was affected from birth through age 14 particularly in the areas of attention, speed of information processing, and learning problems, especially in arithmetic. Goldschmidt et al. (1996) found that children whose mothers consumed seven or more drinks per week during pregnancy had poorer performance in reading and spelling at 7 years of age.

A meta-analytical review by Testa et al. (2003) found effects which differed substantially according to infant age at the time of assessment. While there was a significant negative effect of alcohol exposure on Bayley MDI scores in 12-13 month old infants, there was no association among either 6-8 month olds or 18-24 month olds. Because there are differences in MDI item content for assessing children at different ages, the results may indicate that some skills (i.e., those measured in 1-year olds) are more affected by fetal alcohol exposure than others. Another study (McCarver et al., 1997), which specifically looked at allelic effects, found that drinking during pregnancy was only associated with lower MDI scores in the offspring of mothers without an ADH1B*3 allele.

Important findings have resulted from other, more specific and localized neurocognitive tests that assess specific functional domains of the brain. In one series of studies, infants whose mothers drank seven drinks per week or more on average during pregnancy were more than twice as likely to perform poorly -- in the bottom 16th percentile -- on five different neuropsychological tests, including processing speed on the Fagan Test of Infant Intelligence; elicited (imitative) play on the Belsky scale; and reaction time on the Haith Visual Expectancy Paradigm, as well as the Bayley Mental and Motor Scales; (Jacobson J et al., 1993; Jacobson S et al., 1993; Jacobson S et al., 1994) .

However, research suggests that it is the drinking pattern, rather than average number of drinks per week, which is likely to be the most significant factor affecting adverse pregnancy outcomes. Some investigators who have looked at the distribution of drinking over the week have found that the risk of deficits or defects is highest when women concentrate their weekly drinking by having five drinks or more in one day, while maintaining a weekly consumption of at least seven drinks (Jacobson & Jacobson, 1994; Jacobson J et al., 1998; Streissguth et al., 1993; 1994). Nevertheless, due to individual differences in sensitivity to alcohol, and the likelihood that at certain development time points the fetus is more sensitive to the effects of alcohol, it cannot be assumed that drinking fewer than five drinks per day is a safe threshold.

Impact on Stillbirths:

With respect to risk for the adverse outcome of stillbirth, Kesmodel et al. (2002) found a nearly 3-fold increase in risk of stillbirth among women who reported consuming five or more drinks weekly. The mechanism of action was unclear, as the increased risk could not be attributed to low birth weight, preterm delivery, or malformation, and there was no association between fetal alcohol exposure and risk of first-year death for live-born infants.

Animal Studies:

Animal models have been particularly useful because the dose and pattern of alcohol consumption, as well as many confounding variables associated with human studies, can be more precisely controlled. Nevertheless, the generalizability of findings from animal studies can be

challenging. Studies have used a variety of animal species/strains as well as different alcohol administration paradigms. An important consideration is what constitutes moderate drinking in animal studies versus humans.

The majority of animal studies assessed the effects of moderate alcohol exposure on brain growth, structure, and function. Bonthius and West (1990) demonstrated that a smaller absolute amount of ethanol (4.5 g/kg body wt) administered over a short period of time induced a high blood alcohol concentration (BAC) (mean 362 mg/dl), simulating heavy episodic exposure, whereas a higher daily dose (6.6 g/kg) administered continuously resulted in a low BAC (mean 39 mg/dl). Unlike the high BAC condition, low BACs did not induce microcephaly or cell loss in hippocampus and cerebellum. Another area of active investigation is the impact of moderate alcohol exposure on learning and memory tasks. Recently, Savage et al. (2002) determined that the threshold for maternal BAC that elicits subtle, yet significant learning deficits in adult offspring was 30 mg/dl. This is roughly equivalent to 2 to 3 drinks/day for humans.

Alcohol has been shown to impair the function of the L1 cell adhesion molecule with half maximal inhibition occurring at an alcohol concentration of 7 mM (approximate 35 mg percent), a concentration that can be achieved in blood and brain after one drink. Cell adhesion molecules are critical in the development of the brain as they are involved in the mechanism by which newly developing brain cells migrate to their appropriate location and form appropriate connections to other brain cells. Failure to migrate to the proper location can result in the death of the brain cell by a process called apoptosis. Although this research was conducted in tissue culture systems, the high sensitivity at alcohol dose levels that clearly correspond to low to moderate drinking calls for increased attention, and suggest a potential underlying mechanism explaining the behavioral teratogenic effects of alcohol (Ramanthan et al., 1996).

In research with a chick model of alcohol-induced teratogenic injury, exposure of the developing chick embryo to an alcohol dose equivalent to 35-42 mg/dl, a dose in a human model which would correspond to low to moderate exposure, caused apoptosic cell death of cells from the cranial neural crest. The loss of these specific cells is consistent with the phenotypic characteristics of fetal alcohol injury, including FAS. (Cartwright & Smith, 1995).

Summary – Birth Defects

There is no question about the effects of excessive consumption: heavy drinking during pregnancy can produce a range of behavioral and psychosocial problems, malformations, and mental retardation in the offspring (Kesmodel et al., 2002; Meister et al., 2000; NIAAA, 1992). The question of whether there is a safe level of drinking during pregnancy still remains to be established, with studies indicating that low-to-moderate drinking during pregnancy does not appear to be associated with an increased risk of fetal physical malformations, but may have behavioral or neurocognitive consequences. There is some evidence for a dose-response association, but so far there is not an established threshold level below which consumption is not teratogenic. In the absence of definitive information on low- or moderate-level drinking, in 1981 the Surgeon General recommended that women maintain abstinence during pregnancy.

E. Breastfeeding

Epidemiological data on the effects of moderate drinking throughout the lactation period on the human infant are limited, but there are a larger number of animal studies and human experimental data. Little et al. (1989) reported a slight but statistically significant deficit in motor development at one year of age, as measured by the Bayley Scales of Infant Development, when the lactating mother had an average of 2 drinks daily in the first three months postpartum. The association persisted even after controlling for more than 100 potential confounding variables (e.g., smoking, alcohol/drug exposure during pregnancy, etc.). However, the authors questioned the clinical relevance of the finding, noting that, while very low Bayley scores can be indicative of abnormal developmental progression, small differences (i.e., 7 points, in this case) are not predictive of future patterns, given that infant development scales are not precise measures. Moreover, Little et al. (2002) did not replicate the motor development findings in a later study assessing a larger cohort of infants at 18 months with the Griffiths Scales of Mental Development. The 2002 study actually showed a small but significant “abstainer effect”, with the offspring of non-drinkers having lower scores on 3 of the 5 scales. The discrepant findings may reflect a combination of age differences (12-month -olds in the 1989 study; 18-month-olds in 2002), use of different outcome measures (Bayley Scales versus Griffiths Scale), and the instability of the relatively slight effects found in both studies. Additionally, mothers in the two studies differed in terms of diet, demographics, and lifestyle, adding even more confounds to the mix.

Research indicates that a small amount of alcohol consumed by the mother shortly before the beginning of a breast feeding session can have short term effects on lactational performance and infant behaviors (see review by Mennella, 2001a). Two small human laboratory experimental studies (Mennella and Beauchamp, 1991; 1993) showed that breast-fed infants consumed about 20% less milk on average when the mothers consumed a beverage fortified with 0.3 g ethanol per kg body weight, the equivalent of approximately 1 – 2 drinks. The reduction in milk consumption was due to a slight, but statistically significant, decrease in the mother's milk yield (Mennella 1998), a finding in agreement with an earlier study on rats (Subramanian and Abel, 1988), which demonstrated that the decrease resulted from an inhibition by alcohol of suckling-induced prolactin release. More recently, Mennella (2001b) confirmed the original observation, but reported a compensatory increase in milk consumption by the infant in the 8-16 hr period after exposure to alcohol in the milk resulting from one drink, suggesting that any brief nutritional deficits to the infant are likely to be self-correcting, as long as the mother is only an occasional (as opposed to chronic) moderate drinker. This is confirmed by epidemiologic studies that found no significant differences in weight at 3 and 6 months in infants with lactation-alcohol exposure compared to controls (Flores-Huerta et al. 1992; Villalpando et al. 1993).

The infant’s sleep-wake patterns are also disrupted by acute exposure to 32 mg alcohol in 100 ml of breast milk, resulting in significantly less sleep time during the 3.5 hours immediately after exposure (Mennella and Gerrish, 1998). This finding was replicated in a follow-up study (Mennella and Garcia-Gomez, 2001), which showed that, as with the milk consumption deficits noted above, infants can also compensate for the sleep deficit by increasing the amount of time spent in active (REM) sleep during the 20.5 hr. following the sleep deficit period (i.e., within the same 24-hour cycle). Additionally, as both the reduced milk production/reduced consumption and the infant sleep deficits occur only when breast feeding follows shortly after the mother’s alcohol consumption, it appears that a nursing woman who drinks occasionally can limit her infant’s exposure to alcohol by timing breast feeding in relation to her drinking.

Experience with the sensory qualities of alcohol in mother’s milk during nursing may influence early learning, resulting in altered behavioral responses to alcohol. Mennella (1997) has shown that human infants can detect the flavor of alcohol in milk, even when the alcohol is present in small amounts (32 mg/dL - the average concentration in breast milk one hour after a single drink). Infants who had relatively more exposure to alcohol because of the mother’s drinking pattern responded differently to an alcohol-scented toy than infants with less exposure (Mennella and Beauchamp, 1998). Rodent studies have also shown that exposure to alcohol in a nursing context results in learned, enhanced responses to ethanol in the preweanling animal (Hunt et al., 1993). There as yet have been no longitudinal studies assessing whether this early experience has any effect on the individual’s later (i.e., as an adolescent or an adult) sensitivity or tolerance to alcohol.

The effects of ethanol on the lactation process and on the breast-fed infant have received much less attention than the effects of prenatal alcohol exposure. Several studies (reviewed by Mennella, 2001a), have shown that the concentration and elimination rate of ethanol in human milk closely parallels that of the blood. Peak alcohol levels occur between 0.5 and one hr after a low to moderate dose of alcohol, and the clearance rate is linear. Not surprisingly, variability between individuals on these parameters was observed.

Rodent models of lactational exposure have contributed to our knowledge of the potential effects of chronic exposure to low dose alcohol in breast milk (infant BAC ~ 15-30 mg/dL) on the offspring. However, caution must be exercised in evaluating lactational alcohol effects on brain and behavior of the offspring, because rodent brain development during the immediate postnatal period is more comparable to the third trimester of human gestation, and there are species and strain differences in developmental sensitivity to alcohol. The most consistent finding has been a reduction in overall growth of pups, accompanied by reductions in some organ weights; however, brain weight does not appear to be affected (Lancaster et al., 1984, 1986; Oyama and Oller do Nascimento, 2003). Additional parameters of brain development

have been examined. Alterations in brain myelin content (Lancaster et al., 1984), brain glucose metabolism (Oyama and Oller do Nascimento, 2003), and developmental profiles of enzyme activities in dopaminergic and cholinergic systems of the corpus striatum (Lancaster et al. (1986) have been reported. These changes could potentially give rise to impaired neurotransmitter function leading to altered behavior such as hyperactivity.

Summary – Breastfeeding

Because the level of alcohol in breast milk mirrors the mother's blood alcohol content (i.e., it decreases as time-since-consumption lengthens), nursing mothers can limit their infants' exposure to alcohol by timing their drinking so it does not coincide with feeding schedules. However, while folklore has perpetuated the belief that alcohol is an aid to lactation, and new mothers have often been encouraged to use low or moderate consumption as a way to increase milk production, the research indicates that alcohol ingestion does not enhance lactational performance, and may actually decrease it, at least in the several hours immediately following the consumption period.

F. Aging

Cognitive Effects

Alcohol and its metabolites are known to affect tissues of the central nervous system, and prolonged or excessive alcohol intake has been associated with an increased risk of dementia, both through direct neurotoxic effects and through external causes such as malnutrition and trauma (Brust, 2002; Truelsen et al., 2002). Thus, as life expectancy increases, the effect of alcohol use on cognitive functioning and dementia in older adults has become an important area of investigation. The two most common types of dementia in Western populations are Alzheimer dementia (AD) and vascular dementia (VD). There has been some indication that low to moderate alcohol consumption decreases the risk of VD (Brust, 2002; Peele & Brodsky, 2000; Truelsen et al., 2002). Some studies indicate that levels of consumption less than 1 drink per day, or greater than 4 drinks per day is not significant (Ruitenberg et al., 2002; Mukamal et al., 2003b), while the risk of VD is significantly lowered (RR = 0.30) by consumption of 1 –3 drinks per day (Ruitenberg et al., 2002). Others (Truelsen et al., 2002) found protective effects arising from intake as sporadic as monthly or weekly.

At moderate levels of alcohol consumption (e.g., up to 2 ½ or 3 drinks per day), meta-analyses and epidemiological reviews have failed to find significant effects in relation to AD (English & Holman, 1995; Graves et al., 1991; Tyas, 2001). Among individual studies, consumption levels ranging from 1 drink per week up to 2 drinks per day reduced risk by up to 60% in some studies (Huang et al., 2002; Mukamal et al., 2003b; Orgogozo et al., 1997). No consumption levels demonstrated increased risk.

A number of studies have looked at cognitive function, a different level of assessment than dementia. Moderate alcohol intake was found to be associated with improved cognitive performance (or decreased cognitive impairment) by some researchers (Galanis et al., 2000; Peele & Brodsky, 2000; Zuccala et al., 2001); however, the level of intake that was found to be optimal varies widely across studies, ranging from fewer than 4 drinks per week up to 5 ½ per day for men (up to 2 ½ per day for women). In other studies, levels of consumption from 1 through 30 drinks per week (i.e., as high as 4 drinks per day) were not significantly associated with cognitive impairment or benefit (Broe et al., 1998; Cervilla et al., 2000; Eckardt et al., 1998; Huang et al., 2002).

Macular Degeneration

Because of its relation to vascular risk factors, age-related macular degeneration (AMD) has been postulated to be influenced by moderate alcohol consumption (Ajani et al., 1999; Hiratsuka & Li, 2001). Although one research group (Obisesan et al., 1998) found moderate consumption to be associated with decreased odds of developing AMD, most studies have found no appreciable association in either direction (Ajani et al., 1999; Cho et al., 2000; Moss et al., 1998).

Sensitivity and Tolerance

Tupler et al. (1995) found differences in the pattern but not in the magnitude of skill/task impairment for elderly subjects as compared to younger individuals, with the elderly demonstrating earlier decrements (i.e., impairment), more rapid acute tolerance, and less pharmacodynamic sensitivity. Regression analyses indicated that age and impairment were negatively related, rather than supporting the assumption of synergistic intoxication effects as a function of aging. Although the elderly subjects reached higher BACs than their younger counterparts under equivalent doses, their baseline performance versus their performance at legally intoxicating BACs reflected no age effects. Results failed to confirm that impairment would be either more severe, or more sustained, as a function of age.

Lucey et al. (1997) confirmed the influence of age on blood ethanol response (i.e., BAC level) after a moderate dose of ethanol. However, neither gastric metabolism nor motility accounted for the age/BAC effects, since they were independent of administration route (orally or IV).

Nishimura et al. (2003) compared younger versus older adults stratified according to ALDH2 genotype, looking at ALDH2-normal (NN) versus heterozygote ALDH2-deficient (ND) individuals. Alcohol consumption markedly increased EEG power (especially in theta and slow-alpha power) in the NN subjects but not in the ND subjects of the older group, in comparison to their younger counterparts. As there were no differences between the two age groups in blood ethanol and acetaldehyde concentrations at 30 minutes after alcohol ingestion, the researchers suggest that the rate of alcohol metabolism was not influenced by age. However, sensitivity to alcohol in the central nervous system of the NN subjects seems to have been modified with age, resulting in greater increases in EEG energy after alcohol ingestion in the older group. The findings suggest that both ALDH2 genotype and age modify alcohol sensitivity in the central nervous system.

Summary – Aging

There is some indication that moderate alcohol consumption may reduce risk for vascular dementia, while effects on Alzheimer's dementia and on macular degeneration remain inconclusive. Although elderly drinkers reach higher BACs with lower levels of consumption than their younger counterparts (possibly due to changes in body mass/body water or to decreased hepatic function that affects first pass metabolism), their level of impairment at any given BAC level does not differ from that of younger drinkers.

III. ADDITIONAL AREAS OF POTENTIAL RISKS AND BENEFITS

Alcohol Abuse and Dependence

The likelihood exists that some portion of current abstainers and very infrequent drinkers may succumb to alcohol abuse or dependence if they begin or increase consumption. A number of factors come into play in considering the risk for development of alcohol dependence or abuse, including genetic makeup, environmental contributions, and the interaction of the two. A low estimate might be that 5 to 7 % of current abstainers and/or infrequent drinkers could develop diagnosable alcohol problems with upon beginning usage --- a percentage similar to that in the overall population. However, people's reasons for not drinking vary; if someone abstains because of a family history of alcohol problems or an awareness of their own limitations, he or she is also someone who likely would be at greater than "average" risk for developing dependence (Dawson, 2000; Hasin et al., 2001; Schuckit & Smith, 2001). Epidemiologic data indicate that the greatest risk for the development of alcohol dependence occurs between ages 18 and 25, for the general population as a whole (NIAAA/NLAES, 1992). It is worth noting, however, that most people who do drink have begun by that age, which coincides with legal age requirements, moving away from home, etc. It perhaps is more a situation of the development of dependence occurring within 5-10 years of first regular use, rather than being tied to an actual age. Thus we cannot predict whether abstainers or very infrequent drinkers who take up regular alcohol use later in life based on an expectation of "health benefits" have managed to avoid a developmentally-based high-risk period, or whether the timeframe of risk simply shifts to the 5-10 years that follow their start-up.

Cerebrovascular Effects

Cerebrovascular events (i.e., strokes) are the third leading cause of death and the leading cause of disability in the US (CDC, 2002). The risk of stroke increases with age, with only about 25% of strokes occurring in persons younger than 65 (eMedicine, 2001) . Hemorrhagic strokes account for about 10-15% of all cases, and are more common than ischemic strokes for younger persons (eMedicine 2002). Because blood pressure increases with heavy alcohol consumption, excessive intake levels can be expected to increase the risk of stroke. At lower levels of consumption, however, alcohol's effects on blood lipoproteins and blood clotting might be expected to reduce the risk of ischemic stroke, although the same anti-clotting effects could increase the risk of hemorrhagic stroke (Meister et al., 2000). While heavy alcohol drinking (about 5 drinks/day) is associated with higher relative risk for both ischemic and hemorrhagic stroke, research suggests that moderate drinking lowers the risk of ischemic stroke via a J-shaped curve with the nadir at just under 2 drinks per day, with the consumption of 7 or more drinks per day increasing the risk about 3-fold (Hillbom et al., 1999; Reynolds et al., 2003; Rotondo et al., 2001). The empirical evidence regarding hemorrhagic stroke is mixed; some studies have found no statistically significant association, some have found a J-shaped relationship, and others indicate a linear relationship (Berger et al., 1999; Klatsky, 2002; Reynolds et al., 2003; Rimm, 2000). Overall, the evidence suggests that moderate alcohol intake reduces the risk of stroke in populations where ischemic stroke predominates (i.e., the middle-aged and elderly), but may increase the risk in populations where hemorrhagic strokes are more common, such as young adults (Meister et al., 2000).

Magnetic resonance imaging (MRI) of brains of elderly individuals free of known cerebrovascular disease who consumed moderate amounts of alcohol (1 - <7 drinks/week) showed a lower prevalence of cerebral infarcts and white matter abnormalities (Mukamal et al., 2001a). There is a paucity of experimental data from animal models of ethanol and stroke that might illuminate possible mechanisms underlying these observations.

The vascular endothelium is likely a target for and mediator of many of ethanol's effects both deleterious as well as protective. Low dose alcohol has been shown to be involved in modulating many endothelial cell functions including increased release of nitric oxide, and adhesion receptor expression (Puddey et al., 2001). Treatment with low concentrations of ethanol (2-20 mM) promotes endothelial cell survival (Liu et al., 2002) and may stimulate angiogenesis at 10 and 20 mM (Gu et al., 2001). Studies in animals demonstrate that low dose alcohol augments endothelium-mediated vasodilation whereas higher doses impair endothelium-mediated relaxation. A study examining moderate or heavy alcohol consumption and circulating adhesion molecules (Sacanella et al., 2002) found that moderate drinkers (1½ to 3 drinks /day) showed lower serum adhesion molecule levels than did abstainers and heavy drinkers. The authors suggested that moderate alcohol consumption may have an anti-inflammatory effect on the endothelium, contributing to its vaso-protective effect.

Hepatic Effects

Alcohol abuse is the leading cause of liver-related mortality in the US, accounting for at least 40%, and perhaps as many as 90%, of cirrhosis deaths (Meister et al., 2000). The level of alcohol consumption associated with increased risk for liver disease is uncertain; some studies have suggested levels as low as 14 drinks per week for men and 7 for women (Pequignot and Tuyns, 1980) while others have observed considerably higher thresholds (e.g., see Meister et al., 2000). However, the largest body of evidence suggests that intake of at least 5 drinks/day over a period of at least 5 years is necessary for the development of cirrhosis, while the odds ratio for hepatocellular carcinoma shows a linear increase after more than 4 drinks/day, and becomes statistically significant when consumption levels exceed 5 ½ drinks/day (Donato et al., 2002; Montalto et al., 2002).

Although chronic heavy alcohol consumption leads to the development of alcoholic liver disease, studies in animals showed no significant effects with moderate amounts of alcohol. However, moderate alcohol consumption may potentiate the carcinogenic potency of other hepatotoxins. For example, daily consumption of 1 ½ to 2 drinks per day increased by 35-fold the risk of developing hepatocellular carcinoma induced by dietary aflatoxin B1 (Bulatao-Jayme et al., 1982).

Heavy alcohol intake is also known to impair hepatic regeneration (e.g. Diehl et al., 1990). Recently, data were published reporting the effect of light (1g/kg), moderate (2 g/kg), and heavy (4 g/kg) alcohol intake on hepatic regeneration after partial hepatectomy in rats (Zhang et al., 2000). While heavy alcohol impaired liver regeneration, moderate alcohol had no effect, and light alcohol enhanced liver regeneration. The mechanisms of these effects are not known.

Individuals with Pre-existing Hepatitis C:

Several studies (Bellentani et al., 1999; Harris et al., 2002; Poynard et al., 1997; Thomas et al., 2000) have demonstrated an increased risk for cirrhosis in HCV-infected patients who consume more than 4 drinks per day, with some studies suggesting an increased risk to patients who consume more than 2 drinks per day. However, the hepatotoxic effects of light and moderate amounts of alcohol on HCV infection, progression, and severity need further exploration.

Three studies examined the relationship between moderate levels of alcohol consumption and fibrosis progression in patients with hepatitis C infection (Hezode et al., 2003; Westin et al., 2002; Wiley, et al., 1998). In each instance, moderate alcohol consumption worsened the degree of biopsy confirmed fibrosis. These studies are somewhat comparable since alcohol consumption was defined as less than 2 ½ drinks per day. One study showed a dose dependent increase in fibrosis for an intake between 2 and 3 ½ drinks per day (Hezode et al., 2003).

Oxidative stress is increased in patients with alcoholic liver disease and in patients with HCV. A number of reports link low and moderate alcohol intake with increased oxidative stress and liver fibrosis progression in chronic hepatitis C. Rigamonti et al. (2003) evaluated serum markers of oxidative stress and concluded that moderate alcohol consumption, defined as less than 4 drinks per day, promotes oxidative stress in patients with chronic hepatitis C. However, this was a retrospective analysis on stored serum samples in which auto-oxidation had occurred. Results were compared with freshly obtained control specimens. The presence of steatosis in patients with hepatitis C increased progression rates when low to moderate (1 ½ to 2 drinks per day) amounts of alcohol were consumed (Serfaty et al., 2002).

Oral/Upper Digestive Tract Cancers

Associations have been reported between drinking and cancers of the mouth, pharynx, larynx, and esophagus (Holman et al., 1996; Seitz et al., 1998; 2001). Risk appears to increase directly with consumption level, although it may increase more rapidly at higher drinking levels (Ashley et al., 1997; Gronbaek et al., 1998). In most studies "moderate" drinking per se was not studied, but results were statistically extrapolated; however, some studies have found that alcohol had adverse effects on these diseases even when the usual level of intake was classified as "responsible", with risks increasing by 26% to 83% (Holman et al., 1996).

One hypothesis is that the responsible agent is acetaldehyde, (i.e., the first metabolite of ethanol) which has been shown to be carcinogenic (International Agency for Research on Cancer, 1999). Acetaldehyde can be formed by microbial alcohol dehydrogenases (ADHs) in the upper GI tract (Salaspuro, 1996). High acetaldehyde levels of microbial origin were found in human saliva after a moderate dose of alcohol (Homann et al., 1997). Furthermore, moderate alcohol consumption (0.5 g/kg of body weight) resulted in three-fold higher salivary acetaldehyde levels in Asians with deficient aldehyde dehydrogenase -2 (ALDH2) allele (flushers) than with Asians with normal ALDH2 (nonflushers) (Vakevainen et al., 2000). Higher levels of acetaldehyde in flushers may contribute to the higher incidence of alcohol-associated cancers of the upper digestive tract.

Colorectal Cancer

Estimates of relative risk based on meta-analyses have found modest but statistically significant risk of cancer of the colon and/or rectum at lower levels of alcohol consumption. For consumption up to 2 drinks per day, estimated relative risks range from 1.08 to 1.14 (Bagnardi et al.

2001a; 2001b; English & Holman, 1995). Individual cohort and case/control studies found no increased risk for colon cancer at up to 2 drinks per day, while the risk for rectal cancer was inconsistent across studies, with some studies finding no association and others finding an increased risk for rectal cancer (RR = 1.7) at 1-2 drinks per day (Flood et al., 2002; Ji et al., 2002; Pedersen et al. 2003). Yet another study (Murata et al., 1999) found a significant protective effect for colorectal cancer, as well as colon cancer alone, from consumption of up to 2 drinks per day. Overall, the relationship between moderate alcohol consumption and colorectal cancer is inconclusive, with studies demonstrating some minor effects and many modifying or confounding factors.

Cancer - General

Considering all cancers combined, an American Cancer Society study of middle-aged men found that mortality from cancer was significantly lower among those consuming up to one drink daily, as compared to abstainers (Ashley et al., 1994). However, it seems that any cancer-related benefits conferred occur only at the lower end of the "moderate drinking" range.

Injuries/Accidents

Studies on the role of alcohol in injury from falls and violence/abuse frequently do not distinguish between moderate and excessive drinking (e.g., Cunningham et al., 2003; Humphrey et al., 2003; Vinson et al., 2003; Wells & Graham, 2003; Zautcke et al, 2002). However, many "moderate drinkers" have episodes of high-risk drinking, including heavy episodic drinking and acute intoxication leading to injuries and violence (Gutjahr et al., 2001). Additionally, studies of the acute effects of alcohol show that even moderate-dose consumption compromises brain performance in terms of error detection, processing speed, and response time (Ridderinkhof et al., 2002), impairments that may be particularly important in terms of driving-related risk. Several reports (Deery & Love, 1996; Hingson et al., 1999; Midanik et al. 1996) have indicated that low levels of drinking (e.g., 1 or fewer per day) and BACs below the legal limit of 0.08% (e.g., 0.05%) increase risk of driving-related accidents.

Total (All-Cause) Mortality

A meta-analysis on all cause-mortality (Gmel et al., 2003) using approximately 50 studies demonstrated an inverse association between light to moderate drinking and total mortality under all scenarios, although the extent of the effect (i.e., nadir of risk curve; magnitude of effect) may differ according to demographics (e.g., women versus men; older populations versus younger). The resulting J-shaped curve, with the lowest mortality risk occurring at the level of 1-2 drinks per day, is likely due primarily to the protective effects of alcohol consumption on CHD and ischemic stroke, which comprise the leading cause of death in the US (CDC, 2002).

IV. CONCLUSIONS:

Government dietary guidelines commonly indicate a minimum daily requirement necessary for good health. Health care consumers are familiar with this approach and may easily confuse low-risk guidelines for alcohol use with recommended levels of intake for good health. Thus, "moderate alcohol use" should not be construed as "healthy alcohol use" (Masters 2003).

Furthermore, as described in the "Background" section of this report, the relationship between moderate alcohol consumption and disease outcome is confounded and modified by numerous individual differences – age, gender, genetic susceptibility, metabolic rate, co-morbid conditions, lifestyle factors, and patterns of consumption, just to name a few. Protective and detrimental levels of alcohol consumption cannot be generalized across the population, but instead should be determined by an individual in consultation with her or his physician.

Finally, most of the research refers to the risk of disease occurrence. Some of these illnesses may detract from quality of life without increasing mortality; most differ in prognosis, either via the natural history of the disease or due to currently available treatment options. The potential for moderate alcohol consumption to increase risk for one disease may be offset or outweighed by its potential to decrease risk for another disease, depending on the individual's family history, medical history, genetic makeup, and lifestyle.

While keeping these issues in mind as caveats, the current scientific literature suggests the importance of the following points:

The lowest total all-cause mortality occurs at the level of 1 - 2 drinks per day.

Current scientific data continue to show that moderate levels of alcohol consumption do not increase risk for heart failure/ myocardial infarction or ischemic stroke, and in fact provide protective effects along a J-shaped curve.

There is evidence of a monotonic increase in relative risk of breast cancer with alcohol consumption. Compared to nondrinkers, there appears to be a 10% increase in risk for women averaging 1 drink per day; the risk may be higher for women with a family history of breast cancer and for those on hormone replacement therapy.

The data on the relationship between moderate alcohol consumption and weight gain/obesity are inconclusive. However, there is some evidence for reduced risk of diabetes and metabolic syndrome, which often co-exist with or develop from obesity.

Low-to-moderate drinking during pregnancy does not appear to be associated with an increased risk of fetal physical malformations, but may have behavioral or neurocognitive consequences. There is some evidence for a dose-response association but, so far, there is not an established threshold level below which consumption may be safe. There is no question about the effects of excessive consumption: heavy drinking during pregnancy can produce a range of behavioral and psychosocial problems, malformations, and mental retardation in the offspring.

Alcohol ingestion by nursing mothers does not enhance lactational performance, and may actually decrease it, at least in the several hours immediately following the consumption period. Effects on the infant appear to be short-term and reversible; however, as alcohol dissipates from breast milk over time, the safest course would be to allow sufficient time between drinking occasion and feeding session for the mother to fully metabolize the alcohol.

There is no evidence that cognitive functioning is negatively affected by moderate alcohol consumption as one ages, and there may be a protective effect against vascular dementia.

Summary – Conclusions

The current scientific knowledge on the risks and benefits related to various levels of alcohol consumption does not suggest a need to modify the existing guidelines on moderate alcohol use. Except for those individuals at particular risk (as are described in the current guidelines), consumption of 2 drinks a day for men and 1 for women is unlikely to increase health risks. As risks for some conditions and diseases do increase at higher levels of consumption, men should be cautioned to not exceed 4 drinks on any day and women to not exceed 3 on any day.

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Hepatic gamma-glutamyltransferase activity in alcoholic fatty liver: comparison with other liver enzymes in man and rats

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SUMMARY Compared with controls, patients with alcoholic fatty liver showed a significant increase of gamma-glutamyltransferase activity both in the liver and serum, whereas alkaline phosphatase activity was raised only in the liver but not in the serum. The activities of other enzymes such as aspartate aminotransferase, alanine aminotransferase and glutamate dehydrogenase remained virtually unchanged in the liver of patients with alcoholic fatty liver but were strikingly enhanced in the serum. The hepatic and serum alterations of enzymic activities observed in patients with alcoholic fatty liver could be reproduced in the rat model of alcoholic fatty liver only for gamma-glutamyltransferase but not for the other enzymes tested, substantiating evidence that the animal model may serve as an appropriate tool for studying interactions between alcohol and gamma-glutamyltransferase. The present experiments also indicate that the primary cause for increased serum gamma-glutamyltransferase activities associated with prolonged alcohol consumption is hepatic enzyme induction rather than liver cell injury.

Patients with alcoholic liver disease may exhibit increased serum activities of various enzymes including gamma-glutamyltransferase (GGT),[1] alkaline phosphatase (ALP),[2] aspartate aminotransferase (AST),[1 3] alanine aminotransferase (ALT),[1 3] and glutamate dehydrogenase (GDH).[1] Rises in these enzyme activities are commonly found in the serum of patients with severe alcoholic liver disease, but they may be present already at early stages of the disease such as the fatty liver stage associated with alcohol abuse. In particular, striking rises of serum GGT are common already at the alcoholic fatty liver stage, and the determination of GGT is therefore now preferred to the transaminases for assessing early stages of liver disease due to alcohol abuse.

The pathogenesis of increased serum activities of various clinically used enzymes in alcoholic liver disease is poorly understood and might reflect enhanced leakage of enzymes out of the hepatocytes, hepatic enzyme induction or extrahepatic origin of enzymes because of alterations of other tissues. The aim of the present investigation was therefore to study the relationship of commonly used hepatic enzymes in patients with alcoholic fatty liver by assessing the enzyme activities not only in the serum but also directly in the liver. Moreover, for comparison reasons, similar studies have been carried out in rats with alcoholic fatty liver due to prolonged ethanol intake in order to assess the validity of this commonly used experimental animal model regarding enzyme alterations observed under similar conditions in man.

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Methods

PATIENTS

Nineteen alcoholics (seven women and 12 men, age 45±4 years) with alcoholic fatty liver admitted to the Medical Department of the University of Düsseldorf were included in the study. The results of the clinical as well as laboratory examination had been suggestive of alcoholic liver disease, and blind liver biopsy or laparoscopy was indicated. After informed consent was obtained from each patient, liver specimens were obtained within one week after admission by blind liver biopsy using a Menghini

needle or at laparoscopy. In general, 20–30 mg of liver tissue were obtained, part of which was sent for routine histology. For the histological diagnosis of fatty liver the presence of fat droplets in more than 25% of the hepatocytes were required. The remaining liver specimen was immediately homogenised in 66 mM phosphate buffer (pH 7·4) with a glass homogeniser, and the final homogenate containing 10 mg of liver per millilitre was used for the subsequent enzyme assays. Blood was obtained by venepuncture on the same day of the liver biopsy, and after centrifugation the serum was used for enzyme assays.

The control group consisted of nine patients (five women and four men, 44±4 years) with normal values of serum transaminases and gamma-glutamyltransferase and normal appearances of liver biopsy specimens, who underwent laparoscopy or laparotomy for diagnostic or therapeutic reasons. The alcohol consumption of this group was nil or negligible.

EXPERIMENTAL STUDIES

Sixteen female Sprague-Dawley rats were obtained from Zentralinstitut für Versuchstierzucht in Hannover (Federal Republic of Germany) and fed Altrumin laboratory chow and tap water *ad libitum* until they reached a body weight of 200–220 g which was achieved at an age of about 75 days. The animals were then housed in individual wired bottom cages and fed for six weeks with liquid diets in drinking tubes as the only source of food and water. The rats were pair-fed nutritionally adequate diets which contained either 36% of total calories as ethanol or additional carbohydrates on an isocaloric basis.[4] At the end of the feeding procedure the animals were killed by decapitation, and blood was collected from the neck vessels for the determination of serum enzyme activities. Part of the liver was excised for histological examination by haematoxylin eosin staining, which revealed a fatty liver in the alcohol fed animals. The remaining liver was homogenised to be used for enzyme assays.

BIOCHEMICAL DETERMINATIONS

GGT activity was measured in liver homogenates and serum spectrophotometrically according to the method of Szasz.[5] The activity of ALP was determined using the method of Hausamen *et al*,[6] and for the measurement of AST activity the method of Bergmeyer and Bernt[7] was followed. The activities of ALT and GDH were assayed by the method of Bergmeyer and Bernt[8] and Schmidt,[9] respectively. The determination of protein was performed according to the method of Lowry *et al*,[10] using crystalline human albumin as standard.

STATISTICAL ANALYSIS

Each measurement was carried out in duplicate. The results are expressed as means (± SD), and the significance of the differences was assessed by the Wilcoxon's test.

Results

GAMMA-GLUTAMYLTRANSFERASE

Compared with controls without biochemical and histological signs of liver disease, patients with the histological diagnosis of alcoholic fatty liver showed a striking rise of hepatic GGT activity (Table 1). The enhancement was 150% ($p<0·01$) when expressed per gram of liver wet weight and 119% ($p<0·01$) when calculated per gram of liver protein. Similarly, the hepatic GGT activity was significantly increased in rats with an alcoholic fatty liver after six weeks of alcohol intake when compared with animals fed the control diet (Table 2).

Patients with an alcoholic fatty liver exhibited a striking enhancement of serum GGT activity compared with controls (Table 1). There was also a significant rise of serum GGT activities in the experimental rat model of prolonged alcohol administration when compared with their respective controls pair-fed the control diet (Table 2). As in the hepatic enzyme activities, the basal and alcohol-induced serum levels of GGT activities were considerably lower in rats (Table 2) than in man (Table 1).

ALKALINE PHOSPHATASE

Alcoholic fatty liver in man was associated with a significant rise in hepatic ALP activity when compared with controls, and this alcohol mediated increase of hepatic ALP activity was not associated with any significant change of serum ALP activity (Table 1). Conversely, in rats with an alcoholic fatty liver a significant rise of serum ALP activity could be shown, whereas hepatic ALP activity remained virtually unchanged in comparison with their respective controls (Table 2). The basal levels of hepatic ALP activities were about one order of magnitude higher in man than in rats, whereas the corresponding serum values were in the same order of magnitude in both species (Table 1 and 2).

ASPARTATE AMINOTRANSFERASE

Compared with controls, hepatic AST activity remained virtually unchanged in patients with alcoholic fatty liver (Table 1) but was slightly increased in rats with alcoholic fatty liver when expressed per gram of liver wet weight or per 100 gram of body weight but not when calculated per gram of liver protein (Table 2). Serum AST

Table 1 *Enzyme activities in patients with alcoholic fatty liver*

Assay	*Control*	*Alcoholic fatty liver*
Liver GGT (U/g wet weight)	1·91±0·62	4·78±1·81†
(U/g protein)	16·4±6·6	35·9±16·1†
Serum GGT (U/l serum)	13·7±5·91	195·0±406·7†
Liver ALP (U/g wet weight)	3·58±1·54	5·22±2·62*
(U/g protein)	30·3±16·8	42·2±24·4*
Serum ALP (U/l serum)	110·7±72·2	124·0±34·9 NS
Liver AST (U/g wet weight)	29·2±11·1	35·5±16·5 NS
(U/g protein)	268·0±138·0	257·0±100·3 NS
Serum AST (U/l serum)	11·1±4·6	34·8±24·6†
Liver ALT (U/g wet weight)	19·2±13·8	18·5±37·1 NS
(U/g protein)	149·0±78·0	137·0±74·0 NS
Serum ALT (U/l serum)	16·6±8·2	30·6±20·2‡
Liver GDH (U/g wet weight)	79·2±30·0	80·9±18·3 NS
(U/g protein)	620·0±207·0	591·0±302·4 NS
Serum GDH (U/l serum)	1·92±2·3	9·91±13·2†
Liver protein (mg/g wet weight)	143·0±52·8	137·47±44·11 NS

Enzyme activities of gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glutamate dehydrogenase (GDH) were determined in the liver and serum of patients with alcoholic fatty liver (n=19) and in their respective controls without liver disease (n=9). The results represent means ± SD.
* p<0·05, † p<0·01, ‡ p<0·001.

Table 2 *Enzyme activities in rats with alcoholic fatty liver*

Assay	*Control*	*Alcoholic fatty liver*
Liver GGT (U/g wet weight)	0·07±0·03	0·14±0·06§
(U/g protein)	0·79±0·19	1·19±0·23‡
(U/100g bw)	0·34±0·09	0·80±0·28§
Serum GGT (U/l serum)	2·19±0·31	4·41±1·64‡
Liver ALP (U/g wet weight)	0·40±0·11	0·39±0·08 NS
(U/g protein)	3·66±0·99	3·48±0·59 NS
(U/100g bw)	1·83±0·60	2·25±0·84 NS
Serum ALP (U/l serum)	161·0±42·4	283·0±67·9‡
Liver AST (U/g wet weight)	69·9±6·22	96·1±8·2§
(U/g protein)	729·0±155·6	849·0±87·7 NS
(U/100g bw)	323·6±40·7	498·0±56·5§
Serum AST (U/l serum)	99·0±14·4	108·0±17·3 NS
Liver ALT (U/g wet weight)	11·1±2·0	20·2±0·49§
(U/g protein)	121·0±20·1	169·0±26·0§
(U/100g bw)	55·2±9·2	101·2±12·4§
Serum ALT (U/l serum)	25·0±5·9	59·2±9·6§
Liver GDH (U/g wet weight)	130·0±19·8	168·0±19·8‡
(U/g protein)	1221·0±305·5	1431·0±223·4 NS
(U/100g bw)	558·0±131·4	893·0±182·2§
Serum GDH (U/l serum)	3·87±1·44	6·80±2·57†
Liver protein (mg/g wet weight)	106·1±23·59	117·9±15·74 NS
(mg/100g bw)	470·57±97·31	594·35±115·1†
Liver weight (g)	10·96±1·53	11·91±1·55 NS
(g/100g bw)	4·49±0·64	5·04±0·59*
Body weight (g)	244·75±11·85	222·38±45·14 NS

Enzyme activities of gamma-glutamyltranferase (GGT), alkaline phosphatase (ALP), aspartate aminotranferase (AST), alanine aminotransferase (ALT), and glutamate dehydrogenase (GDH) were determined in the liver and serum of rats with alcoholic fatty liver (n=8) after feeding of a nutritionally adequate liquid alcohol diet for 6 weeks and in their pair-fed controls (n=8). The results represent means ± SD.
* p<0·05, † p<0·025, ‡ p<0·01, § p<0·001.

activities were significantly enhanced in the fatty liver group only in man (Table 1) but not in rats (Table 2) when compared with their respective control groups. Basal AST activities were considerably higher in rats than in man, both in serum and liver (Table 1 and 2).

ALANINE AMINOTRANSFERASE

In comparison with controls, patients with alcoholic fatty liver failed to exhibit significant alterations of hepatic ALT activities (Table 1) whereas rats with an alcoholic fatty liver showed a pronounced increase of enzymic activity (Table 2). In the serum of both man (Table 1) and rats (Table 2) there was a significant rise of ALT activity in the alcoholic fatty liver group when compared with the corresponding control group. In addition, basal ALT activities of liver and serum were in the same order of magnitude for man and rats (Table 1 and 2).

GLUTAMATE DEHYDROGENASE

GDH activity was similar in the liver of patients with alcoholic fatty liver and of their corresponding controls, whether the data were given per gram of liver wet weight or per gram of liver protein (Table 1). Compared with controls, alcoholic fatty liver in rats was associated with a rise in hepatic GDH activity which was significant when expressed per gram of liver wet weight or per 100 gram of body weight but not when calculated per gram of liver protein (Table 2). In both man (Table 1) and rats (Table 2) with alcoholic fatty liver, however, there was a striking enhancement of serum GDH activities when compared with their respective control groups. Finally, basal values for hepatic and serum GDH activities were in the same order of magnitude both in man and rats (Table 1 and 2).

ENZYME RATIO

The serum enzyme ratio for AST/ALT in patients with alcoholic fatty liver was more than doubled compared with the respective controls, but the difference failed to achieve the level of statistical significance ($1{\cdot}64 \pm 1{\cdot}57$ *vs* $0{\cdot}72 \pm 0{\cdot}24$; NS).

Discussion

The present study shows that patients with alcoholic fatty liver exhibit increased GGT activities not only in the serum but also in the liver when compared with their respective controls (Table 1). Conversely, enhanced hepatic ALP activities in this patient group are not associated with a concomitant rise of ALP activity in the serum. Moreover, the activities of other enzymes such as AST, ALT, and GDH remained virtually unchanged in the liver of patients with alcoholic fatty liver but were strikingly enhanced in the serum (Table 1). These data therefore show that GGT is a unique enzyme in the sense that its activity is augmented in both liver and serum of patients with alcoholic fatty liver, whereas ALP, AST, ALT, and GDH activities are increased either in the liver or in the serum.

There has been considerable debate concerning the mechanism of increased serum GGT activities owing to chronic alcohol consumption.[11–14] Studies in experimental animals have shown that chronic alcohol consumption depresses hepatic GGT activities, suggesting that increased serum GGT activities might be because of an enhanced release of hepatic GGT into the bloodstream as a consequence of liver cell injury.[14] This hypothesis, however, could not be substantiated in the present study showing an increased rather than decreased hepatic GGT activity after prolonged alcohol intake both in man (Table 1) and rats (Table 2), agreeing thereby with the interpretation of other reports.[11 15–23] This study also clearly indicates that the commonly used rat model for alcohol feeding of DeCarli and Lieber[4] is an appropriate tool for studying interactions between alcohol and GGT, as alterations of the GGT enzyme pattern in the serum and liver because of alcohol are similar in both species. Indeed, it has been speculated that enhanced activities of GGT observed after experimental alcohol feeding might exclusively be because of dietary imbalance with respect to carbohydrates rather than to alcohol itself,[12] but this thesis was disproved by other studies.[15 18 19 22 23]

The observation of increased hepatic GGT activities due to prolonged alcohol intake both in man (Table 1) and experimental animals (Table 2) raises the question of the subcellular site of enhanced GGT activity in the liver cell. In experimental studies in rats, hepatic GGT activities have been found to be increased because of chronic ethanol consumption in microsomal fractions[11 15 24] as well as plasma membrane fractions of the hepatocytes, both free of as well as rich in bile canaliculi.[16 17] It is reasonable to assume that the hepatic induction of GGT activity after ethanol administration occurs primarily at the site of the endoplasmic reticulum.[17] The enzyme could then be translocated to plasma membranes, possibly by means of the Golgi apparatus. The possibility cannot be ruled out, however, that part of the GGT activity recovered in the microsomal fraction is due to plasma membrane GGT, as contamination of microsomal fractions by plasma membranes during the course of subcellular fractionation is unavoidable.[16 17]

Contrasting with the values obtained for GGT,

ALP activities remained unchanged in the serum of patients with alcoholic fatty liver but were significantly enhanced in the liver (Table 1), whereas the reversed constellation was found in the experimental rat model with alcoholic fatty liver (Table 2). In addition, it is of particular interest that alcohol leads to a striking induction of ALP activity in the human liver, a condition not associated with an increased activity of the corresponding serum enzyme, suggesting that at the alcoholic fatty liver stage hepatic enzyme induction of ALP by itself is not sufficient to cause an increase of enzyme activity in the serum. As striking rises of serum ALP activities can be found in patients with severe stages of alcoholic liver disease, additional factors apart from hepatic enzyme induction have to be incriminated, and different isoenzymes may be also be involved.

Patients with alcoholic fatty liver failed to show significant alterations of hepatic AST, ALT, and GDH activities (Table 1). With respect to hepatic AST and ALT activities, similar results have been published in patients with fatty liver in the absence of alcohol abuse when compared with healthy controls.[25] In patients with late stages of alcoholic liver disease, however, such as alcoholic hepatitis or cirrhosis, and associated striking increases of serum AST and ALT activities, a fall of hepatic AST and ALT activities was shown.[25] It appears from these studies[25] as well as the present data (Table 1) that slight increases of serum AST and ALT activities are not necessarily associated with a significant change of hepatic enzyme activity, whereas this may not pertain to severe liver disease.[25 26] Under the latter conditions, hepatic AST and ALT may be released from the liver in amounts sufficient to cause a striking decrease of hepatic enzyme activity. It is also noteworthy that the data in man regarding AST, ALT and GDH activities (Table 1) could not be universally reproduced in the experimental rat model of chronic alcohol administration (Table 2).

Previous studies have shown that a AST/ALT ratio in the serum greater than two is highly suggestive for alcoholic hepatitis and cirrhosis.[26] Conversely, the present study indicates that this statement may not be applicable for patients with alcoholic fatty liver. Indeed, in this patient group the mean value of AST/ALT ratio in the serum was only 1·64, and dissociation from other forms of hepatobiliary diseases[26] by the use of the serum enzyme ratio is therefore not possible.

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Hepatic gamma-glutamyltransferase activity in alcoholic fatty liver: comparison with other liver enzymes in man and rats.

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Fundamental and Molecular Mechanisms of Mutagenesis

Effects of antioxidants on streptonigrin-induced DNA damage and clastogenesis in CHO cells

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Abstract

The effect of several free radicals scavengers on DNA damage and clastogenesis induced by streptonigrin (SN) in CHO cells was investigated.The addition of the antioxidant enzymes superoxide dismutase and/or catalase on CHO cell cultures did not prevent the induction of DNA and chromosome damage by SN. In fact, when superoxide dismutase was added to the culture medium an increase on the frequency of SN-induced chromosome aberrations was observed. Moreover, the addition of the hydroxyl radicals scavenger mannitol caused a significant increase in DNA and chromosome damage induced by SN. On the contrary, when all the antioxidants mentioned above were added – alone or in different combinations – encapsulated into liposomes, a significant decrease in the yield of SN-induced chromosome aberrations and DNA damage was observed. These findings indicate that free radicals are involved in the production of DNA and chromosome damage by SN and that this damage can be partially inhibited through the incorporation of antioxidants by the cells.

Keywords: Streptonigrin; Superoxide dismutase; Catalase; Mannitol; DNA damage; Chromosomal aberration

1. Introduction

Streptonigrin (SN) is a metabolite of *Streptomyces flocculus* that possesses antitumor activity. It has been demonstrated that in non-cellular systems SN is reduced by NADH, giving rise to a semiquinone radical which can undergo autoxidation resulting in the generation of the free radical superoxide (O_2^-) [1]. The sequence of reactions is the following:

$$SN + NADH \rightarrow SNH_2 + NAD \quad (\text{reduction of SN}) \tag{1}$$

$$SNH_2 + O_2 \rightarrow SNH^{\cdot} + OH_2^{\cdot} \quad (\text{generation of semiquinone radical}) \tag{2}$$

$$OH_2^{\cdot} \rightarrow O_2^- + H^+ \quad (\text{generation of superoxide radical}) \tag{3}$$

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We have recently reported preliminary data on the action of SN on chromosomes from CHO cells [2]. We suggested that SN induces a persistent process of clastogenesis that continues even after the drug is washed out from the culture medium and we demonstrated that SN has a delayed clastogenic effect that lasts for at least three cell cycles [3]. To explain this finding, we postulated that SN very likely induces direct DNA degradation by free radical production and indirect DNA damage by the formation of stable SN-DNA complexes. Very recently, we demonstrated that DNA substitution with halogenated deoxyuridine potentiates the chromosome damage induced by SN [4]. The mechanisms of SN-induced DNA and chromosome damage modulation in cellular systems are at the present time unknown. It has been reported that the addition of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) to non-cellular systems totally inhibits the DNA cleavage induced by SN [1]. In this report, we investigated whether the addition of antioxidants into the culture medium can modulate the DNA and chromosome damage induced by SN in a cellular system (CHO cells). We demonstrate that DNA damage and clastogenesis induced by SN can be partially inhibited by the addition of liposome-entrapped antioxidants into the culture medium, thus indicating that free radicals are involved in the production of DNA and chromosome damage by SN.

2. Materials and methods

2.1. Cytogenetic analysis

CHO cells were grown in Ham F10 medium (Sigma) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). During the log phase of growth the cells were treated for 20 min with SN (CAS No. 3930-19-6) (Sigma) at the single dosis of 250 ng/ml. Bovine SOD (Cu,Zn-SOD, CAS No. 9054-89-1) (100 μg/ml), CAT (CAS No. 9001-05-2) (100 μg/ml) and mannitol (MAN) (CAS No. 69-65-8) (0.05M) (all purchased from Sigma) were added to the cultures 30 min before the addition of SN. Different treatments as indicated in Tables 1 and 2 were performed. At the end of the pulse treatment the SN and the antioxidants were removed by washing the cells twice with Hanks' balanced solution and then fresh culture medium was added. Cultures were kept at 37°C and harvested at 18 h after the end of treatments. During the last 2.5 h of culture the cells were exposed to colchicine (0.1 μg/ml) (CAS No. 64-86-8) (Sigma). Chromosome preparations were made according to the flame-drying method. One hundred metaphases per treatment were scored for chromosomal aberrations.

Statistical significance of the comparison of aberration frequencies among different treatments was

Table 1
-Chromosomal aberrations induced by SN in the presence of antioxidants

Treatment	MI	Aberrations (absolute frequency per 100 cells)							Total aberrations	Damaged metaphases (%)
		Chromatyd-type			Chromosome-type					
		Breaks	Exch.	Total	Dic.	Rings	Del.	Total		
Control	9.9	3	0	3	1	0	0	1	4	4
SN	5.3	22	3	25	7	1	5	13	38	25
SN/SOD	4.9	41	9	50	7	3	6	16	66	44
SN/CAT	4.1	16	4	20	6	2	0	8	28	20
SN/SOD/CAT	4.8	18	3	21	3	2	3	8	29	17
SN/MAN	2.4	117	26	143	13	2	12	27	170 [a]	69
SOD	10.8	4	0	4	0	0	0	0	4	3
CAT	5.7	1	0	1	1	1	0	2	3	3
SOD/CAT	6.8	1	0	1	1	0	0	1	2	2
MAN	7.8	2	0	2	1	0	0	1	3	3

SN, streptonigrin; MAN, mannitol; MI, mitotic index; Exch., exchanges; Dic., dicentrics; Del., deletions. [a] $p < 0.05$.

Table 2
-Chromosomal aberrations induced by SN in the presence of antioxidants encapsulated into liposomes

Treatment	M.I.	Aberrations (absolute frequency per 100 cells)							Total aberrations	Damaged metaphases (%)
		Chromatyd-type			Chromosome-type					
		Breaks	Exch.	Total	Dic.	Rings	Del.	Total		
Control	8.6	13	1	14	0	0	0	0	14	13
SN	6.8	52	3	55	4	2	6	12	67	40
SN/SOD	6.5	20	1	21	0	0	2	2	23 [b]	21
SN/CAT	6.5	17	0	17	3	1	3	7	24 [b]	21
SN/SOD/CAT	7.0	16	0	16	1	1	4	6	22 [b]	16
SN/MAN	8.5	14	0	14	0	0	0	0	14 [b]	12
SOD	35.2	12	0	12	0	0	1	1	13	11
CAT	7.6	13	1	14	0	0	0	0	14	12
SOD/CAT	13.0	9	1	10	0	0	0	0	10	9
MAN	10.9	10	0	10	2	1	1	4	14	12

SN, streptonigrin; MAN, mannitol; MI, mitotic index; Exch., exchanges; Dic., dicentrics; Del., deletions. [b] $p < 0.01$.

obtained by comparing Poisson distributions with 95% confidence intervals [5].

2.2. DNA analysis

Exponentially growing CHO cells were labeled for 24 h with 0.1 uCi/ml of [^{3}H]TdR (New England Nuclear, sp. act. 6.7 Ci/mM) and chased for 3 h with 2×10^{-6} M cold TdR. Afterwards the cells were resuspended in fresh culture medium at a concentration of 2.5×10^5 cells/ml. SN treatments were performed at this stage. Antioxidants were added to the cultures 30 min before the addition of SN at the same doses as indicated in cytogenetic analysis. At the end of the 20-min treatment the cells were washed twice with cold Hanks' saline. Alkaline unwinding was performed immediately after the washing [6]. Aliquots of 10^6 cells were lysed in 0.03 M NaOH, 0.01 M Na_2HPO_4, 0.9 M NaCl, pH 12.2 for 30 min at room temperature in the dark. The samples were afterwards neutralized with an equal volume of 0.03 N HCl and disrupted by sonication. Lysis was completed by adding 8.7 mM SDS. The samples were stored at −20(C until further processing. Chromatography was performed in hydroxyapatite columns. Single-stranded (ss) and double-stranded (ds) DNA were eluted with 0.08 M and 0.2 M potassium phosphate buffer, respectively. The amounts of both forms of DNA in each experimental point were determined by liquid scintillation counting, and the mass of ssDNA was estimated as follows:

$$\text{ssDNA} = \frac{(\text{cpm})\text{ssDNA}}{(\text{cpm})\text{ssDNA} + (\text{cpm})\text{dsDNA}}$$

An increase in the mass of ssDNA indicates DNA degradation [6]. Each experiment was performed in duplicate and two sets of samples were run simulta-

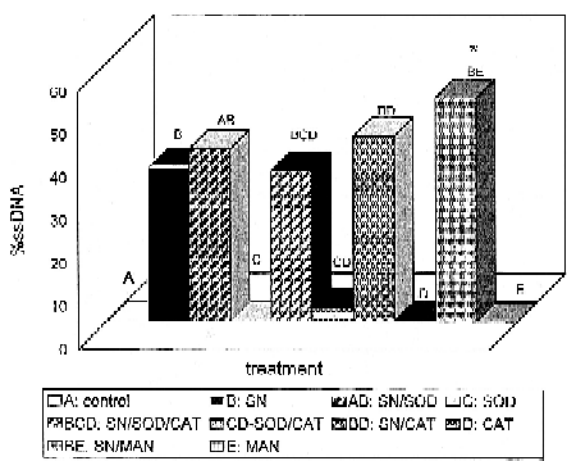

Fig. 1. DNA damage analysis of control CHO cells and CHO cells treated with SN (250 ng/ml) alone or in the presence of free antioxidants. The percentage of ssDNA observed for each treatment performed is indicated. SN, streptonigrin; SOD, superoxide dismutase; CAT, catalase; MAN, mannitol. * $p < 0{,}05$.

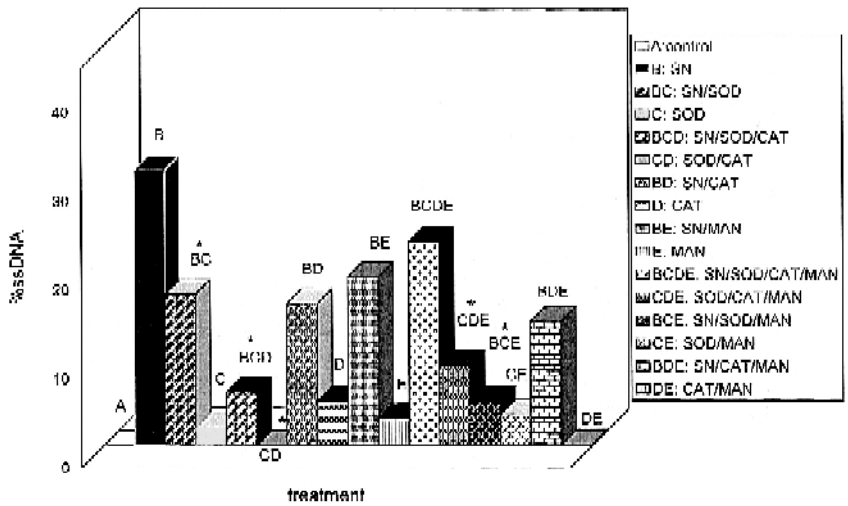

Fig. 2. DNA damage analysis of control CHO cells and CHO cells treated with SN (250 ng/ml) alone or in the presence of liposome-entrapped antioxidants. The percentage of ssDNA observed for each treatment performed is indicated. SN, streptonigrin; SOD, superoxide dismutase; CAT, catalase; MAN, mannitol. * $p < 0{,}05$.

neously for each experiment and each endpoint. The different treatments performed are indicated in Figs. 1 and 2.

In order to determine the main effects and interactions between SN and the antioxidants, Multifactorial Analysis of Variance (MANOVA) was used. The level of significance chosen was $p < 0.05$.

2.3. Preparation and utilization of liposomes

In order to facilitating the delivery of the antioxidants to CHO cells, in a second set of experiments the cells were treated with SOD, CAT and mannitol entrapped into liposomes and the corresponding cytogenetic and DNA analysis were made as indicated above. The antioxidants were encapsulated into small unilamellar liposomes by a previously published method [7]. Briefly, L-α-phosphatidylcholine dimyristoyl (C14:0) (CAS No. 80724-31-8) (Sigma) and L-α-phosphatidic acid dimyristoyl (C14:0) (CAS No. 18194-24-6) (Sigma) were mixed at a molar ratio of 3:1, respectively, and were first partially dried in a nitrogen atmosphere and then brought to complete dryness in a vacuum. Twenty milligrams of this dried mixture was added to 1 ml of the antioxidant solutions at the concentrations mentioned above, and the mixture was centrifuged at $12\,000 \times g$ for 5 min at 4°C and the supernatant discarded. Afterwards, the resultant pellet was resuspended in Hanks' balanced solution and sonicated for 15 min at 37°C. Residual large vesicles were removed by centrifugation at $12\,000 \times g$ for 5 min at 4°C. This procedure gives rise to a small unilamellar vesicles preparation with a mean diameter ranging from 0.02 to 0.05 μm. CHO cells in logarithmic growth phase were treated with liposome entrapped antioxidants. Control cultures were treated with Hank's saline encapsulated into liposomes.

3. Results

3.1. Cytogenetic analysis

The scoring of aberrations was carried out 18 h after the end of the pulse treatment with SN. Previous experience [3,4] shows that at this harvesting time most CHO treated metaphases cells are in first mitosis. The total frequency of chromatid and chromosome aberrations, the percentage of damaged cells and the mitotic index for each treatment and each set of experiments are shown in Tables 1 and 2. There was a clear predominance of chromatid over chromosomal damage (Tables 1 and 2). The Papworth

test showed that the frequencies per cell of both types of aberrations fit a Poisson distribution for the majority of the treatments. Table 1 shows that the addition of CAT or CAT/SOD caused a slight decrease in the frequency of SN-induced chromosomal aberrations ($p > 0.05$). Neither SOD nor mannitol prevented the chromosome damage induced by SN. What is more, when SOD ($p > 0.05$) or MAN ($p < 0.05$) were added to the culture medium an increase in the frequency of SN-induced chromosomal aberrations was observed (Table 1). On the contrary, when the antioxidants were added encapsulated into liposomes, a significant decrease in the yield of SN-induced chromosome aberrations for all the treatments was observed ($p < 0.01$) (Table 2). The effect was more evident with MAN – which completely inhibited the chromosome damage induced by SN – than with SOD and/or CAT (Table 2y). No differences between control and positive control cultures (treated with Hanks' saline entrapped into liposomes) were found ($p > 0,05$).

3.2. DNA analysis

Two sets of experiments with alkaline unwinding were performed to analyze the extent of DNA degradation after 20 min treatments with SN and the previous addition of the same antioxidants used in the cytogenetic analysis. Figs. 1 and 2 illustrate the results of these experiments. Fig. 1 shows that the addition of antioxidant enzymes or mannitol did not protect from SN-induced DNA breaks. The combination of SN with SOD or MAN produced an increase in the amount of ssDNA which resulted statistically significant in the latter case ($p < 0.05$; Fig. 1).

However, when antioxidants were added encapsulated into liposomes, a decrease in the SN-induced DNA damage was observed (Fig. 2). In order to analyze main effects and interactions, all possible combinations were performed. Statistical analysis of data showed that the main effects in regard to DNA damage were due to SN and SOD and that the combinations SN/SOD, SN/SOD/CAT and SN/SOD/MAN produced a significant decrease in the amount of ssDNA ($p < 0.05$). The combinations SOD/CAT and SOD/CAT/MAN were also statistically different ($p < 0.05$) indicating that some sort of interaction must exist between these antioxidants.

4. Discussion

Recently, we proposed [3] that SN induces indirect DNA damage by the formation of stable SN-DNA complexes and direct damage on DNA by free radical production. To test whether free radicals are involved in the clastogenesis induced by SN in eucaryotic cells we studied the effect of the antioxidant enzymes SOD (which converts O_2^- into H_2O_2) and CAT (which scavenges H_2O_2) and the $OH^\cdot$ radical scavenger mannitol on SN-induced chromosome and DNA damage in CHO cells. In a previous report, De Graaf et al. [8] showed that neither exogenously added SOD nor CAT protects V79 cells from SN-induced chromosome damage. Our present results with CHO cells indicate that the addition of these antioxidant enzymes or mannitol directly to the cell cultures did not protect the chromosomes from the damage induced by SN. Moreover, the combination of SN/SOD or SN/MAN paradoxically enhanced the chromosome and DNA damage above the levels produced by SN alone. It is well known that semiquinones can reduce transition metals and react with H_2O_2 to produce $OH^\cdot$ radical in an 'organic Fenton reaction' [9] and that SN in non-cellular systems is reduced by NADH giving rise to a semiquinone radical which undergoes autoxidation producing superoxide [1]. SOD, which cannot pass cell membranes, by catalysing the dismutation of O_2^- generates H_2O_2 extracellularly. This active oxygen species – as opposed to O_2^- – difuses freely into the cell [10], and reacts with DNA associated transition metals like iron and copper, giving rise to the extremely dangerous $OH^\cdot$ radical (Fenton reaction), which directly attacks DNA. Thus, the enhancement by SOD of the clastogenesis by SN may be due to a rise in the intracellular amount of H_2O_2 which reacts with the SN-derivated semiquinone radical or with O_2^- in the presence of transition metals to form $OH^\cdot$ radicals, leading to DNA damage. This assumption is further supported by the report of Duell et al. [11] who found that SOD directly added to human lymphocyte cultures potentiated the chromosome damage produced by the active oxygen species generated by a xanthine–xanthine oxidase system. On the other hand, we find difficult to explain why mannitol increased the damage induced by SN.

Antioxidants showed a remarkable capacity to

neutralize the SN damage to chromosomes and DNA when they entered the cell into liposomes. In particular, SN/SOD, SN/SOD/CAT and SN/SOD/MAN were the best combinations to produce a significant decrease in the amount of ssDNA while for chromosome aberrations all the combinations were effective.

The addition of SOD and CAT to non-cellular systems has been shown to totally inhibit DNA cleavage by SN [1]. Our present results with CHO cells demonstrate that DNA degradation by free radicals produced by SN can also be prevented in cellular systems by the addition of liposome-entrapped antioxidant compounds to the culture medium. In this way, SOD exerts its protective effect eliminating the O_2^- originated during SN reduction or semiquinone autoxidation thus preventing the reduction of metal ions and Fenton reaction. On the other hand, CAT by removing H_2O_2 avoids Fenton reaction and $OH^{\cdot}$ radicals production which are responsible of site-specific damage to DNA. In addition, MAN prevents DNA degradation in a direct way by scavenging the $OH^{\cdot}$ released in the cytoplasm as well as those originated by SN-DNA redox cycling complexes in the nucleus.

Therefore, we may conclude that chromosome and DNA damage are the result of an oxidative stress induced by SN; and that the $OH^{\cdot}$ radical released from SN-DNA complexes might be involved in a 'site specific' mechanism of damage.

Acknowledgements

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Subchronic Oral Toxicity Studies with Erythritol in Mice and Rats

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Erythritol is a sugar alcohol (polyol) with potential applications as a low-calorie, bulk sweetener. Ingested erythritol is efficiently absorbed and excreted unchanged via the urine since it is not metabolized systemically by the animal or human body. Erythritol was administered to four groups of 10 male and 10 female Swiss CD-1 mice and four groups of 15 male Wistar Crl:(WI) WU BR rats at dietary levels of 0, 5, 10, or 20% for 90 days. A fifth group of rats received a diet containing 20% erythritol on a time-restricted basis (6 hr/day), and a sixth group received a diet containing 20% mannitol for comparison. There were no treatment-related mortalities in either mice or rats. Soft stools and occasional diarrhea were observed in rats fed diets with 20% erythritol or mannitol but not in mice. Body weights were slightly yet significantly reduced in rats fed 20% erythritol or mannitol and in male mice of the 20% dose group. Erythritol intake in the high-dose group was approximately 12 g/kg body wt in rats and 44 and 45 g/kg body wt in male and female mice, respectively. Hematological and clinicochemical examinations of blood and plasma did not reveal any treatment-related effects. Urine output increased with increasing erythritol dose. In male and female mice of the 20% erythritol group, the creatinine-normalized urinary excretion of protein, K-glutamyltransferase (GGT), and electrolytes (Na^+, K^+, Ca^{2+}, P_i, citrate) was significantly increased while urinary *N*-acetylglucosaminidase (NAG) remained unchanged. At the 10% level, significantly increased urinary protein (both sexes) and GGT (males only) excretion were seen. In rats, the creatinine-normalized urinary excretion of GGT, NAG, and some electrolytes (Na^+, K^+, and Ca^{2+}) was increased in some erythritol groups but a clear dose–response relationship was evident only for calcium. On termination of the study, cecal enlargement was seen in rats of the 10 and 20% dose groups and in mice of the 20% dose group. Increased relative and absolute kidney weights were observed in both sexes of mice in the 20% erythritol group, in male mice of the 5 and 10% groups, and in rats of the 10 and 20% erythritol groups. Histopathological examination did not reveal any treatment-related abnormalities in either mice or rats. In conclusion, the ingestion of erythritol for 90 days at dietary levels of up to 20% did not produce signs of toxicity in mice or rats. In particular, the morphological integrity of the kidneys was not adversely affected by the treatment in either species. The increases in urinary excretion of protein, GGT, NAG, and electrolytes were considered to result from extensive osmotic diuresis and a potential overload of the renal excretory system at the high dose levels employed.

INTRODUCTION

Sugar alcohols (polyols) such as sorbitol, mannitol, xylitol, maltitol, isomalt, or lactitol have been used as bulk sweeteners for many years, for example, for the production of energy-reduced and noncariogenic confectionery or for products that may be included in a diabetic diet (Imfeld, 1993; Bär, 1991). More recently, erythritol has been proposed as another, new bulk sweetener. This C-4 sugar alcohol, which occurs in small amounts in the human body (Pitkänen and Servo, 1973), has a lower physiological energy value and a higher gastrointestinal tolerance than existing products (Goossens and Röper, 1994). Erythritol owes these advantages to its high intestinal absorption and the fact that it is not metabolized systemically in rats and humans (Noda and Oku, 1990, 1992; Oku and Noda, 1990; Bornet *et al.*, 1992; Hiele *et al.*, 1993). These properties of erythritol have been known for over 50 years (Beck *et al.*, 1938) but practical interest in its physiological and technological properties arose only a few years ago when a commercially feasible process for its fermentative production from glucose was developed.

To further examine the tolerance to high oral doses

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of erythritol, two subchronic (13-week) studies were performed in mice and rats.

MATERIALS AND METHODS

Animals

SPF male and female Swiss-outbred (CD-1) mice and male Wistar-outbred [Crl:(WI) WU BR] rats were obtained from Charles River Wiga GmbH, Sulzfeld, Germany. On arrival, the mice and rats were 3–4 and 11–12 weeks old, respectively. After a short acclimatization period (mice, 9 days; rats, 5–6 days), the animals were randomly allocated to the different study groups and treatment was started.

The animals were housed under conventional conditions, male mice singly and female mice and rats in groups of 5 animals/cage. The mice were kept in macrolon boxes with sterilized sawdust; the rats were housed in suspended, stainless steel cages with wire-screen bottoms and fronts. The animal rooms were maintained at about 20–24°C. Relative humidity varied between 50 and 70% for mice and 50 and 75% for rats. Artificial light was provided for 12 hr, for rats during the nighttime and for mice during the daytime.

Diets and Test Materials

Mice received a pelleted diet, which for untreated controls consisted of whole ground wheat (28.1%), soya bean oil meal (20%), whole ground maize (10%), fish meal (8%), meat and bone scraps (4%), brewer's yeast (3%), grass meal (3%), mineral and vitamin premixes (0.9%), soya bean oil (3%), and wheat starch (20%). The diet also contained 25 g/kg molasses which was added together with water during the pelleting process. For the different treatment groups, erythritol was added at the expense of wheat starch. The pellets were offered *ad libitum* in the covers of the macrolon cages; tap water was supplied in glass bottles. During the period of urine collection, nonpelleted diets were used.

Rats received a pelleted, semipurified diet, which for the untreated controls consisted of vitamin-free raw rice starch (54.97%), vitamin-free casein (18.75%), cellulose (Dicacel 10) (15%), cocos fat (2.9%), minerals (2.83%), soybean oil (2%), micronutrient premix (2%), vitamin premix (1%), glycine (0.25%), L-methionine (0.2%), and choline chloride (0.1%). The test compounds (erythritol or mannitol) were incorporated in the diets at the expense of rice starch. Water (11.2%) was added during the pelleting process. Food and water were offered to the rats *ad libitum*, except for one group which had time-restricted access to the food. The food was offered in stainless steel cans and the tap water in glass bottles. All diets were stored at −20°C until used.

Erythritol and mannitol were obtained from Cerestar, Vilvoorde, Belgium. Erythritol was reported to have a purity of >99.9% on a dry matter basis.

The actual content of the test compounds in the different test diets of each batch was determined by HPLC (mice, 3 batches; rats, 1 batch). The homogeneity of the mixtures was examined by analyzing five samples of each test diet of the first batch. The stability of the test compound was determined after storage of the test diets of the first batch for 21 days at 22°C. The actual polyol concentrations varied within 85–120% of the nominal concentrations for mice and 85–97% for rats. The rather low polyol levels of the test diets for rats may be attributed to the admixture of water during the pelleting process. There was a good homogeneous distribution of the test compounds and there were no losses due to instability under the conditions of the study.

Analyses of the drinking water for contaminants are performed at the study site twice per year.

Experimental Design

The mouse study was composed of four treatment groups, each consisting of 10 males and 10 females receiving diets containing 0 (controls), 5, 10, or 20% erythritol, respectively.

The rat study consisted of six treatment groups of 15 males each. Only male rats were included since in this species the male kidney appears to be more prone for nephropathy as indicated, for example, by an earlier occurrence of chronic progressive nephrosis (Gray, 1986). In addition, a disposition study of erythritol in rats did not reveal sex-related differences (van Ommen *et al.*, 1996). Four groups of rats received diets containing 0, 5, 10, or 20% erythritol, respectively. A fifth group had access to the 20% erythritol diet for only 6 hr per day (restricted group). It was estimated that in this group a faster food intake would result in higher postprandial erythritol concentrations in plasma and urine than in the *ad libitum* fed rats. The sixth group received a diet containing 20% mannitol as a comparison group. Mannitol was considered appropriate for comparison since, like erythritol, it is not metabolized by rats yet is fermented to short-chain fatty acids by the gut flora.

For adaptation to the high polyol intakes, the dietary concentrations were increased stepwise in the mid and high-dose groups (in mice in 3-day steps and in rats in weekly steps from 5 to 10 and finally to 20%).

The general condition and behavior of the animals were examined twice daily on working days and once daily on weekends. The individual body weights of all animals were recorded initially and then weekly (mice) or biweekly (rats). The animals also were weighed on the day of termination in order to calculate relative organ weights.

Food consumption was recorded weekly except during the weeks in which the animals were housed in metabolism cages (food consumption measured during the 48-hr period of urine collection). Water consumption was measured in mice over three periods of 4 days each in Weeks 4, 8, and 12 (no measurement was made during urine collection). In rats, water consumption was not measured except during urine collection.

Blood samples were obtained from mice via the orbital plexus on Day 91 after overnight fasting and from rats via the tip of the tail on Day 86. The samples were examined for hemoglobin concentration, packed cell volume, red and white blood cell counts, differential white blood cell counts, thrombocyte counts, prothrombin time, red blood cell distribution width, mean platelet volume, platelet distribution width, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. In addition, blood glucose was measured in the blood samples of mice.

At necropsy (rats on Days 91–93; mice on Days 95 and 96), blood samples were collected from the orbita (mice) or aorta (rats) (ether anesthesia). The heparinized blood samples were centrifuged and the plasma was analyzed for total protein (TP), alkaline phosphatase (AP), alanine aminotransferase, aspartate aminotransferase, γ-glutamyltransferase (GGT), bilirubin (total), creatinine, potassium (K^+), inorganic phosphate (P_i), calcium (Ca^{2+}), and sodium (Na^+). In addition, aldosterone and magnesium were measured in the plasma of rats, and albumin, urea, chloride, cholesterol, and triglycerides were measured in the plasma of mice.

For collecting urine and feces, all mice and rats were kept for 4-day periods in metabolism cages. During the first 2 days of each period, the animals were acclimatized to these cages and during the second 2 days urine and feces were collected. Excreta of male and female mice were collected on Days 69/70 and 76/77, respectively. In rats, excreta were collected twice, on Days 37/38 (i.e., Week 6) and Days 65/66 (i.e., Week 10).

The urine samples were collected in dry ice-cooled beakers. Immediately after completion of the collection period, the samples were thawed and vigorously mixed. Volume and pH were measured. Then, the samples were centrifuged (750*g*, 5 min). In the supernatants, density, osmolality, TP, GGT, *N*-acetylglucosaminidase (NAG), and the test compounds were determined. In addition, Na^+ and K^+ were analyzed in mice, and low-molecular-weight proteins (LMP) were analyzed in rats. The remaining supernatants were acidified with concentrated hydrochloric acid (0.2 ml concentrated HCl per 10 ml), stored at −20°C, and analyzed later for Ca^{2+}, P_i, citrate, and creatinine. In rats, Na^+ and K^+ concentrations also were determined. The feces were weighed, freeze-dried for 24 hr, homogenized, and extracted with 0.1 *N* HCl. Following centrifugation, the extracts were analyzed for the test compounds by HPLC (for description of analytical methods, see Lina *et al.*, 1996).

In rats (but not mice), a urine concentration test was performed on Day 79 with all surviving animals of the control and the two 20% erythritol groups (*ad libitum* and restricted feeding). To accomplish this, rats were kept individually in metabolic cages. They were deprived of water for 24 hr and of food for the last 16 hr. Urine was collected in calibrated tubes during the 16 hr of fasting, and volume and density were measured on the following day.

After completion of the 13-week treatment period, all surviving animals were killed and examined grossly for pathological changes. The weights of the brain, cecum, heart, kidneys, liver, spleen, and testes were recorded. In rats, the adrenals, femur, urinary bladder, thymus, and thyroids (with parathyroids) also were weighed. The contents of the cecum were suspended in 5 ml distilled water for determination of the fecal pH.

In mice, the liver, kidneys, adrenals, cecum, and spleen of all animals of the control and high-dose groups and the liver and kidneys of the low- and mid-dose groups were processed and subjected to detailed histopathological examination.

For rats of the control, 20% erythritol (*ad libitum* and restricted), and 20% mannitol groups, the liver, kidneys, urinary bladder, stomach, duodenum, cecum, spleen, and brain were subjected to detailed histopathological examination. All other tissues were stored for possible later examinations.

Statistical Analysis

Data on body weights were evaluated by one-way analysis of covariance, followed by Dunnett's multiple comparison tests. Food and water intakes were evaluated by analysis of variance (ANOVA), followed by either Dunnett's multiple comparison tests (unit: the animal) or the least-significant-difference tests (unit: the cage). Hematological and clinicochemical data, urinary values (except pH), and organ weights were evaluated by ANOVA followed by Dunett's multiple comparison tests. Urinary pH was analyzed by the Kruskal–Wallis test followed by the Mann–Whitney *U* test. Histopathological changes were examined by Fisher's exact probability test. All analyses were two-sided.

RESULTS

The ingestion of erythritol was generally well tolerated by mice and rats. Transient diarrhea (i.e., excretion of soft fecal matter) was observed in some rats of the 20% erythritol and mannitol groups. It appeared that this phenomenon was more pronounced in manni-

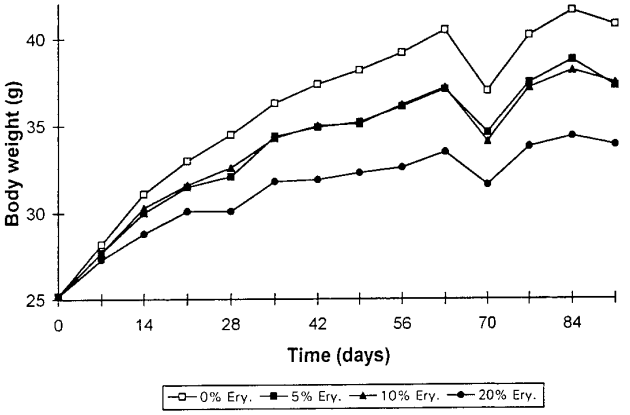

FIG. 1. Body weights of male Swiss CD-1 mice fed diets containing up to 20% erythritol for 13 weeks. Note that the decrease of body weights on Day 70 was the result of a low food intake during the preceding 4-day housing in metabolism cages.

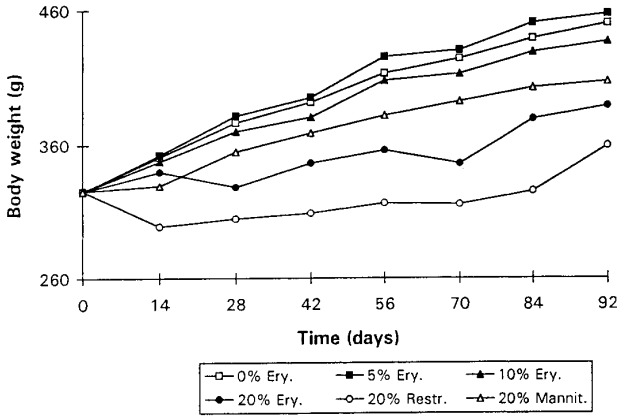

FIG. 3. Body weights of male Crl:(WI) WU BR rats fed diets containing up to 20% erythritol, or 20% mannitol, for 13 weeks.

tol- than erythritol-fed rats. Soft, i.e., not formed, stools and diarrhea were not seen in mice. No apparent differences of food spillage were noted between the different treatment groups.

No mortality occurred in the mouse study. In the rat study, one animal of the 20% erythritol group was found dead on Day 65 because of a mechanical trauma in the metabolism cage. Tissues of this rat were retained for histopathological examination but blood and urine samples were not obtained.

In both mice and rats body weights were reduced in the 10 and 20% erythritol groups. In male mice and rats, this effect was statistically significant at the 20% (but not 10%) level (Figs. 1–3). Rats with restricted access to the 20% erythritol diet exhibited the lowest body weights. Significantly reduced body weights were also seen in rats of the 20% mannitol group. During the periods of urine collection, body weights decreased in male and female mice due to a reduced food intake in all groups (Figs. 1 and 2).

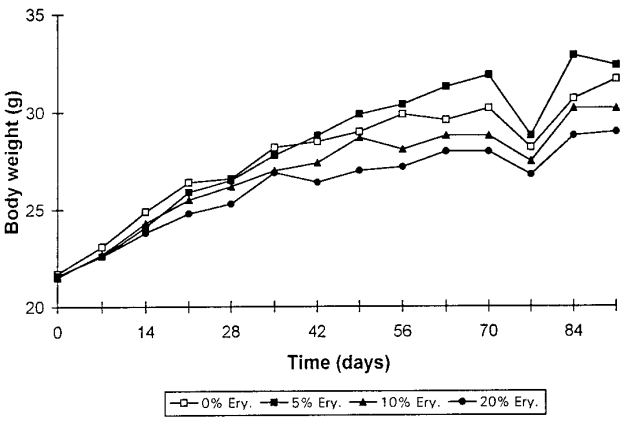

FIG. 2. Body weights of female Swiss CD-1 mice fed diets containing up to 20% erythritol for 13 weeks. Note that the decrease of body weight on Day 77 was the result of a low food intake during the preceding 4-day housing in metabolism cages.

Accurate measures of individual food consumption could not be obtained because of food spillage and because female mice and rats were not housed individually. However, animals given high erythritol or mannitol doses appeared to eat more food in an attempt to compensate for caloric dilution. From the data on body weight, food consumption, and measured dietary erythritol concentration, the erythritol intake of high-dose group animals is estimated at about 12 g/kg body wt for rats and 44 and 45 g/kg body wt for male and female mice, respectively. Mean water intake of mice appeared to increase with increasing consumption of erythritol. A similar observation was made for rats during the periods of urine collection.

Hematological examination of mice did not reveal any treatment-related changes of red and white blood cell variables or blood clotting potential (data not shown). In rats, the red blood cell distribution width was slightly but significantly increased in the 20% erythritol (*ad libitum*) group, significantly decreased in the 20% mannitol group, and not different from controls in all other groups. Prothrombin time was significantly increased from 38.7 sec (controls) to 42.3 sec (20% erythritol, *ad libitum*) and 44.8 sec (20% mannitol). White blood cell and lymphocyte counts were slightly, yet significantly, increased in the 20% mannitol group. Otherwise, there were no statistically significant differences between controls and the treated groups.

The clinicochemical plasma analyses did not reveal any treatment-related changes in mice (data not shown). In rats, plasma AP activity was slightly yet significantly increased in the group with restricted access to the 20% erythritol diet and in the 20% mannitol

TABLE 1
Urinary Parameters of Swiss CD-1 Mice during a 2-Day Period of a 13-Week Oral Toxicity Study on Erythritol

Treatment	Volume ml/24 hr	Osmolality mosm/kg	pH[a]	Creatinine μM/24 hr	Protein mg/mmol[b]
Controls					
Males	2.2 ± 0.1	2202 ± 59	6.8	13.8 ± 0.7	239 ± 7
Females	1.8 ± 0.1	2627 ± 67	6.6	13.5 ± 0.5	136 ± 7
5% erythritol					
Males	2.5 ± 0.3	2506 ± 43**	6.8	13.6 ± 1.5	265 ± 9
Females	1.7 ± 0.2	2814 ± 73	6.6	11.7 ± 1.2	156 ± 4
10% erythritol					
Males	4.1 ± 0.2**	2163 ± 37	6.7	16.0 ± 0.8	307 ± 12**
Females	3.8 ± 0.3**	2292 ± 68**	6.6	16.3 ± 1.1	200 ± 10**
20% erythritol					
Males	10.7 ± 0.6**	1443 ± 40**	6.7	19.7 ± 1.2**	544 ± 22**
Females	9.6 ± 0.5**	1498 ± 31**	6.6	19.8 ± 0.8**	343 ± 21**

Treatment	GGT U/mmol[b]	NAG U/mmol[b]	Sodium mol/mol[b]	Potassium mol/mol[b]	Calcium mol/mol[b]	Phosphate mol/mol[b]	Citrate mol/mol[b]
Controls							
Males	14 ± 2	14.48 ± 1.57	34.25 ± 0.84	43.53 ± 0.87	0.27 ± 0.04	9.69 ± 0.49	1.351 ± 0.120
Females	17 ± 2	2.11 ± 0.10	31.71 ± 0.84	40.38 ± 0.71	0.42 ± 0.07	7.67 ± 0.30	2.077 ± 0.156
5% erythritol							
Males	26 ± 4	16.79 ± 1.21	35.70 ± 0.94	45.22 ± 1.13	0.25 ± 0.03	10.11 ± 0.70	1.503 ± 0.115
Females	44 ± 8*	2.26 ± 0.17	30.27 ± 0.88	38.78 ± 1.02	0.48 ± 0.16	7.53 ± 0.53	1.899 ± 0.189
10% erythritol							
Males	29 ± 4*	14.97 ± 1.00	36.49 ± 0.57	44.55 ± 0.74	0.26 ± 0.04	10.76 ± 0.35	1.730 ± 0.117
Females	37 ± 4	2.02 ± 0.12	33.12 ± 0.67	40.63 ± 0.73	0.89 ± 0.21	8.88 ± 0.31	2.380 ± 0.191
20% erythritol							
Males	32 ± 4**	17.38 ± 1.19	41.81 ± 0.63**	47.28 ± 0.85*	0.57 ± 0.14*	12.38 ± 0.37**	2.052 ± 0.171**
Females	46 ± 10**	1.98 ± 0.16	37.99 ± 0.67**	43.88 ± 0.90*	1.14 ± 0.17**	9.85 ± 0.26**	2.795 ± 0.215*

Note. Values are means ± SEM (n = 10 animals/sex/group). Urine of males and females was collected on Days 69–70 and 76–77, respectively.

[a] Mean pH represents the logarithm of the group mean hydrogen ion concentration. Because of this calculation mode, a SEM cannot be given.

[b] Values are expressed per mole or millimole of creatinine.

Statistics: For all parameters except pH [Anova + Dunnett's tests (two-sided)]: *$P < 0.05$, **$P < 0.01$. For pH [Kruskal–Wallis Anova + Mann–Whitney U test (two sided)]: *$P < 0.05$, **$P < 0.02$.

group. Plasma GGT was significantly decreased in the 20% erythritol (*ad libitum*) and 20% mannitol groups. The P_i concentration was slightly yet significantly increased in the group with restricted access to the 20% erythritol diet.

The urine volumes in mice and rats increased with increasing erythritol consumption (Tables 1 and 2). Up to the 10% level, the osmolality of the urines remained essentially unaffected by the erythritol treatment. However, at 20% erythritol, urine osmolality dropped below control levels. In mice, but not rats, the 24-hr creatinine output increased with increasing erythritol dose (Table 1). This increase could be a consequence of a more complete collection of urine in the 10 and 20% dose groups. Presumably, a larger fraction of urine was lost in the control and low-dose groups due to adhesion and evaporation of urine on the collecting funnels than in the mid and high-dose groups in which urine production was higher. To balance for incomplete urine recovery, the values of urinary proteins, enzymes, and electrolytes were normalized to creatinine in mice and, in order to facilitate between-species comparison, also in rats.

In mice of both sexes, the creatinine-normalized urinary excretions of TP, GGT, creatinine, and electrolytes were significantly increased in the 20% erythritol group (Table 1). In the 10% erythritol group, the excretions of TP (both sexes) and GGT (males) were increased significantly. In the 5% erythritol group, GGT excretion was increased only in females.

In rats, similar changes were seen for some, but not all urinary parameters (Table 2). For example, GGT excretion was significantly increased in Week 6 at the 5 and 10% dose level. On the other hand, NAG excretion was elevated significantly only in Week 10 in the 20% erythritol groups (*ad libitum* and restricted).

TABLE 2
Urinary Parameters of Male Crl:WT (WU) BR Rats during Two 2-Day Periods of a 13-Week Subchronic Toxicity Study on Erythritol

Treatment	Volume (ml/24 hr	Osmolality mosm/kg	pH[a]	Creatinine μM/24 hr
Controls				
Week 6	10.4 ± 1.6	1863 ± 159	6.4	120.9 ± 2.4
Week 10	8.0 ± 0.7	2148 ± 112	6.1	131.5 ± 3.8
5% erythritol				
Week 6	11.2 ± 0.8	1978 ± 122	6.4	121.1 ± 3.0
Week 10	10.8 ± 0.5	2338 ± 89	6.1**	148.9 ± 5.1**
10% erythritol				
Week 6	15.4 ± 1.1	1791 ± 77	6.4	118.4 ± 6.7
Week 10	15.4 ± 1.3**	1968 ± 96	6.2***	141.8 ± 3.6
20% erythritol				
Week 6	25.0 ± 2.2**	1473 ± 74	6.4	114.8 ± 3.1
Week 10	27.2 ± 2.0**	1552 ± 76**	6.2***	126.9 ± 3.7
20% erythritol (restricted)				
Week 6	22.9 ± 1.8**	1129 ± 68**	6.5	100.0 ± 2.4**
Week 10	20.6 ± 2.0**	1346 ± 103**	6.3***	105.2 ± 3.1**
20% mannitol				
Week 6	8.8 ± 0.7	1618 ± 114	6.4	109.6 ± 3.2
Week 10	9.6 ± 0.7	1626 ± 111**	6.0	127.9 ± 3.2

Treatment	Protein g/mmol[b]	LMP g/mmol[b]	GGT U/mmol[b]	NAG U/mmol[b]	Sodium mol/mol[b]
Controls					
Week 6	0.125 ± 0.012	0.201 ± 0.012	105 ± 4	2.15 ± 0.09	5.08 ± 0.21
Week 10	nd	nd	223 ± 11	2.07 ± 0.09	5.00 ± 0.17
5% erythritol					
Week 6	nd	nd	200 ± 17**	2.40 ± 0.07	5.94 ± 0.23
Week 10	nd	nd	244 ± 13	2.13 ± 0.10	5.55 ± 0.25
10% erythritol					
Week 6	nd	nd	171 ± 12**	2.40 ± 0.09	6.07 ± 0.16*
Week 10	nd	nd	246 ± 12	2.39 ± 0.11	5.92 ± 0.20*
20% erythritol					
Week 6	0.146 ± 0.011	0.252 ± 0.22	137 ± 14	2.68 ± 0.17	5.84 ± 0.30
Week 10	nd	nd	202 ± 12	3.03 ± 0.18**	5.12 ± 0.35
20% erythritol (restricted)					
Week 6	nd	nd	156 ± 15*	2.80 ± 0.37	4.92 ± 0.36
Week 10	nd	nd	279 ± 12**	2.84 ± 0.10**	4.58 ± 0.22
20% mannitol					
Week 6	nd	nd	85 ± 7	2.27 ± 0.10	3.71 ± 0.25**
Week 10	nd	nd	214 ± 9	2.14 ± 0.09	3.91 ± 0.24**

Among the electrolytes, consistent increases were noted only for Ca^{2+} (20% erythritol groups) and citrate (20% mannitol group).

The urinary excretion of erythritol (expressed in percentage of intake) was higher in mice than in rats, while the fecal excretion was similarly low. In the 10 and 20% erythritol groups, about 80–100% and 50–55% of ingested erythritol was excreted in the urine of mice and rats, respectively. The feces of either species contained about 3–5% of the ingested dose. In rats of the mannitol group, only very small amounts of this polyol were detected in urine (<2.5%) and feces (<0.4%).

The mean urine volume produced by control rats during the urine concentration test was 2.8 ± 0.1 ml. The animals of the 20% erythritol groups produced significantly more urine, namely 4.9 ± 0.4 ml (*ad libitum*) and 4.5 ± 0.8 ml (restricted). The densities (in g/liter) of the samples did not differ significantly (data not shown).

At termination, male mice of the 20% erythritol group exhibited significantly increased absolute kidney and liver weights and female mice increased absolute kidney and cecum (full) weights (data not shown). All relative organ weights of mice are presented in Table 3. A dose-related increase of relative kidney weights is apparent for male and female mice. The cecum weights were also increased in both sexes in the 20% erythritol

TABLE 2—*Continued*

Treatment	Potassium mol/mol[b]	Calcium mol/mol[b]	Phosphate mol/mol[b]	Citrate mol/mol[b]
Controls				
Week 6	11.24 ± 0.31	0.11 ± 0.01	6.34 ± 0.17	0.464 ± 0.032
Week 10	10.18 ± 0.33	0.09 ± 0.01	6.28 ± 0.18	0.466 ± 0.038
5% erythritol				
Week 6	12.00 ± 0.30	0.11 ± 0.01	6.81 ± 0.19	0.645 ± 0.055
Week 10	10.93 ± 0.30	0.09 ± 0.00	6.48 ± 0.14	0.608 ± 0.058
10% erythritol				
Week 6	12.46 ± 0.37	0.15 ± 0.01	6.93 ± 0.15	0.744 ± 0.052
Week 10	10.94 ± 0.27	0.12 ± 0.01	6.42 ± 0.18	0.619 ± 0.048
20% erythritol				
Week 6	11.70 ± 0.50	0.29 ± 0.04**	6.18 ± 0.20	0.652 ± 0.084
Week 10	12.04 ± 0.44**	0.37 ± 0.04**	6.12 ± 0.23	0.501 ± 0.055
20% erythritol (restricted)				
Week 6	10.29 ± 0.40	0.35 ± 0.07**	5.98 ± 0.24	0.447 ± 0.079
Week 10	10.27 ± 0.34	0.27 ± 0.03**	6.59 ± 0.16	0.372 ± 0.052
20% mannitol				
Week 6	11.63 ± 0.28	0.17 ± 0.01	5.13 ± 0.26**	1.068 ± 0.139**
Week 10	10.64 ± 0.26	0.14 ± 0.02	5.14 ± 0.15**	0.839 ± 0.074**

Note. Values are means ± SEM ($n = 15$; except for 20% erythritol, $n = 14$). Abbreviation: nd, not determined. Restricted implies restricted access to food (6 hr/day).

[a] Mean pH represents the logarithm of the group mean hydrogen ion concentration. Because of this calculation mode, a SEM cannot be given.

[b] Values are expressed per mole or millimole of creatinine.

Statistics: Mean pH values were calculated as the negative logarithm of the mean of the hydrogen ion concentrations measured in the individual samples. For all parameters except pH [Anova + Dunnett's tests (two-sided)]: $*P < 0.05$, $**P < 0.01$. For pH [Kruskal–Wallis Anova + Mann–Whitney U test (two sided)]: $*P < 0.05$, $**P < 0.02$, $***P < 0.002$.

group. The relative liver and spleen weights were increased significantly in females of the high-dose group. The increased relative brain weight of males of this group probably reflects the significantly lower body weights (Feron *et al.*, 1973).

In rats, body weights were significantly reduced at termination in the 20% erythritol (*ad libitum* and restricted) and 20% mannitol groups. In these groups, the absolute weights of the heart and liver were also decreased, while the weight of the cecum was increased (data not shown). The relative organ weights of rats are presented in Table 4. The relative weights of the brain, spleen, and testes were increased in the 20% erythritol groups (*ad libitum* and restricted feeding). The relative weights of the adrenals and bladder also tended to be increased at that dose level (significant in groups with restricted feeding only). The relative kidney weights exhibited a dose-related, significant increase at the 10 and 20% erythritol doses. The relative weight of the urinary bladder was increased in the 20% erythritol group with restricted feeding. The cecum weights were increased in the 10 and 20% erythritol and 20% mannitol groups.

Gross examination of all mice at necropsy and microscopic examination of their adrenals, cecum, kidneys, liver, and spleen did not reveal treatment-related changes. Since kidney weights of mice were increased in the 20% erythritol group, the detailed renal histopathological observations are reported in Table 5.

Gross examination of rats revealed mucosal edema in the glandular stomach of two rats of each of the 10 and the 20% erythritol groups (*ad libitum* and restricted). No edema were seen in controls. Dilation of the cecum was observed in 1 of 14 rats of the 20% erythritol group (*ad libitum*) and in 9 of 15 rats of the 20% mannitol group. All other gross changes were about equally distributed between controls and test rats, occurred in a single animal only, or are commonly seen in rats of this strain and age. Microscopic examination of the cecum revealed no changes other than an increased number of goblet cells in 1 rat of the 20% mannitol group. In the stomach, no abnormality was detected in any of the rats. Treatment-related microscopic changes were not detected in rat kidneys (Table 6) or in any of the other organs examined.

DISCUSSION

During the periods of urine collection, about 50–55% of ingested erythritol was recovered from the urine of rats and about 80–100% from the urine of mice (10 and 20% dose groups). Erythritol excretion with feces varied between about 3 and 5%. In general, these data confirm that erythritol is efficiently absorbed and ex-

TABLE 3
Mean Terminal Body Weights (g) and Relative Organ Weights (g/kg Body Wt) of Swiss CD-1 Mice Fed Diets Containing up to 20% Erythritol for 13 Weeks

Body weights/ organ weights	Controls	Erythritol (% of diet) 5	10	20
Body weight				
Males	42.6 ± 1.7	39.4 ± 1.3	39.7 ± 1.5	35.0 ± 0.9**
Females	31.8 ± 1.2	32.5 ± 1.3	31.1 ± 1.4	30.2 ± 0.9
Cecum full				
Males	12.3 ± 1.0	15.0 ± 0.8	14.0 ± 0.8	18.8 ± 1.8**
Females	10.1 ± 0.5	10.7 ± 0.9	12.4 ± 0.7	16.0 ± 0.8**
Cecum empty				
Males	3.1 ± 0.3	3.3 ± 0.1	3.5 ± 0.2	4.3 ± 0.2**
Females	5.2 ± 0.5	5.2 ± 0.3	6.1 ± 0.5	7.1 ± 0.7
Liver				
Males	53.7 ± 1.4	51.5 ± 1.0	51.6 ± 1.1	56.2 ± 0.9
Females	45.1 ± 1.6	43.9 ± 1.3	49.5 ± 0.7	52.4 ± 1.4**
Kidneys				
Males	12.75 ± 0.60	14.36 ± 0.37*	14.69 ± 0.53*	17.08 ± 0.23**
Females	11.68 ± 0.57	11.36 ± 0.33	12.54 ± 0.39	13.97 ± 0.55**
Heart				
Males	4.80 ± 0.19	4.98 ± 0.14	4.70 ± 0.20	5.21 ± 0.12
Females	4.80 ± 0.26	4.60 ± 0.17	4.88 ± 0.10	5.03 ± 0.25
Spleen				
Males	4.36 ± 0.27	3.59 ± 0.16	4.83 ± 0.63	4.63 ± 0.33
Females	4.98 ± 0.29	6.11 ± 0.39	6.11 ± 0.24	6.41 ± 0.39*
Brain				
Males	11.35 ± 0.48	12.10 ± 0.49	11.80 ± 0.37	13.47 ± 0.44**
Females	15.87 ± 0.78	15.24 ± 0.73	16.74 ± 0.79	15.83 ± 0.56
Testes				
Males	6.10 ± 0.38	6.36 ± 0.47	6.31 ± 0.37	7.36 ± 0.40
Females	nd	nd	nd	nd

Note. Values are means ± SEM (n = 10 mice/sex/group). nd, not determined.
Statistics [ANOVA + Dunnett's tests (two-sided)]: *$P < 0.05$, **$P < 0.01$.

creted unchanged with the urine. The higher urinary excretion and the lower cecal enlargement in erythritol-fed mice suggest that erythritol absorption is more complete in mice than in rats.

Reduced body weight gains have often been observed in rodent feeding studies with polyols, particularly if high dietary levels (20%) were consumed. However, the administration of these polyols did not usually lead to a significantly increased food intake (Sinkeldam *et al.*, 1992; Smits-van Projie *et al.*, 1990). In the present rat study, the animals of the 20% mannitol group also had significantly reduced weight gains (Fig. 3), while food intake was similar to controls. Rats of the 10 and 20% erythritol groups, however, increased their food intake somewhat, probably in an attempt to compensate for the low energy value of the ingested erythritol. In mice, body weights were significantly reduced in males but not females of the 20% erythritol group. Food intake was more difficult to measure in this species because of increased food spillage.

The hematological and clinicochemical examinations did not reveal treatment-related changes in either rodent species. A few statistically significant changes were observed but these were not dose-related or occurred in only one of the two high dose erythritol groups. Indeed, none of these changes was reproduced in a subsequent chronic toxicity study with erythritol in male and female rats of the same strain (Lina *et al.*, 1996). Even at the 20% erythritol level, plasma electrolyte concentrations of mice and rats remained unchanged despite substantially increased urine production and increased urinary excretion of electrolytes in mice. Correspondingly, plasma aldosterone also did not differ between erythritol-fed rats and the controls.

Erythritol consumption had some effect on urine production and composition, but not all changes exhibited a consistent pattern. A common and expected observation was the increase of urine volumes with increasing erythritol intake. However, despite this dose-related increase, the osmolality of the urine remained at normal levels up to the 10% erythritol level, indicating that the concentrating power of the kidneys was intact. Only at 20% erythritol was urine osmolality decreased significantly, consistent with the marked increase of

TABLE 4

Mean Terminal Body Weights (g) and Relative Organ Weights (g/kg Body Wt) of Male Crl:WI (WU) BR Rats Fed Diets Containing up to 20% Erythritol, or 20% Mannitol, for 13 Weeks

Body weights/ organ weights	Controls	Erythritol (% of diet) 5	10	20 (*Ad libitum*)	20 (Restricted)	Mannitol (%) 20
Body weight	450.2 ± 7.7	457.2 ± 8.2	436.9 ± 5.5	388.8 ± 7.6**	359.0 ± 5.2**	407.0 ± 5.3**
Cecum full	6.2 ± 0.4	7.1 ± 0.3	10.9 ± 0.8*	19.3 ± 1.4**	22.3 ± 1.7**	29.1 ± 1.5**
Cecum empty	1.6 ± 0.1	1.9 ± 0.1	2.4 ± 0.1*	3.8 ± 0.2**	3.9 ± 0.2**	5.5 ± 0.3**
Liver	27.8 ± 0.5	28.6 ± 0.6	28.3 ± 0.5	27.2 ± 0.4	28.3 ± 0.6	27.0 ± 0.5
Kidneys	5.30 ± 0.09	5.32 ± 0.10	5.75 ± 0.14*	5.94 ± 0.09**	6.21 ± 0.08**	5.46 ± 0.10
Urinary bladder	0.34 ± 0.01	0.30 ± 0.02	0.38 ± 0.03	0.40 ± 0.02	0.42 ± 0.02*	0.35 ± 0.02
Heart	2.88 ± 0.04	2.93 ± 0.08	2.94 ± 0.05	2.89 ± 0.08	2.96 ± 0.05	2.85 ± 0.04
Spleen	1.47 ± 0.03	1.58 ± 0.05	1.58 ± 0.06	1.85 ± 0.05**	1.68 ± 0.05*	1.59 ± 0.03
Brain	4.41 ± 0.07	4.40 ± 0.06	4.56 ± 0.07	5.13 ± 0.08**	5.43 ± 0.08**	4.91 ± 0.07**
Testes	7.26 ± 0.15	7.51 ± 0.12	7.63 ± 0.13	8.60 ± 0.28**	9.23 ± 0.14**	8.41 ± 0.15**
Thyroids	0.055 ± 0.002	0.060 ± 0.003	0.059 ± 0.001	0.064 ± 0.004	0.065 ± 0.004	0.059 ± 0.003
Adrenals	0.106 ± 0.003	0.106 ± 0.003	0.106 ± 0.004	0.120 ± 0.004	0.123 ± 0.005*	0.110 ± 0.003
Thymus	0.77 ± 0.04	0.81 ± 0.05	0.83 ± 0.05	0.83 ± 0.03	0.78 ± 0.04	0.71 ± 0.03
Femur	3.99 ± 0.09	3.92 ± 0.15	4.31 ± 0.12	4.29 ± 0.12	4.39 ± 0.10	4.22 ± 0.19

Note. Values are means ± SEM (n = 15 rats/group).
Statistics [ANOVA + Dunnett's test (two-sided)]: *$P < 0.05$, **$P < 0.01$.

urine flow. This change could be the result of an osmotic diuresis. Higher urinary calcium and citrate excretions are usually seen in polyol-fed rodents and represent a generic effect to the feeding of low digestible carbohydrates (Bär *et al.*, 1995). In the absence of morphological changes in the kidneys, the occasional increase of urinary GGT and NAG in both mice and rats may be an indirect consequence of the treatment-related diuresis. The increased urinary protein excretion of mice may reflect an increased glomerular filtration rate and/or a decreased tubular protein reabsorption. Interestingly, the relative increase of protein excretion above baseline values was similar in male and female mice despite the sex-related difference of urinary proteins in this species (Alt *et al.*, 1985). In rats, urinary TP and LMP excretions remained unchanged which indicates that in this species glomerular filtration and tubular protein reabsorption are not affected by the erythritol treatment.

Among the observed changes of organ weights, cecal enlargement represents a normal consequence of the ingestion of incompletely absorbed yet fermentable substrates (Newberne *et al.*, 1988). The increased kidney weights, on the other hand, may reflect the increased diuresis and workload that is associated with the elimination of high amounts of absorbed erythritol (Bär *et al.*, 1995; Ogino *et al.*, 1994; Sterck *et al.*, 1992). The higher relative weights of the urinary bladder in rats of the 20% erythritol group may also be attributed

TABLE 5

Histopathological Changes in Kidneys of Swiss CD-1 Mice Fed Diets Containing up to 20% Erythritol for 13 Weeks

		Incidence of lesions (numeric)							
		Males fed erythritol				Females fed erythritol			
Changes	Treatment (%)	0	5	10	20	0	5	10	20
Kidneys		(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)
Basophilic tubules		3	3	0	4	2	3	3	3
Tubular dilatation		2	2	2	4	2	2	1	3
Focal mononuclear-cell infiltrate		2	3	3	3	1	3	5	4
Corticomedullary mineralization		0	0	0	1	0	0	0	0
Cortical mineralization		0	1	0	0	0	0	0	0

Note. Numbers in parentheses represent numbers of animals examined.

TABLE 6
Histopathological Changes in Kidneys of Male Crl:WI (WU) BR Rats Fed Diets Containing up to 20% Erythritol, or 20% Mannitol, for 13 Weeks

Changes	Controls	Treatment with: 20% erythritol, *Ad libitum*	Treatment with: 20% erythritol, Restricted	Treatment with: 20% mannitol
Kidneys	(15)	(14)	(15)	(15)
Unilateral pelvic dilatation	0	1	0	0
Glomerular degeneration	0	0	0	2
Unilateral hydronephrosis	0	0	1	0
Cast(s)	1	1	2	0
Tubular nephrosis	4	2	6	4
Very slight	1	0	0	0
Slight	1	2	6	4
Moderate	2	0	0	0
Epithelial proliferation	0	0	1	0
Mononuclear-cell infiltrate	2	2	4	3
Medullary mineralization	1	0	0	0

Note. Numbers represent numbers of animals affected. Numbers in parentheses represent total numbers of animals examined.

to the higher urine flow (Anderson, 1988). The increased relative weights of the brain and testes in the 20% erythritol groups are probably an indirect result of the lower body weights (Feron *et al.*, 1973).

In terms of safety assessment, it is most important that neither in mice nor in rats were there any treatment-related histopathological changes of the kidneys. At the 20% dietary level, the male and female mice ingested erythritol at doses of about 44 and 45 g/kg body wt/day, respectively. In rats, exposure at that dietary erythritol level was 12 g/kg body wt/day. If the renal exposure is calculated from the kidney weights and the amounts of erythritol excreted, values of nearly 2000 g/kg kidney/day result for mice and 950 g/kg kidney/day for rats. The absence of any detectable adverse effects on kidney morphology at such levels attests to the lack of any direct effect of erythritol.

CONCLUSION

The ingestion of erythritol at dietary levels of up to 10% was tolerated by mice and rats without signs of toxicity. The observed, apparent, treatment-related effects either are known to occur commonly in rodents in response to the feeding of polyols (e.g., cecal enlargement, reduced body weight gains and associated changes of certain relative organ weights, increased urinary calcium output) or can be attributed to the increased urine output which is associated with the renal elimination of absorbed erythritol (e.g., increased kidney weights, increased urinary excretions of protein and GGT). Although the concentrating capacity of the kidneys may have been exceeded at the high dose level in mice and rats (as suggested by a decreased urinary osmolality), it is remarkable that the morphological integrity of this organ was not adversely affected in either species.

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ORIGINAL ARTICLE

Biochemical Evaluation of Patients of Alcoholic Liver Disease and Non-alcoholic Liver Disease

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Abstract Alcoholic liver disease (ALD) is due to excessive alcohol intake for long duration. Distinguishing ALD from non-ALD (non-alcoholic steatohepatitis, hepatitis of viral origin) is difficult as patient may deny alcohol abuse. Clinical examination, histology and serology may not differentiate these conditions. Accurate diagnosis is important as management of ALD differs from non-ALD patients. The aim of our study was (1) To evaluate the patients of ALD and non-ALD by biochemical parameters compared to controls, (2) To assess whether these parameters can differentiate ALD from non-ALD. Study was carried out on 50 patients of ALD in group I and 35 patients of NASH (non-alcoholic steatohepatitis) and acute viral hepatitis each in group II. Age matched healthy controls $n = 50$. Selection criteria—history of alcohol intake (amount and duration), clinical examination, sonography of abdomen, serum alanine transaminase (ALT) and bilirubin levels. Blood samples were analyzed for bilirubin, aspartate transaminase (AST), ALT, alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) by kinetic method. Statistical analysis was done by Student unpaired '*t*' test. Patients of ALD have raised AST/ALT ratio (De Ritis ratio) (>2), ALP and GGT compared to controls ($P < 0.01$).There is significant difference in AST/ALT ratio, serum GGT and ALP in ALD group compared to that in NASH and acute viral hepatitis ($P < 0.05$). This study suggests that De Ritis ratio >2 in ALD patients may be due to alcohol induced hepatic mitochondrial injury and pyridoxine deficiency. High GGT and ALP values may indicate enzyme induction by alcohol and mild cholestasis. Thus ALD patients have severe hepatic damage. De Ritis ratio <1 and normal to mild elevation in GGT level in NASH and acute viral hepatitis suggest mild hepatic injury of non-alcoholic origin. Our study concludes that ALD patients can be differentiated from NASH and acute viral hepatitis with certainty by measuring serum AST/ALT ratio, GGT and ALP. These biochemical parameters may help clinicians to support the diagnosis of ALD and non-ALD.

Keywords Alcoholic liver disease · Aspartate transaminase · Alanine transaminase · Gamma glutamyl transpeptidase

Introduction

Alcoholism is worldwide social and medical problem. Over past few years, alcohol consumption has increased in quantity and frequency. The age at which people start drinking has also declined. The population at risk is those undergoing rapid socio-economic and cultural changes. With the development of economy and the improvement in living standards of people, the incidences of diseases caused by alcohol abuse have been increasing.

Alcoholic liver disease (ALD) remains one of the most common causes of chronic liver disease. ALD has a known cause but a complex process. The amount and duration of ethanol ingestion rather than the type of alcoholic beverage or the pattern of ingestion, appears to be important determinant of liver injury. A substantial proportion of adult population in India uses alcohol. Globally 5 million people die per year due to alcohol related problems [1]. Seventy percent of deaths are due to liver related disease. About

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half of all cirrhosis in the world are alcohol induced and about 10–20 % of all alcoholics are cirrhotic. Alcoholic cirrhosis has 5 year mortality of 77 % and at 10 years, survival in only 7 %. In men, ethanol intake of 40–80 g/day produces fatty liver and that of 160 g/day for 10–20 years causes hepatitis or cirrhosis. Only 15 % of alcoholics develop ALD. The threshold for developing ALD in men is an intake of >60–80 g/day of alcohol for 10 years. Social, nutritional, immunologic and host factors play important role in development of pathogenic process.

In recent years, conventional biochemical markers and potential ones have aroused the interest of researchers to study the damages caused by ethanol in liver. Alanine transaminase (ALT) and aspartate transaminase (AST) are predominant ones in aminotransferases reflecting the damage to hepatocytes. Physicians have long sought an accurate and inexpensive means of identifying persons who consume excessive amounts of ethyl alcohol. Chronic alcoholism is diagnosed on the basis of clinical history, questionnaire about alcohol consumption and a number of laboratory investigations.

NASH is associated with raised levels of plasma free fatty acids resulting from adipose tissue or from hydrolysis of lipoprotein TG by LPL in extrahepatic tissues. It leads to fatty liver. Metabolic block in production of plasma lipoproteins and deficiency of lipotropic factors like choline can cause fatty liver. Increased TG in liver is seen in starvation, high fat diet, uncontrolled diabetes mellitus, pregnancy, ketoacidosis causing hepatic dysfunction.

Distinguishing non-ALD from ALD has generously been difficult. The history can be unreliable as many patients may deny alcohol abuse. Furthermore histology may not distinguish non-ALD from ALD with certainty. In hepatocyte, ALT is exclusively found in cytosol while different isoenzymes of AST exist in mitochondria and cytosol [2, 3]. About 80 % of AST activity in human liver is contributed by mitochondrial isoenzyme, whereas most of the circulating AST activity in normal people is derived from cytosolic enzyme [4, 5]. This mitochondrial AST is elevated in liver diseases with prolonged duration [6]. In alcoholic hepatitis AST/ALT ratio may be >2 [7–11]. AST/ALT ratio is typically <1 in other causes of fatty liver [8, 12, 13]. When AST/ALT ratio is >1.5, it is considered as highly suggestive that alcohol is the cause of liver injury [14]. Patients with alcoholic hepatitis often demonstrate an AST to ALT ratio of >2 with absolute aminotransferase levels of <300 IU/l. Although some authors have stated that no consistent relationship exists between serum AST and ALT levels in non-alcoholic steatohepatitis, others have implied that ALT level is generally greater than AST level [12, 15].

Activity of alkaline phosphatase (ALP) arises from bone, intestine, liver and placenta [16]. In the absence of bone disease or pregnancy, elevated levels of ALP activity usually reflect impaired biliary tract function. Slight to moderate increases in ALP occur in many patients with liver disorders such as hepatitis and cirrhosis [17–20]. Striking increase in ALP (>10 times) occur more consistently with extrahepatic biliary tract obstruction or with intra hepatic cholestasis.

Measurement of gamma glutamyl transferase (GGT) activity in serum has been found useful in screening alcohol abuse. A sudden rise in serum GGT activity in known alcoholics is suggestive of a recent bout of alcohol [10, 21]. Elevated GGT associated with an increase in serum ALP shows that ALP in serum is of hepatic origin, as GGT is not raised in bone diseases [22].

No study however, has formally assessed the utility of serum AST/ALT ratio, GGT and ALP to differentiate alcoholic from non-ALD.

Aims and Objectives

(1) To evaluate the patients of ALD and non-ALD by biochemical parameters compared to controls.
(2) To assess whether these parameters can differentiate the patients of ALD from non-ALD.

Materials and Methods

The present study was carried out in the Department of Biochemistry. To evaluate biochemically, male patients with presumptive diagnosis of ALD ($n = 50$) and non-ALD ($n = 35$ each of NASH and acute viral hepatitis) admitted in medicine ward were included in the study. All the patients in our study were male. Most of the patients in this hospital are from rural areas with orthodox beliefs where drinking of alcohol by a female is not socially acceptable. Hence number of females with ALDs reporting to hospital is negligible owing to social stigma attached to alcoholism. Age matched 50 healthy male individuals in the age group of 18–65 years were selected as controls.

Patients of ALD in the age group of 18–65 years were diagnosed on the basis of history of alcohol intake (amount and duration), clinical examination and sonography of abdomen, with raised serum ALT and serum bilirubin. None of these were suffering from NASH, viral hepatitis, heart or kidney disease, diabetes mellitus, hepatic tumors. They had history of alcohol intake for >10 years with average daily drinking of >60 g of alcohol. Most of them were having country liquor. They were on abstinence since hospitalization.

Patients of NASH and acute viral hepatitis in the age group of 18–65 years were diagnosed on the basis of history, clinical examination, viral antigen/antibody study and

sonography of abdomen, with raised serum ALT and serum bilirubin. Patients with history of alcoholism, heart or kidney disease and hepatic tumors were excluded from the study.

Serum samples from the patients were analyzed for total bilirubin (normal 0.1–1.2 mg%) and direct bilirubin (0–0.3 mg%) by Diazo method, AST (normal up to 40 IU/l), ALT (normal up to 40 IU/l), ALP (normal 25–90 IU/l) and GGT (normal 0–45 IU/l) by kinetic method in clinical biochemistry laboratory using autoanalyzer (Erba Excel-300) and semiautoanalyzer (Erba chem 5-plus). Parametric data was analyzed by Student unpaired '*t*' test. A '*P*' value of <0.05 was considered to be statistically significant. $P < 0.01$ was taken as highly significant.

Results

Data regarding biochemical evaluation of patients of ALD and non-ALD (NASH and acute viral hepatitis) are presented below in Tables 1, 2 and 3. Comparison between the groups is shown in Tables 4 and 5.

Table 1 Biochemical evaluation of patients of ALD (group I)

Parameters	Control ($n = 50$) (mean ± SD)	ALD ($n = 50$) (mean ± SD)	*P* value
Bil. (T) (mg%)	1.14 ± 0.219	3.94 ± 2.88	<0.01, S
Bil. (D) (mg%)	0.506 ± 0.17	2.01 ± 1.86	<0.01, S
ALT (IU/l)	24.22 ± 7.31	49.32 ± 20.99	<0.01, S
AST (IU/l)	23.78 ± 5.56	125.58 ± 57	<0.01, S
ALP (IU/l)	49.68 ± 13.48	117.52 ± 43.93	<0.01, S
GGT (IU/l)	24 ± 7.16	55.12 ± 17.02	<0.01, S
AST/ALT ratio	1.04 ± 0.32	2.54 ± 0.77	<0.01, S

SD standard deviation

$P < 0.01$: highly significant

Table 2 Biochemical evaluation of patients of NASH (group IIA)

Parameters	Control ($n = 50$) (mean ± SD)	NASH ($n = 35$) (mean ± SD)	*P* value
Bil. (T) (mg%)	1.14 ± 0.21	2.56 ± 0.71	<0.05, S
Bil. (D) (mg%)	0.50 ± 0.17	1.47 ± 0.57	<0.05, S
ALT (IU/l)	24.22 ± 7.31	60.28 ± 14.13	<0.01, S
AST (IU/l)	23.78 ± 5.56	53.12 ± 17.40	<0.01, S
ALP (IU/l)	49.68 ± 13.48	96 ± 25.06	<0.01, S
GGT (IU/l)	24 ± 7.16	34.12 ± 5.89	<0.01, S
AST/ALT ratio	1.04 ± 0.32	0.88 ± 0.17	NS

NS non-significant

$P < 0.05$,S: statistically significant; $P < 0.01$: highly significant

Discussion

Alcoholism is diagnosed on the basis of clinical history, questionnaire about alcohol consumption and many laboratory investigations. Distinguishing ALD from non-ALD has important implications for treatment and management. But many times it becomes difficult, as history can be unreliable. So we evaluated the patients of ALD, NASH

Table 3 Biochemical evaluation of patients of acute viral hepatitis (group IIB)

Parameters	Control ($n = 50$) (mean ± SD)	Acute viral hepatitis ($n = 35$) (mean ± SD)	*P* value
Bil. (T) (mg%)	1.14 ± 0.21	5.796 ± 6.1	<0.01, S
Bil. (D) (mg%)	0.50 ± 0.17	3.38 ± 4.68	<0.01, S
ALT (IU/l)	24.22 ± 7.31	401.32 ± 379.13	<0.01, S
AST (IU/l)	23.78 ± 5.56	363.44 ± 366.6	<0.01, S
ALP (IU/l)	49.68 ± 13.48	207.4 ± 166.98	<0.01, S
GGT (IU/l)	24 ± 7.16	45.36 ± 25.18	<0.01, S
AST/ALT ratio	1.04 ± 0.32	0.83 ± 0.29	NS

Table 4 Comparison between patients of groups I (ALD) and IIA (NASH)

Parameters	ALD ($n = 50$) (mean ± SD)	NASH ($n = 35$) (mean ± SD)	*P* value
Bil. (T) (mg%)	3.94 ± 2.88	2.56 ± 0.715	NS
Bil. (D) (mg%)	2.01 ± 1.86	1.47 ± 0.578	NS
ALT (IU/l)	49.32 ± 20.99	60.28 ± 14.13	<0.01, S
AST (IU/l)	125.58 ± 57	53.12 ± 17.40	<0.01, S
ALP (IU/l)	117.52 ± 43.93	96 ± 25.06	<0.01, S
GGT (IU/l)	55.12 ± 17.02	34.12 ± 5.89	<0.01, S
AST/ALT ratio	2.5462 ± 0.77	0.88 ± 0.176	<0.05, S

Table 5 Comparison of patients of groups I (ALD) and IIB (acute viral hepatitis)

Parameters	ALD ($n = 50$) (mean ± SD)	Acute viral hepatitis ($n = 35$) (mean ± SD)	*P* value
Bil. (T) (mg%)	3.94 ± 2.88	5.79 ± 6.1	NS
Bil. (D) (mg%)	2.01 ± 1.86	3.38 ± 4.68	NS
ALT (IU/l)	49.32 ± 20.99	401.32 ± 379.13	<0.01, S
AST (IU/l)	125.58 ± 57	363.44 ± 366.63	<0.01, S
ALP (IU/l)	117.52 ± 43.93	207.4 ± 166.98	<0.01, S
GGT (IU/l)	55.12 ± 17.02	45.36 ± 25.18	<0.05, S
AST/ALT ratio	2.546 ± 0.77	0.83 ± 0.29	<0.05, S

and acute viral hepatitis by various biochemical laboratory parameters.

Table 1 shows higher levels of serum bilirubin (total [T] and direct [D]), ALT, AST, ALP and GGT in ALD patients compared to controls and it is statistically significant ($P < 0.01$). It also shows AST/ALT ratio of 2.54 ± 0.77 in ALD patients which is higher (>2) compared to 1.04 ± 0.32 in controls and this difference is statistically significant ($P < 0.01$). Biochemical evaluation of patients of ALD revealed that they had severe hepatic damage compared to control.

Table 2 shows raised levels of serum bilirubin (T) and (D), ALT, AST, ALP and GGT in NASH patients compared to controls and it is statistically significant ($P < 0.01$).

Table 2 also shows slight difference in AST/ALT ratio in NASH patients and control which is statistically non-significant ($P > 0.05$).

Table 3 shows raised levels of serum bilirubin (total and direct), ALT, AST, ALP and GGT in acute viral hepatitis patients compared to controls and these are statistically significant ($P < 0.01$). But there is minimal difference in AST/ALT ratios of these two groups, which is statistically non-significant ($P > 0.05$).

Biochemical evaluation of patients of NASH and acute viral hepatitis revealed mild hepatic injury (AST/ALT ratio <1, normal to minimal rise in serum ALP and GGT) in them compared to controls. Some patients of acute viral hepatitis showed raised ALP which may be due to cholestasis.

In ALD AST/ALT ratio may be >2 [7, 9, 10]. This appears to be the result of reduction in hepatic ALT content due to a deficiency in the cofactor pyridoxine-5-PO_4. AST/ALT ratio is typically <1 in other causes of steatohepatitis [8, 12, 13]. An elevated serum AST in relation to serum ALT has been proposed as an indicator that alcohol has induced organ damage. When AST/ALT ratio is >1.5, it is considered as highly suggestive that alcohol is the cause of liver injury [14].

We compared the patients of ALD and NASH by various biochemical parameters in Table 4. We observed a rise in AST/ALT ratio in ALD compared to NASH with $P < 0.05$. Transaminase levels in ALD patients were AST dominant while those in NASH patients were ALT dominant.

One study [23] observed AST/ALT ratio of 0.9 in NASH patients and 2.6 in ALD patients. Aminotransferase levels were significantly different in two groups ($P < 0.05$). They observed AST/ALT ratio of 0.7, 0.9 and 1.4 in NASH patients with no fibrosis, mild fibrosis and cirrhosis respectively ($P < 0.05$).They concluded that a ratio of <1 suggests NASH and >2 suggests ALD [11, 23, 24]. The rise in AST/ALT ratio in ALD patients compared to NASH patients in our study may be due to pyridoxine-5-PO_4 deficiency, decreased hepatic ALT activity and alcohol induced mitochondrial damage leading to release of mitochondrial AST in serum [25].

In our study (Table 4) we found a rise in serum ALP and GGT in ALD patients compared to NASH patients. This rise is statistically significant ($P < 0.01$). Elevated GGT associated with an increase in serum ALP shows that ALP in serum is of hepatic origin, as GGT is not raised in bone diseases [22]. The rise in serum GGT in alcoholic patients may be due to hepatic microsomal enzyme induction by alcohol [18, 21, 26–28].

This comparison in our study provides information that the patients of ALD can be differentiated from NASH patients based on biochemical parameters like AST/ALT ratio, GGT and ALP.

Many times alcoholic patients present with clinical presentation similar to that of viral hepatitis. Also history of alcoholism may not be reliable. Antigens and antibodies against viruses may not be detectable in early cases. These conditions need to be distinguished at the earliest to have proper diagnosis and management. Focusing this need we compared the patients of ALD and acute viral hepatitis by biochemical parameters in Table 5.

We observed (Table 5) a significant and proportionate rise in serum ALT and AST in acute viral hepatitis patients compared to that in ALD patients ($P < 0.01$). AST/ALT ratio in acute viral hepatitis patients is 0.83 ± 0.29 whereas that in ALD patients is 2.54 ± 0.77 ($P < 0.05$).

AST rise is less than ALT in liver diseases including viral hepatitis except ALD where AST rise is 2–3 times more than ALT [8, 9, 11, 29].

Slight to moderate increase in ALP occurs in many patients with liver disorders such as viral hepatitis and cirrhosis [17–19, 30]. We observed (Table 5) a significant rise in serum ALP in viral hepatitis patients compared to that in ALD patients ($P < 0.01$). Rise in serum ALP is a better indicator of cholestasis than GGT [31].There is a rise in serum GGT level in ALD patients compared to that in acute viral hepatitis patients ($P < 0.05$). Raised GGT in ALD patients may be due to alcohol induced enzyme release [10, 18, 21, 26, 32].

The results in our study suggested that we can differentiate patients of ALD from those of acute viral hepatitis based on parameters like AST/ALT ratio, ALP and GGT.

This study concluded that ALD patients can be differentiated from patients of NASH and acute viral hepatitis by measuring serum AST/ALT ratio, GGT and ALP. Our study suggests that these biochemical parameters may be used prior to invasive and expensive investigations like biopsy and sonography to support the clinical diagnosis. So that early diagnosis and treatment can be done to prevent the further complications. Overall, there are poor chances of recovery in patients of ALD. Timely intervention in the form of alcohol

abstinence and supportive treatment should be emphasized to halt the disease process.

Patients of NASH and acute viral hepatitis can be said to have fair prognosis with much less mortality after proper diagnosis and treatment.

Limitation of our study is that we evaluated very few biochemical parameters. Moreover these parameters may lack sensitivity when used singly. Further studies in this regard, using other biochemical parameters to differentiate ALD from non-ALD with certainty, are undoubtedly warranted.

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Glycyrrhizic acid modulates t-BHP induced apoptosis in primary rat hepatocytes

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ABSTRACT

Glycyrrhizic acid (GA) is the main bioactive ingredient of licorice (*Glycyrrhiza glabra*). The object of this study was to evaluate the protective effects of GA on tert-butyl hydroperoxide (t-BHP) induced oxidative injury leading to apoptosis in cultured primary rat hepatocytes. Throughout the study silymarin was used as positive control. Molecular mechanisms involved in apoptotic pathways induced in hepatocytes by t-BHP at 250 μM were explored in detail. DNA fragmentation, activation of caspases and cytochrome *c* release were demonstrated. In addition, changes in the mitochondrial membrane potential and ROS generation were detected confirming involvement of mitochondrial pathway. Pre-treatment with GA (4 μg) protected the hepatocytes against t-BHP induced oxidative injury and the results were comparable to the pre-treatment with positive control, i.e. silymarin. The protective potential against cell death was achieved mainly by preventing intracellular GSH depletion, decrease in ROS formation as well as inhibition of mitochondrial membrane depolarization. GA was found to modulate critical end points of oxidative stress induced apoptosis and could be beneficial against liver diseases where oxidative stress is known to play a crucial role.

1. Introduction

Hepatocytes make up 60–65% of the cells in the liver and play a pivotal role in the metabolism of exogenous chemicals and toxins, thus making liver a target for toxic substances. Reactive oxygen and nitrogen species (ROS/RNS) are generated during detoxification which leads to oxidative stress. These free radicals are considered critical molecules as they take part in a variety of cellular functions (Das et al., 2004). Imbalance in the cellular redox status, the increased levels of oxidants overwhelm the capacity of the antioxidant defense network resulting into oxidative stress. Involvement of ROS/RNS in the pathogenesis of certain human diseases, including cancer, diabetes, cataract, neuronal disorders and artherosclerosis is increasingly being recognized. Upsetting this balance causes oxidative stress, which can lead to cell death/injury. Natural antioxidants that can inhibit lipid peroxidation or are able to protect the system from the damage caused by free radicals are being explored for strengthening antioxidant defense. Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases, used as a chemopreventive agent in cancers and neurodegenerative disorders (Di Matteo and Esposito, 2003).

Tert-butyl hydroperoxide (t-BHP) is an organic lipid hydroperoxide analogue, used as pro-oxidant to evaluate mechanisms involving oxidative stress in cells and tissues. t-BHP has been shown to induce cell death in a variety of cells via apoptosis including U937 macrophages, PC-12 cells, SH-SY5Y neuroblastoma cells and HepG2 cells (Amoroso et al., 2002; Kanupriya et al., 2007). Two distinctive pathways are involved in the metabolism of t-BHP in hepatocytes (Haidara et al., 2001). The first employs microsomal cytochrome P-450 system leading to the production of ROS such as peroxyl and alkoxyl radicals. These radicals initiate peroxidation of the cell membrane phospholipids and accumulation of lipid peroxides which are expected to alter the membrane fluidity and permeability, consequently leading to disruption of membrane structure and function. The second pathway involves

Abbreviations: $\Delta\Psi_m$, mitochondrial membrane potential; AIF, apoptosis inducing factor; APAF-1, apoptosis protease activating factor-1; CAD, caspases-activated DNase; CMF-DA, 5′-chloromethylfluorescein diacetate; DCF, dichlorofluorescein; $DCFH_2$, dichlorodihydrofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; GA, glycyrrhizic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; JC-1, 5′,5′,6′,6′-tetrachloro-1′,1′,3′,3′-tetraethylbenzamidazolcarbocyanine iodide; LDH, lactate dehydrogenase; LPO, lipid peroxidation; MDA, malonyldialdehyde; MFI, mean fluorescence intensity; MPT, mitochondrial permeability transition; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; *p*-NA, *p*-nitro aniline; PARP, poly ADP ribose polymerase; PBS, phosphate buffer saline; PVDF, polyvinylidene fluoride; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, 2′-thiobarbituric acid; TBARS, thiobarbituric acid reactive substance; t-BHP, tert-butyl hydroperoxide; TCA, trichloroacetic acid.

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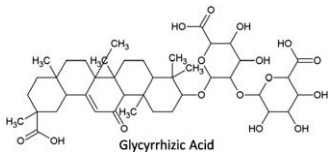
GSH peroxidase and its substrate GSH, which converts t-BHP to t-butanol and GSH to GSSG. The GSSG is then reduced to GSH by GSH reductase, resulting in NADPH oxidation. Decreased GSH and oxidized NADPH contribute to altered Ca^{2+} homeostasis, which is considered to be a major event in t-BHP-induced toxicity (Shimizu et al., 1998). It has been documented that GSH depletion and MDA increase, events observed frequently during oxidative damage, are inducers of mitochondrial permeability transition (MPT). Loss of $\Delta\Psi_m$ is directly associated with apoptosis. Apoptosis permits the removal of damaged, senescent or unwanted cells in the multicellular organisms, without damaging the microcellular environment and can be induced by various extracellular or intracellular factors. It results in several morphological changes which are characteristic feature of apoptosis such as cell shrinkage, membrane blebbing along with biochemical changes like DNA fragmentation, cleavage of wide variety of cellular proteins including PARP and lamin. In apoptosis, the $\Delta\Psi_m$ is lost, releasing small apoptogenic molecule, cytochrome *c*, leading to the formation of apoptosome. Apoptosome is high in molecular weight and consists of cytochrome *c*, dATP, apoptosis protease activating factor-1 (APAF-1) and procaspases-9. Once the signal is received the initiator caspase activates the downstream caspases, like caspase-3. Caspases are synthesized as pro-enzymes, which are cleaved at internal sites to form an active enzyme. These are group of cystein proteases which can be either initiator (caspase-2, -8, -9, -10) or effector (caspase-3, -6, -7).

Exogenous dietary antioxidants capable of scavenging free radicals are of great interest in combating oxidative stress induced cell damage. Plants containing high content of polyphenols, flavanoids are considered as potential antioxidants and can be used as adjuvant therapy. These plant polyphenols and flavanoids are multifunctional and can act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal ion chelators (Gassen and Youdim, 1999). Some hepatoprotective plants as well as formulations used in traditional medicine have been pharmacologically evaluated for their efficacy such as *Andrographis panniculata* (Singh et al., 2001) and *Eclipta alba* (Saxena et al., 1993) to name a few. Amongst all these medicinal plants *Glycyrrhiza glabra* (licorice) is a plant with a rich ethno-botanical history. The roots are used as a folk medicine both in Europe and in Eastern countries. The main components are the triterpene, saponins, glycyrrhizin/glyccyrrhizic acid and glycyrrhetic acid. Glycyrrhizic acid (GA), a biologically active constituent of licorice root with a structure of 20β-carboxy-11-oxo-30-norolean-12en-3-β-yl-2-o-β-D-glucopyranosiduronic acid, is believed to be partly responsible for anti-ulcer, anti-inflammatory, anti-diuretic, anti-epileptic, anti-allergic, anti-dote, anti-tumor, anti-viral, anti-hypotensive and several other properties of the plant (Baltina, 2003). Its hypocholesterolaemic and hypoglycemic activities have been reported (Sitohy et al., 1991).



Different polyphenols, which make up 1–5% of the root of *G. glabra*, have been evaluated for antioxidant and anticarcinogenic properties. GA is a powerful sweetener and 50 times as potent as sucrose. This is also used in herbal teas and in herbal formulations. A nutritive substance with additional properties such as antioxidant and hepatoprotective can be routinely taken as a food supplement. The aim of the present study was therefore to assess the anti-apoptotic property of GA against t-BHP induced oxidative stress in relation with mitochondria-mediated cell death process in primary hepatocytes.

Silymarin, a known hepatoprotectant, was used as a positive control throughout the study. It is known to have clinical applications in the treatment of toxic hepatitis, fatty liver, cirrhosis, ischemic injury, radiation toxicity, and viral hepatitis via its antioxidative, anti-lipid peroxidative, antifibrotic, anti-inflammatory, immunomodulating, and liver regenerating effects.

2. Materials and methods

2.1. Test substances and reagents

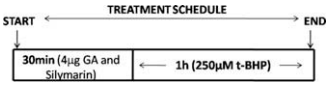
Tert-butyl hydroperoxide (t-BHP) (EC No. 200-915-7; CAS No. B2633-100ML) and glycyrrhizic acid (GA) (EC No. 258-887-7; CAS No. G2137-25G) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was procured from Calbiochem. DNA 50bp ladder was procured from Fermentas whereas agarose was obtained from GE. Mouse polyclonal antibody against cytochrome *c* and horse-radish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ECL kit for detection of western blot was purchased from Amersham Biosciences. All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise mentioned.

2.2. Animals

Sprague–Dawley male rats weighing 180 ± 20 g were taken from Indian Institute of Toxicology Research (IITR) animal colony and used for the experiment. Animals were kept under standard conditions of humidity (60–70%), temperature (25 ± 2 °C) and a controlled 12 h light/dark cycle. Rats were fed Ashirwad pellet diet and water *ad libitum*. All the guidelines of Institutional Animal Ethics Committee (ITRC/IAEC/20/2006) were followed while handling the animals and chloroform was used for euthanasia.

2.3. Primary cell-culture

Hepatocytes were isolated from liver of overnight fasted rat after subjecting it to two-stage collagenase perfusion with HEPES buffer (Seglen, 1976). Cell viability was checked by trypan blue dye exclusion test within an hour of cell isolation. Only preparations with cell viability greater than 95% were used for subsequent experiments. Hepatocytes were maintained in RPMI-1640 media supplemented with heat-inactivated 10% fetal bovine serum and 1% of 10,000 units Penicillin,10 mg Streptomycin and 25 μg Amphotericin-B at 37 °C in a 5% CO_2 −95% air incubator (Thermo-forma) with controlled humidity. The cells were seeded at a density of 1.0×10^4 cells in 0.1% collagen pre-coated 96-well plate, and used for the drug exposure experiments after being cultured overnight. Further treatment schedule was followed as described.



2.4. MTT assay

Cell viability was determined by a colorimetric MTT assay, as described by Mosmann (1983). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase, which demonstrates functional mitochondrial dehydrogenase, i.e. functional mitochondria. Twenty four hours old hepatocytes, were exposed to various concentrations of t-BHP and glycyrrhizic acid. At the end of the incubation period, the culture medium was removed and 0.1 ml of MTT (from a stock of 5 mg/ml) was added to each well. After 4 h incubation the medium was removed and to each well 0.2 ml DMSO was added. Optical density (OD) was measured at 530 nm using a Spectramax PLUS 384 microplate reader (Soft max pro version 5.1; Molecular Devices, USA). The linear relationship between OD and cell density was taken into account. The data are expressed as a percentage of control viability measurement in untreated cells.

2.5. GSH content

5′-Chloromethylfluoroscein diacetate was used to measure the total GSH in the cells. The dye passes freely through the cell membrane, but once inside the cells they are transformed into cell impermeant reaction products. CMF-DA is colourless and nonfluorescent until cytosolic esterases cleave off the acetates, and the bright fluorescence coloured product is formed which is detected by flowcytometer. The treated cells were incubated with the fluorescent dye (5 μg/ml) for 30 min in dark at 37 °C (Okada et al., 1996) and the analysis of treated/ control cells was carried out using flowcytometer (BD-LSR), cell quest software.

2.6. Measurement of intracellular ROS

ROS levels were determined using 2′7′-dichlorofluorescein diacetate (DCFH-DA), which is de-estrified to 2′7′-dichlorodihydrofluorescein ($DCFH_2$) by cellular esterases. This $DCFH_2$ is further oxidized to DCF by ROS and increase in fluorescence intensity is used to quantify the generation of intracellular ROS. Control cells as well as cells with treatment were incubated for 30 min with DCFH-DA (5 μg/ml) at 37 °C in dark. To evaluate ROS mediated oxidation of DCFH-DA to the fluorescent compound DCF, samples were analyzed at an excitation wavelength of 480 nm and an emission wavelength of 525 nm by flowcytometer (BD-LSR). Each determination is based on mean fluorescence intensity of 10,000 events (Mohammad et al., 2001).

2.7. SOD activity

SOD activity was determined spectrophotometrically by measuring inhibition of nicotinamide adenine dinucleotide (reduced)–phenazine methosulfate–nitroblue tetrazolium reaction system by the method of Kakkar in 1984 after adoption on microplate. Superoxide radical is produced *in situ*, which is involved in the NBT reduction leading to the formation of blue formazan, which is read at 560 nm. Fifty percent inhibition of formazan formation in 1 min is taken as 1 unit activity/min (Kakkar et al., 1984).

2.8. MDA determination

Thiobarbituric acid reactive substance (TBARS) formation as a product of lipid peroxidation was estimated in pre-treated hepatocytes by using the method of Wallin in 1993. In this method, oxidation of phospholipids and evaluation of TBARS is achieved in single 96-well microplate. Major oxidative product of phospholipids, i.e. malondialdehyde (MDA) was estimated by measuring the amount of MDA formed as a breakdown product at 530 nm. The lipids were isolated by precipitating the cell lysate with TCA and then indirectly the lipid peroxide concentration was measured with TBA reaction. The amount of MDA formed as a breakdown product was measured at 530 nm and 600 nm (Wallin et al., 1993).

2.9. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in the $\Delta\Psi_m$ in treated hepatocytes were monitored after staining them with JC-1. 5′,5′,6′,6′-Tetrachloro-1′,1′,3′,3′-tetraethylbenzamidazolcarbocyanine iodide commonly known as JC-1 which is specific for mitochondria. In cells not undergoing apoptosis, the $\Delta\Psi_m$ remains intact and the dye accumulates to form an aggregate that gives red fluorescence. In mitochondrial membrane where potential is compromised the formation of JC-1 aggregate is prevented and the fluorescence shifts from red to green. Treated hepatocytes were incubated with JC-1 for 15 min at 37 °C in a CO_2 incubator. After washing the hepatocytes with PBS, change in the $\Delta\Psi_m$ was assessed by comparing the two fluorescence 590 nm (red)/527 nm (green) using flowcytometer (BD-LSR) (Cossarizza et al., 1996).

2.10. Preparation of cytosolic fraction

Cytosolic fractions were prepared by the method described by Zhang et al. (1999) with some modification. Briefly, cells were harvested, washed and suspended in ice-cold buffer containing 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1 mM EGTA and 1 mM PMSF and sonicated for 10 s (Sartorius, Labsonic M). The lysate were now centrifuged at 800*g* for 4 min at 4 °C under cold conditions. The supernatant was again centrifuged at 22,000*g* for 15 min at 4 °C in a refrigerated centrifuge (Sigma 3K18) and the resulting supernatant was used as cytosolic fraction.

2.11. Western blot analysis

The protein content corresponding to each treatment was quantified using Lowry's method (Lowry et al., 1951). Samples were boiled with Lammeli's buffer for 5 min and immediately kept in ice. 20 μg of each protein sample was separated by SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gel and electroblotted on PVDF membrane (Amersham). After blocking non-specific sites washing was performed using PBS containing tween-20. The membrane was then incubated for 1 h with goat polyclonal IgG antibody in dilution 1:500 (Santa Cruz Biotechnology, Inc.). Then it was washed with PBS and incubated with horse-radish peroxidase-conjugated rabbit anti-goat IgG secondary antibody in dilution 1:1000 at 1 h for room temperature. Again it was rewashed and the immunoblot was revealed using ECL chemiluminescent detection kit according to the manufacturer's instructions. β-Actin was used as internal standard. The bands obtained were analyzed using NIH software Image J version 13.2.

2.12. Caspases activity

To investigate the role of mitochondria in the cytotoxicity caused by t-BHP caspase-3 and -9 activity were measured. Caspase activities were determined by a colorimetric assay based on the ability of caspases-3, -9 to change acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-*p*NA) and acetyl-Leu-Glu-His-Asp-*p*-nitroanilide (Ac-LEHD-*p*NA) into yellow formazan product (*p*-Nitro aniline), respectively. An increase in absorbance at 405 nm was used to quantify the activation of caspase activities. In brief, isolated hepatocytes were pre-incubated with GA as well as t-BHP for specified time. After incubation, the medium was discarded and adherent cells were harvested in PBS and sedimented by centrifugation at 600g for 3 min. The pellets were then resuspended in lysis buffer for 20–30 min on ice. The lysed cells were centrifuged at 10,000g for 3 min and to the supernatant, reaction mixture and buffer were added. The concentration of the *p*-NA released from the substrate was calculated from the absorbance value at 405 nm using a calibration curve.

2.13. DNA fragmentation assay

DNA was isolated from control and treated hepatocytes. After incubation, cells were washed with PBS and lysed with lysis buffer containing 250 mM Sucrose, 5 mM Tris–HCl (pH 8.0), 1 mM EDTA, 20% SDS and incubated overnight at 37 °C. Subsequently, RNase A was added for 1 h at 37 °C followed by addition of 8 M potassium acetate. The lysate was extracted twice with an equal volume of chloroform/isoamylalcohol (24:1) and centrifuged at 1000g for 5 min. The upper aqueous layer was taken and two volumes of absolute ethanol was added to it and incubated at −20 °C in order to precipitate the DNA. The pellet obtained after centrifugation at 14,000*g* for 15 min was air dried and then dissolved in Tris–EDTA buffer. DNA quantification was done spectrophotometrically at 260/280 nm. DNA samples were finally separated on 1.8% agarose gel with Tris–Borate/EDTA buffer and analyzed on an Alfa-innotech image analyzer (Hermann et al., 1994).

2.14. Analysis of hepatocyte nuclear morphology

Changes in the nuclear morphology were observed using bisbenzimide (Hoechst 33258) fluorochrome that binds with DNA. Primary hepatocyte monolayers were fixed in ice cold methanol/acetic acid (3:1) for 5 min. Cells were stained with Hoechst 33258 (5 μg/ml) for 10 min and washed. Cells were mounted in a solution of 20 mM citric acid, 50 mM di sodium orthophosphate, and 50% glycerol (pH 5.5) and examined at a wavelength of 350–460 nm using a Nikon microscope with fluorescence attachment (Bayly et al., 1994).

2.15. LDH activity-based cytotoxicity assay

LDH (lactate dehydrogenase) was measured using standardized kit from Sigma–Aldrich. LDH activity was measured both in floating dead cells and viable adherent cells. The floating cells were collected from culture medium by centrifugation (240*g*) at 4 °C for 5 min and LDH content from the pellet was used as an index of apoptotic cell death (LDHp). The LDH released in the culture medium (LDHe; extracellular LDH) was used as an index of necrotic cell death and the LDH present in the adherent viable cells as intracellular LDH (LDHi). The percentage of apoptotic and necrotic cell death was calculated as follows:

$$\% \text{ Apoptosis} : \text{LDHp}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$
$$\% \text{ Necrosis} : \text{LDHe}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$

2.16. Annexin V-FITC binding assay

Annexin is a 35 to 36 kDa Ca^{2+} dependent, phospholipid binding protein that has high affinity for phosphotidylserine and binds to cells with exposed phosphotidylserine. In the early stages of apoptosis membrane phosphotidylserine is translocated from inner side to outer side of the plasma membrane. The annexin V assay was carried out in conjugation with PI (propidium iodide) staining in order to distinguish between apoptosis and necrosis, because PI staining can detect DNA that has leaked from the necrotic cells. (a) Viable hepatocytes are negative for both annexin V and PI, (b) early apoptotic hepatocytes were labeled with annexin V while negative with PI, (c) late apoptotic cells were labeled with both annexin V and PI, and (d) necrotic cells were labeled with PI but negative with annexin (Liu et al., 2003).

2.17. Statistical analysis

Data are expressed as mean ± S.E. Data were analyzed on SPSS software version 14.0 using one way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ were used as the criterion for significance.

3. Results

3.1. GA increased survival of t-BHP stressed primary hepatocytes

The preventive effect of GA was studied on the cytotoxicity of t-BHP, a chemical that generates alkoxyl/peroxyl radicals leading to oxidative stress in the cellular system. Inhibitory concentration (IC_{50}) was determined using MTT cytotoxicity assay. Only 95% viable primary hepatocyte population was used throughout the study as evident from the trypan blue dye exclusion assay (data not shown). It was observed that t-BHP at 250 μM, reduced the cell survival to 52% ± 0.12 ($P < 0.001$), i.e. the inhibitory concentration (IC_{50}) of t-BHP was found to be 250 μM (Fig. 1a). This concentration (250 μM) of t-BHP was used for further experiments in the presence of GA. Isolated primary rat hepatocytes were also treated with varying concentrations of GA (2–12 μg) as shown in Fig. 1b to study its effect on cell viability. At 4 μg of GA the increase in survival was 58.18% ± 0.02 ($P < 0.001$) with respect to control, so for further experiments 4 μg of GA was taken up as the selected dose for treating cells. At the same concentration (4 μg) of silymarin, a positive control, cell survival rate was found to increase by 43.36% ± 0.05 ($P < 0.001$). During the pre-treatment schedule, 24 h cultivated hepatocytes were incubated with GA (4 μg) for 30 min before subjecting them to oxidative stress of t-BHP (250 μM) for an hour. A positive correlation between dose-response in terms of viability was seen. The cells which were pre-incubated with GA showed significant increase in survival by 44.89% ± 0.03 ($P < 0.001$), when compared to control cells whereas cells treated with silymarin showed increase by 38% ± 0.08 ($P < 0.001$) (Fig. 1c).

3.2. Restoration of antioxidant status in stressed rat hepatocytes by GA treatment

3.2.1. GSH content

The fluorescence intensity of CMF dye was captured using flow-cytometer, reflecting GSH content in the cellular system and the results mentioned here are the mean fluorescence intensities (MFI). Cells when stressed with t-BHP (250 μM), showed decrease in GSH content by 0.6-folds ($P < 0.05$). Cells pre-treated with GA at the selected dose (4 μg) were found to restore the GSH content by 1.58-folds ($P < 0.01$) or inhibited the depletion of GSH which was comparable to control cells. Silymarin (4 μg) was also found to inhibit GSH depletion in the stressed cells (1.56-folds) as shown in Table 1. The data indicates that GA treatment is effective in abrogating oxidative damage that further results in GSH depletion by t-BHP.

3.2.2. ROS generation

The extent of ROS generation during t-BHP induced stress in hepatocytes, was monitored by flow cytometry using DCFH-DA dye. Hepatocytes stressed with t-BHP showed an increase in the ROS generation by 2.3-folds as compared to untreated cells. Whereas in the cells that were pre-treated with GA 0.51-folds decline in the ROS generation was observed as compared to t-BHP stressed cells. The decline in ROS generation (Table 1) in pre-treated cells was very much comparable with silymarin, i.e. 0.5-folds (positive control).

3.2.3. SOD activity

Cultured hepatocytes subjected to t-BHP stress showed SOD activity 4.6 units/min/10^4 cells which was 0.7-folds less than the

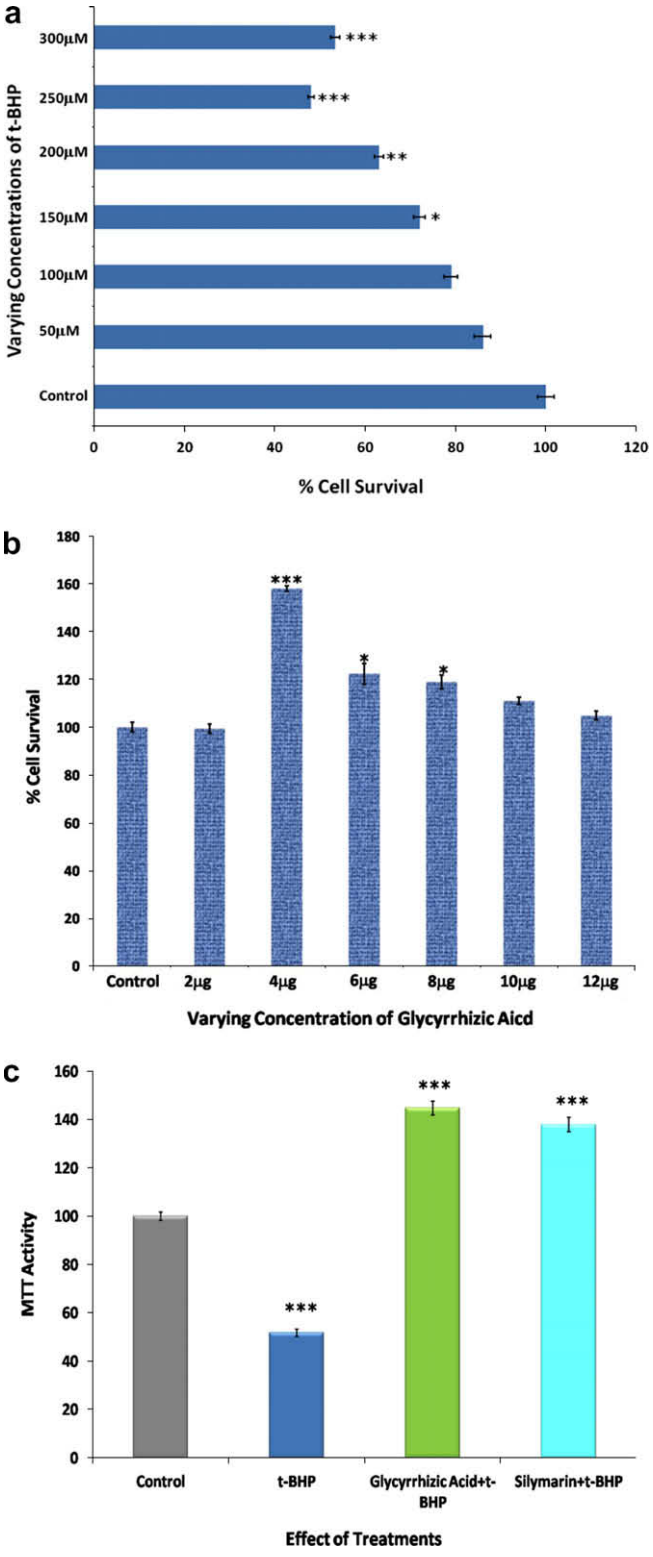


Fig. 1. Effect of treatments on the viability of cultured primary rat hepatocytes: (a, b) Different concentrations of (50–300 μM) t-BHP and glycyrrhizic acid (2–12 μg) were administered to primary rat hepatocytes (1×10^4 cells/well of collagen coated 96-well cell-culture plate in 100 μl of assay medium) for 1 h and 30 min, respectively. At 250 μM concentration of t-BHP the viability of cells decreased to 51%. Viability of hepatocytes was maximum in the presence of 4 μg GA. (c) Hepatocytes were pre-treated with GA (4 μg) and silymarin (4 μg), respectively, for 30 min and then were treated with t-BHP (250 μM) for 1 h. Values are mean ± S.E. of 5 determinations in each case. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 1
Antioxidant status and ROS generation under oxidative stress in hepatocytes: hepatocytes were treated with the selected concentration of t-BHP (250 μM); glycyrrhizic acid (4 μg); silymarin (4 μg); glycyrrhizic acid + t-BHP and silymarin + t-BHP. Total cellular GSH content and intracellular ROS generation was measured by CMF-DA and DCFH-DA fluoroprobes, respectively, using flow cytometry whereas antioxidant potential (SOD activity and lipid peroxidation) was measured using biochemical assays. Cells treated with glycyrrhizic acid and silymarin alone were compared to control cells, whereas cells pre-treated with GA and silymarin followed by t-BHP treatment were compared to cells treated with t-BHP alone. Values are mean ± S.E. of 5 determinations in each case.

Treatments	ROS (DCF mean fluorescence intensity)	GSH content (CMF mean fluorescence intensity)	SOD activity (unit/min/10^4 cells)	Lipid peroxidation (nMMDA formation/10^4 cells)
Control	100.8 ± 1.7	228.0 ± 3.1	12.4 ± 0.64	0.23 ± 0.02
t-BHP	232.9 ± 4.9**	139.7 ± 2.6*	4.6 ± 0.91***	0.41 ± 0.04**
Glycyrrhizic acid	82.0 ± 1.4**	310.0 ± 4.9***	20.4 ± 0.63**	0.14 ± 0.05**
Silymarin	94.5 ± 1.6*	340.0 ± 3.1***	21.3 ± 0.44**	0.11 ± 0.01**
Glycyrrhizic acid + t-BHP	119.3 ± 1.9***	221.0 ± 5.1**	15.6 ± 0.24***	0.19 ± 0.04***
Silymarin + t-BHP	119.8 ± 1.5***	219.0 ± 4.5**	18.0 ± 0.92***	0.15 ± 0.01***

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

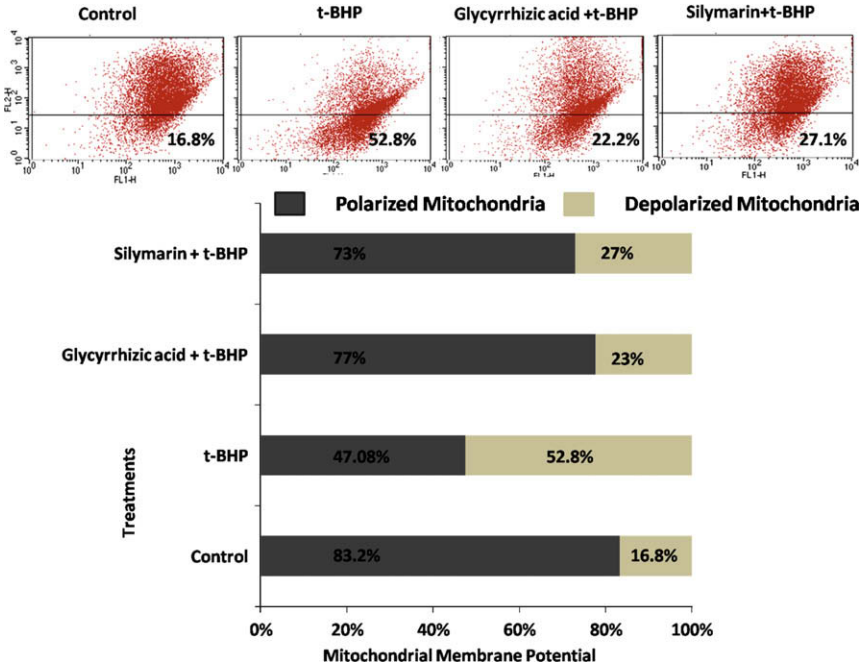


Fig. 2. Mitochondrial membrane potential: changes in mitochondrial membrane potential ($\Delta\Psi_m$) observed using mitochondria specific fluorescent probe JC-1. Monomer green fluorescence increased as MMP dropped. Graph indicates percentage mitochondrial population differentiated by flow cytometer, whereas, in dotplot, quadrant shows the two populations having green fluorescence (depolarized mitochondria) and red fluorescence (polarized mitochondria). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

untreated cells. Cells that were pre-incubated with GA (4 μg) showed 3.4-folds increase in superoxide dismutase activity (table 1) whereas cells pre-incubated with silymarin at the same concentration showed, 3.91-folds increase in superoxide quenching capacity. Thus, effect of GA compared well with a known hepatoprotectant, i.e. silymarin.

3.2.4. LPO inhibitory potential

Oxidative stress induced in the hepatocytes by free radical generation due to t-BHP caused 0.71 ± 0.005 ($P < 0.01$) nM MDA formation per 1.0×10^4 cells. In hepatocytes pre-treated with GA, the peroxidative decomposition of phospholipids was reduced to 0.198 ± 0.036/4 μg ($P < 0.001$). Treatment with silymarin reduced MDA formation to 0.15 ± 0.02/4 μg ($P < 0.001$), indicating strong antioxidant action of both GA and silymarin (Table 1).

3.3. t-BHP induced loss of $\Delta\Psi_m$ which is recovered by GA treatment

Generation of ROS and alteration in mitochondrial functions are well correlated by Kakkar and Singh (2007). Disruption of the mitochondrial membrane potential is one of the earliest indicator of induction of cellular damage. Under experimental conditions if the hepatocytes are not undergoing apoptosis, the mitochondrial membrane remains polarized ($\Delta\Psi_m$) and JC-1 dye gets accumulated and j-aggregates are formed due to which red fluorescence occurs. When the mitochondrial $\Delta\Psi_m$ is lowered the JC-1 aggregate dissipates into monomers and lead to shift from red to green fluorescence captured on flow cytometer. Cultivated hepatocytes when treated with 250 μM of t-BHP showed 52.80% green fluorescence as compared to control or untreated cells with 16.80% green fluorescence. In the cells pre-treated with GA, there was considerable abolition of t-BHP

induced lowering in $\Delta\Psi_m$ and the hepatocytes containing mitochondria with green fluorescence were found to be only 22.22%. GA alone did not alter $\Delta\Psi_m$, confirming the protective effect of GA pre-treatment. Hence, the results indicate that mitochondrial membrane is depolarized when treated with t-BHP and GA accords protection by preventing the mitochondrial depolarization (Fig. 2).

3.4. GA reduced cytochrome c released by t-BHP stress

Cytochrome *c* is located between the inter-membrane spaces of mitochondria where it assists in the production of life sustaining ATP by participating in electron transport. Several studies have shown that release of cytochrome *c* from mitochondria is associated with opening of mitochondrial permeability transition pores (Kroemer et al., 2007). Following the exposure of cells to apoptosis stimuli, cytochrome *c* is rapidly released from the mitochondria to cytosol which further activates cell death proteases (caspases). Thus, release of cytochrome *c* from mitochondria to cytosol is a trigger in the induction of apoptosis. Level of cytochrome *c* release was not so significant in the cytosolic fraction of cells treated with GA and silymarin (Fig. 3a). When stressed cells were analyzed there was an increase in the level of cytochrome *c* release by 4.75-folds as compared to unstressed cells, whereas GA pre-treated cells were observed the level decreased to 0.51-folds. The decrease in the cytochrome *c* level of cells pre-treated with GA was comparable to the positive control, i.e. 0.49-folds.

3.5. GA treatment lowered caspase-activation

To investigate the involvement of different caspases in t-BHP-induced apoptosis, we focused on initiator caspase-9, in the light of prior information that t-BHP induced pro-apoptotic events at

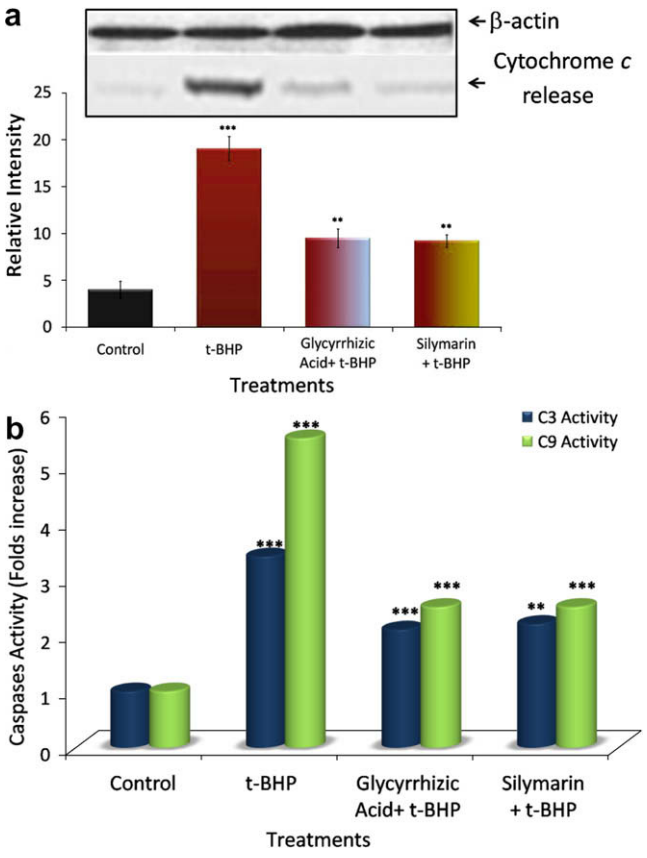


Fig. 3. Effect of treatments on cytochrome *c* release and caspases activation: (a) cytochrome *c* release was assessed from oxidatively stressed hepatocytes which were well protected by pre-treatment of glycyrrhizic acid and silymarin. The sequence of samples in cytochrome *c* blot is (from left to right) control, t-BHP, glycyrrhizic acid + t-BHP and silymarin + t-BHP. β-Actin was used as internal control. Values are mean ± S.E. of 3 determinations in each case. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (b) Caspase-3 and Caspase-9 activity in primary hepatocytes exposed to t-BHP, glycyrrhizic acid + t-BHP and silymarin + t-BHP. Values are mean ± S.E. of 5 determinations in each case. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

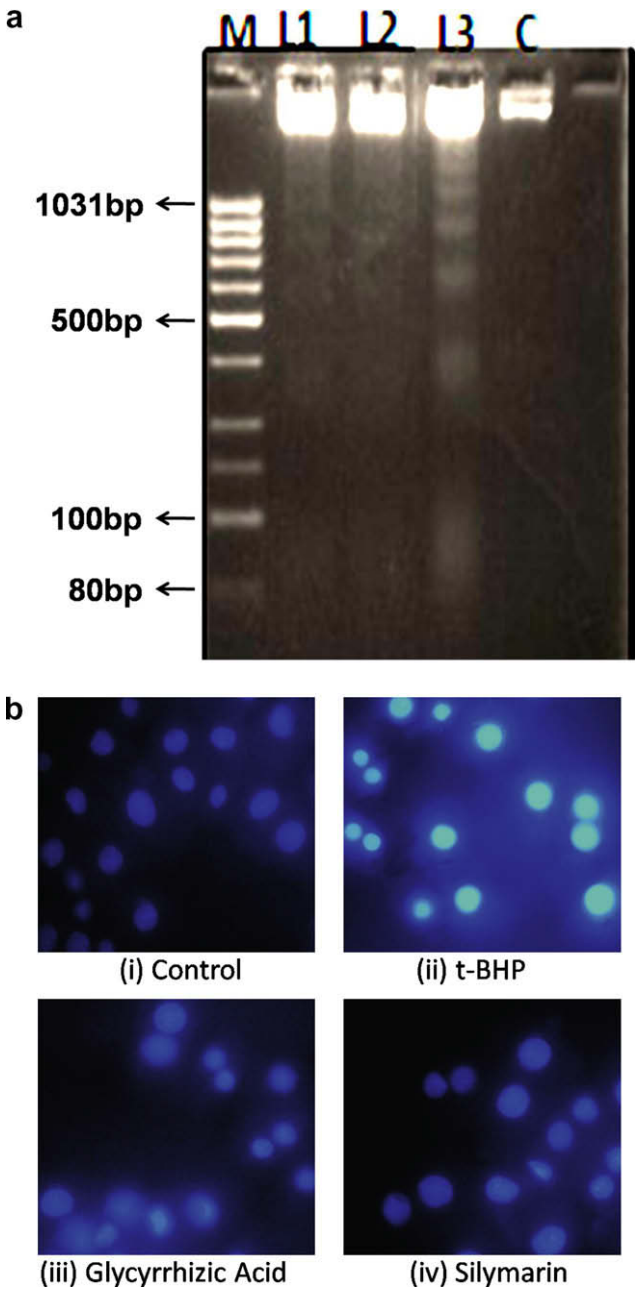


Fig. 4. DNA damage: (a) glycyrrhizic acid inhibits t-BHP-induced apoptosis in primary rat hepatocytes. Cells were incubated with 250 μM t-BHP and 4 μg of glycyrrhizic acid DNA fragmentation was measured by agarose gel electrophoresis (M – 100bp marker, L1 – glycyrrhizic acid pre-treated, L2 – silymarin pre-treated and C – control.). (b) Glycyrrhizic acid inhibits t-BHP-induced apoptosis in primary rat hepatocytes. Cells were incubated with 250 μM t-BHP and 4 μg of glycyrrhizic acid and nuclear morphology was assessed using Hoechst 33258. (i) Control hepatocytes; (ii) cells treated with 250 μM of t-BHP for an hour; (iii) Cells pre-treated with glycyrrhizic acid; (iv) cells pre-treated with silymarin.

the mitochondrial level (Haidara et al., 2001). Caspase-3 is also an important effector protease, activated by cleavage as a step in certain apoptosis-signaling pathway. t-BHP caused significant increase in caspase-3 and caspase-9 activity which was 3.4- and 5.5- ($P < 0.001$) folds higher when compared to control. These enzyme activities were found to decrease significantly when GA and silymarin treated hepatocytes were assessed, i.e. with the pre-treatment of GA, caspase-3 and -9 activity was found to be lowered by 2.6- and 2.2-folds, respectively. Pre-treatment with silymarin showed results comparable with GA, i.e. 2.5- and 2.2-folds lowering in activity, respectively (Fig. 3b).

3.6. t-BHP caused apoptosis in hepatocytes which is prevented by GA

Morphological assessment of rat hepatocytes was done using two methods: DNA fragmentation and condensation of nuclear chromatin to assess the cell damage. DNA fragmentation, is an important hallmark for apoptosis, where DNA is degraded by caspases-activated DNase (CAD), a nuclease enzyme showing characteristic ladder pattern on agarose gel. No fragmentation was observed in control cells. Ladder pattern was observed when cells were treated with 250 μM of t-BHP (Fig. 4a). On pre-treatment of cells with GA no such pattern was observed.

Nuclear chromatin condensation is a morphological assessment, which is characteristic of apoptosis and can be visualized using Hoechst 33258. Condensation of nuclear chromatin was found in the cells treated with t-BHP. This was observed by an increase in fluorescence intensity as compared to untreated cells. Cells which were pre-treated with GA showed low intensity of fluorescence as compared to stressed cells. This indicates that apoptosis is involved in the molecular mechanism of action of t-BHP induced toxicity in rat hepatocytes which can be prevented by glycyrrhizic acid (Fig. 4b).

3.7. LDH release in cells treated with t-BHP is prevented by GA

Cell death through oxidative stress may be accomplished by two distinct mechanisms, necrosis or apoptosis. To further characterize the possible mechanism involved in t-BHP induced cell death and efficacy of GA as protective agent, the ratios of necrosis and apoptosis in primary hepatocytes was analyzed using LDH activity-based assay. Intracellular LDH release was evaluated as a result of the breakdown of plasma membrane and alteration of its permeability. The cells were exposed to t-BHP for 1 h -and LDH leakage was taken as the cell death indicator. As shown in Fig. 5a, LDH leakage increased significantly in the presence of 250 μM t-BHP and showed 51.04% ($P < 0.001$) apoptotic and 29.68% ($P < 0.001$) necrotic cells indicating apoptosis as a predominant mechanism responsible for cell death. Cells pre-incubated with GA showed decrease in the number of apoptotic (20.23%) ($P < 0.001$) as well as necrotic cells (10.68%) ($P < 0.001$) whereas silymarin decreased the number of apoptotic cells by (28.99%) ($P < 0.01$) and necrotic (18.99%) ($P < 0.01$). Results indicate that 4 μg of GA/10^4 cells showed significant decrease in the number of apoptotic and necrotic cells induced by t-BHP.

3.8. Annexin V/PI staining and flow cytometric analysis

t-BHP treated cells were monitored for expression of phosphotidylserine in the outer cell membrane, and percentage of viable, early and late apoptotic and necrotic population was assessed as shown in Fig. 5b. From the dot plots (divided in four quadrants), viable hepatocytes were negative for both annexin V and PI (lower left quadrant). The cells that were annexin V positive and PI negative represent the population in early apoptosis (upper left quadrant). The cells that showed annexin V positive and PI positive represent population with late apoptosis (upper right quadrant) and the cells that were negative for annexin and positive for PI are shown in lower right quadrant. The viable hepatocytes in oxidatively challenged cells were 59.66% that was 35% less than untreated cells. Whereas when GA and silymarin, pre-treated cells were taken into consideration the viability observed was 89.16% and 87.78%. The number of apoptotic population also decreased from 42% (t-BHP treated) to 7.45% and 8.71%, respectively, in GA and silymarin pre-treated cells.

4. Discussion

In this study, we demonstrated increase in ROS generation, GSH depletion, increased MDA formation and apoptosis initiated by mitochondria in t-BHP treated rat primary hepatocytes. Our results collectively indicate that t-BHP induced apoptosis is dependent on ROS production as well as GSH depletion. Involvement of mitochondrial pathway in t-BHP induced apoptosis was also investigated by measuring caspases-3, caspase-9 and cytochrome *c* release along with DNA fragmentation and chromatin condensation. Rat hepatocytes, treated with t-BHP caused DNA fragmentation (laddering) confirming the presence of apoptotic phenomenon. Cytochrome *c* release was detected in the cytosol after 1h treatment of t-BHP (250 μM) in rat hepatocytes. This corresponds to an early response in apoptosis. The upstream caspase-9 was activated in t-BHP induced apoptosis. These finding demonstrate that t-BHP activates the mitochondrial pathway in hepatocytes.

Phenolic compounds, which are widely distributed in plants, have been considered to play an important role as dietary antioxidants for the prevention of oxidative damage in living system. GA exhibits a number of pharmacological effects including anti-inflammatory and is used in hepatoprotective formulations. Pre-treatment with GA has been reported to show protective action against carbon tetrachloride (CCl_4)-induced liver injury in rats (Wang and Han, 1993). GA protects against aflatoxin-induced oxidative stress (Chan et al., 2003). GA is also a potent inhibitor of bile acid-induced apoptosis and necrosis (Gumpricht et al., 2005).The results of the present study suggest that GA is capable of ameliorating hepatocyte lipid peroxidation caused by t-BHP. It is well established that intracellular GSH, the most important biomolecule protecting against chemically induced oxidative stress, can participate in the elimination of reactive intermediates by conjugation and hydroperoxide reduction. These metabolic pathways could increase cellular reactive metabolites, which may attack membrane phospholipids, proteins, and nucleic acids. Thus, antioxidants which can inhibit free radical generation are important in terms of protecting the liver from chemical-induced damage by stabilizing antioxidant systems in the cell. The present study showed that treatment with t-BHP stimulates ROS overproduction, mitochondrial membrane depolarization (loss of $\Delta\Psi_m$), which in turn activates caspase cascade and cause reduction in the level of GSH. The data further showed that cells treated with GA displayed a reduction of t-BHP-induced ROS generation. Moreover, GSH depletion was inhibited when the cells were pre-incubated with GA. The results strongly suggest that the protection accorded by GA against t-BHP-induced hepatotoxicity might be related to its ability to reduce oxidative stress.

In summary, the present study indicates the protective effect of GA on cytotoxicity induced by t-BHP in cultured hepatocytes. t-BHP toxicity is associated with depletion of GSH levels, elevated ROS level, lipid peroxidation, disruption of intracellular antioxidant defense, depolarization of mitochondrial membrane, DNA fragmentation, chromatin condensation as well as release

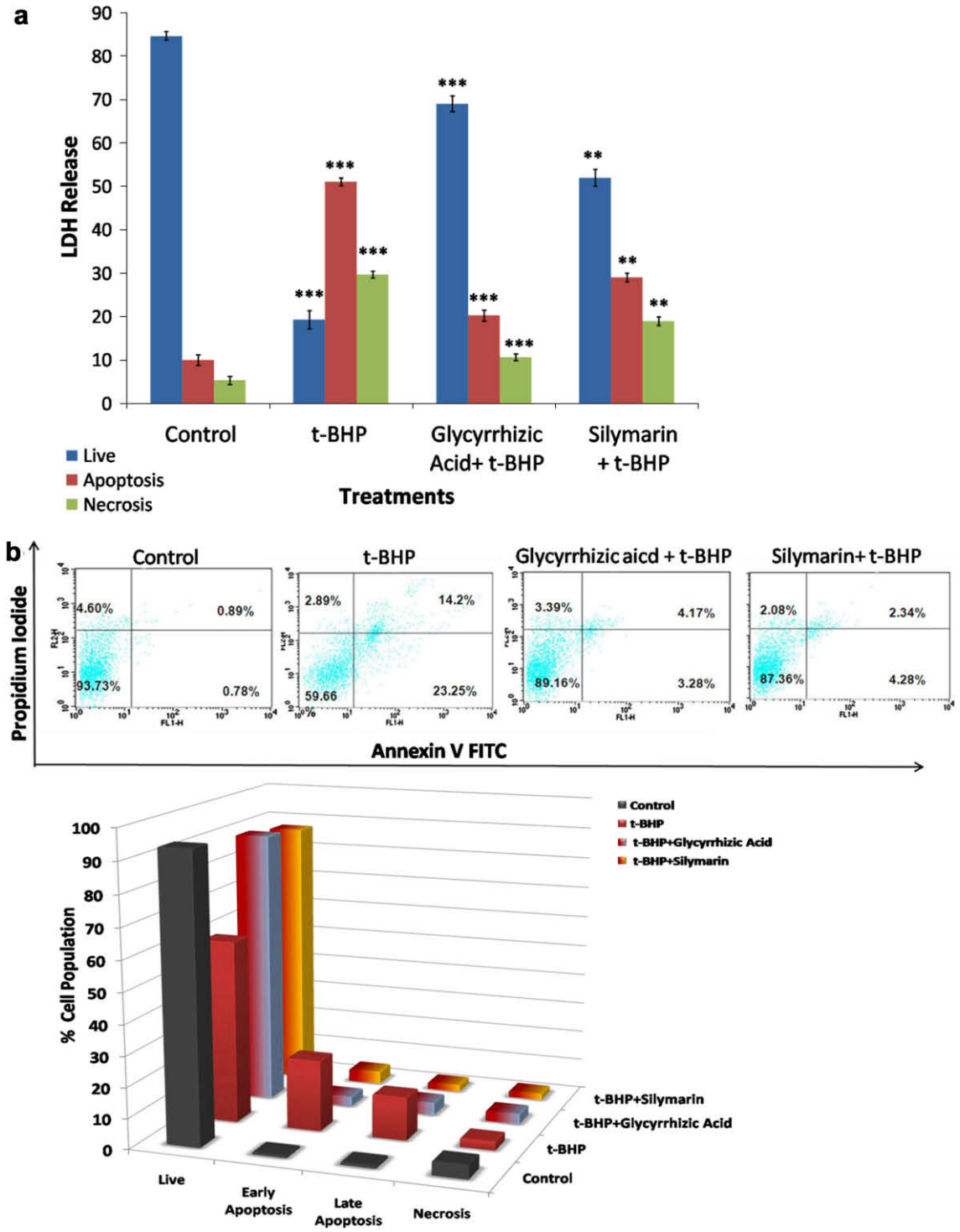


Fig. 5. (a) Effect of glycyrrhizic acid on t-BHP induced cell death: monolayer cultures of rat hepatocytes were exposed to t-BHP (250 μM) and 4 μg of Glycyrrhizic acid (37 °C; in cell-culture medium). The occurrence of apoptosis was assessed by the release of lactate dehydrogenase (LDH). Values are mean ± S.E. of 5 determinations in each case. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (b) Apoptosis determination: Dual-parameter flow cytograms of FITC-labeled annexin V vs. PI staining of the control as well as treated cells. Viable hepatocytes were negative for both annexin V and PI (lower left quadrant); early apoptotic hepatocytes were labeled by annexin V, while negative for PI (upper left quadrant); late apoptotic hepatocytes were positive for both annexin V and PI (upper right quadrant); necrotic hepatocytes were labeled by PI, while negative for annexin V (lower right quadrant).

of cytochrome *c* and caspase-activation as evident from this study. Pre-treatment of GA protected against all these alterations induced by t-BHP. Quenching the radical species could be one of the mechanisms involved in its protective action. This effect was comparable to silymarin, which was used as a positive control. The present findings suggest that GA may be used as a natural antioxidant to protect against oxidative cellular damage caused by toxic chemicals.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Effects of Mannitol or Catalase on the Generation of Reactive Oxygen Species Leading to DNA Damage by Chromium(VI) Reduction with Ascorbate

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Interaction of Cr(VI) and ascorbate in vitro generates Cr(V), Cr(IV), Cr(III), carbon-based alkyl radicals, $COO^{\bullet-}$, $^{\bullet}OH$, and ascorbate radicals and induces DNA interstrand cross-links at guanines. To determine which specific Cr species and free radicals cause DNA damage, we investigated the effects of mannitol and catalase on the formation of reactive intermediates, Cr−DNA associations, DNA polymerase-stop sites, and 8-hydroxydeoxyguanosine (8-OHdG) adducts induced by Cr(VI)/ascorbate in a Hepes buffer. EPR spectra showed that mannitol trapped reactive Cr(V), forming a stable Cr(V)−diol complex, and inhibited the radicals induced by Cr(VI)/ascorbate, whereas catalase or heat-denatured catalase enhanced the levels of Cr(V) without altering the radical signals. Mannitol markedly inhibited the retarded gel electrophoretic mobility of supercoiled plasmids and the formation of DNA polymerase-stop sites induced by Cr(VI)/ascorbate, but catalase did not. On the other hand, mannitol reduced only 32% of the Cr−DNA adducts induced by Cr(VI)/ascorbate, suggesting that Cr monoadducts (possibly DNA−Cr−mannitol adducts) are the major lesions generated in the Cr(VI)/ascorbate/mannitol/DNA solution. Native catalase but not heat-denatured catalase protected ∼25% of the Cr−DNA adducts induced by Cr(VI)/ascorbate, suggesting that hydrogen peroxide may be involved. Mannitol could not completely inhibit the formation of 8-OHdG adducts induced by Cr(VI)/ascorbate, indicating that this DNA damage may be generated before the action of mannitol to trap Cr(V) and reactive oxygen species. Alternatively, Cr−peroxide intermediates may also lead to 8-OHdG formation to account for the incomplete prevention by mannitol. Catalase or heat-denatured catalase partially protected the formation of 8-OHdG adducts induced by Cr(VI)/ascorbate, suggesting an effect of proteins. Together, the results from this study suggest that the primary species generated during the reduction of Cr(VI) by ascorbate are hydroxyl radicals and Cr(V) species, responsible for the formation of 8-OHdG and DNA cross-links, respectively.

Introduction

Cr exists in many oxidation states, of which hexavalent Cr is the most effective form for inducing genotoxicity and carcinogenicity (*1*, *2*). Cr(VI) compounds significantly increase the risk of respiratory tract cancers (*3*, *4*). Cr(VI) also induces chromosomal abnormalities, cell transformations, apoptosis, signal transductions, and gene mutations in cultured mammalian cells (*5*−*12*). However, Cr(VI) compounds do not directly attack DNA or nuclei in vitro (*13*−*15*). The genotoxic and carcinogenic effects of Cr(VI) compounds are associated with their ability to enter cells rapidly and to be activated through intercellular reduction (*16*, *17*). The cellular components involved in reducing Cr(VI) include ascorbate, glutathione, hydrogen peroxide, cysteine, DT-diaphorase, cytochrome P450 reductases, and the mitochondrial electron transport chain (*16*−*25*). Upon activation of Cr(VI), several reactive species are generated, e.g., Cr(V), Cr(IV), Cr(III), reactive oxygen species (ROS),[1] and other free radicals (*16*−*21*). Those Cr intermediates, radicals, and ROS can subsequently attack macromolecules and lead to DNA damage, e.g., strand breaks, DNA−protein cross-links, DNA−DNA cross-links, Cr−DNA adducts, and base modifications in cells (*10*, *21*, *26*−*30*).

Ascorbate is the major reductant of Cr(VI) in rat kidneys, liver, and lung ultrafiltrates (*22*). Pretreatment of Chinese hamster V79 cells with ascorbate increases the number of Cr(VI)-induced DNA−protein cross-links, and decreases the number of alkali-labile sites (*21*). Ascorbate decreases the clastogenicity of lead chromate possibly due to eliminated extracellular dissolution and Cr(VI) uptake in Chinese hamster ovary cells (*31*). Interaction of Cr(VI) and ascorbate in vitro generates Cr(V), Cr(IV), Cr(III), carbon-based alkyl radicals, $COO^{\bullet-}$, $^{\bullet}OH$, and ascorbate radicals (*32*−*34*). The relative amounts of Cr species and radicals that are produced are dependent on the ratios of ascorbate to Cr(VI) and the reaction buffer (*32*, *33*). For example, Cr(V), $COO^{\bullet-}$, $^{\bullet}OH$, and carbon-based alkyl radicals are induced by Cr(VI)/ascorbate at roughly equal concentrations, whereas only

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[1] Abbreviations: ROS, reactive oxygen species; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 8-OHdG, 8-hydroxydeoxyguanosine; DMPO, 5,5-dimethyl-1-pyrroline 1-oxide; ICP-MS, inductively coupled plasma-mass spectrometer; dG, deoxyguanosine; ECD, electrochemical detection.

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ascorbate radicals are observed when the amount of ascorbate exceeds that of Cr(VI) (*32*). Cr(VI)/ascorbate in a phosphate buffer enhances DNA strand breaks (*35*) and apurinic/apyrimidinic sites (*36*), whereas in a Hepes buffer, Cr(VI)/ascorbate induces DNA interstrand cross-links at guanines (*37*). The levels of Cr(VI)/ascorbate-induced DNA strand breaks (*35*) and DNA interstrand cross-links (*37*) decreased when the amount of ascorbate increased.

The reactive intermediates generated by Cr(VI)/ascorbate are considered to be the etiology for the induction of DNA lesions. However, what Cr species and free radicals cause specific types of DNA damage remain to be elucidated. In this study, we examined the ability of Cr(VI)/ascorbate to induce 8-OHdG in DNA, an important oxidative DNA adduct leading to mutagenesis and carcinogenesis. We also investigated the effects of mannitol and catalase on the generation of reactive intermediates, Cr−DNA adducts, DNA polymerase-stop sites, and 8-OHdG adducts induced by Cr(VI)/ascorbate to gain more insight into the species responsible for the DNA damage. The results have suggested that ROS and Cr(V) may be the primary species for inducing 8-OHdG adducts and DNA cross-links during the interaction of Cr(VI) and ascorbate in a Hepes buffer.

Experimental Procedures

Materials. Potassium dichromate (>99.95% pure), mannitol (D-form), and Hepes were purchased from Merck (Darmstadt, Germany). L-Ascorbate, 5,5-dimethyl-1-pyrroline 1-oxide (DMPO), and calf thymus DNA (activated type XV) were purchased from Sigma (St. Louis, MO). Plasmids pSP189 and pZ189 were provided by M. M. Seidman (Otsuka Pharmaceuticals, Rockville, MD). Plasmid pGEM3Zf(+) was purchased from Promega (Madison, WI). Chelex 100 resin (200−400 mesh, sodium form) was purchased from Bio-Rad Laboratories (Richmond, CA). Sephadex G-50 columns and catalase were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). All of the materials were freshly prepared in Chelex 100-treated MilliQ water. Hepes was dissolved in MilliQ water at a concentration of 1 M, and then passed through Chelex 100 resin twice.

EPR Spectroscopy. Mannitol (50 mM) or catalase (800 units/mL) was delivered to an aqueous solution containing 100 mM DMPO, 1 mM $K_2Cr_2O_7$ [equivalent to 2 mM Cr(VI)], and 2 mM ascorbate. The reaction mixtures were kept at room temperature for 4 min before the EPR spectra were recorded using a Bruker ER (type EMX 10/12) spectrometer with a 100 kHz field modulation, a 20 mW microwave power, and a 100 G scan width. The g values were calculated from calibration against 1,1-diphenyl-2-picrylhydrazyl ($g = 2.0037$) (*38*). All of the materials for the EPR analysis were freshly prepared.

Agarose Gel Electrophoresis. Supercoiled plasmids pGEM3Zf(+) (0.4−2.8 μg) were mixed with $K_2Cr_2O_7$ and/or ascorbate in 10 mM Hepes (pH 7.0) in a total volume of 25 μL. The reaction mixture was incubated at 37 °C for 30 min. In experiments aimed at determining the effect of ROS scavengers, either mannitol or catalase was delivered to the DNA solution before adding $K_2Cr_2O_7$ and/or ascorbate. At the end of incubation, DNA samples were analyzed using electrophoresis in 0.8% agarose gels containing ethidium bromide (0.5 μg/mL).

Polymerase-Stop Assay of Cr(VI)/Ascorbate-Treated DNA. Plasmids pZ189 were treated with *Eco*RI and purified as described previously (*39*). The DNA sample (1.7 μg) was then treated with Cr(VI)/ascorbate in the presence of either mannitol, catalase, or heat-denatured catalase in a total volume of 15 μL as described above. At the end of the reaction, the DNA samples were drop dialyzed against a 10 mM Hepes buffer for 4 h, and used as templates for the polymerase-stop assay. The in vitro DNA polymerase-stop assay was performed using the template, [γ-^{32}P]ATP (specific activity of 5000 Ci/mmol), end-labeled primer 5′-ACGGGGTCTGACG, Sequenase, and four deoxyribonucleotides as described previously (*39*).

Determination of the Amount of Cr Bound to DNA. Immediately after treatment, plasmids pSP189 were filtered through Sephadex G-50 columns at 2700 rpm for 4 min. The amount of Cr bound to the plasmid was determined using an ICP-MS (SCIEX ELAN 5000, Perkin-Elmer, Norwalk, CT). The ICP-MS conditions were as follows: power of 5000 W, plasma flow rate of 15 L/min, auxiliary flow rate of 0.8 L/min, and sample flow rate of 0.8 L/min. One set of control experiments was conducted in which DNA samples were excluded. The number of Cr molecules bound per 1000 nucleotides was calculated as the data obtained in the reaction mixtures containing DNA samples subtracted from those obtained in mixtures without DNA.

Determination of the Amounts of dG and 8-OHdG. Calf thymus DNA (100 μg) was treated with Cr(VI)/ascorbate in the presence of either mannitol, catalase, or heat-denatured catalase in 10 mM Hepes (pH 7.0) in a total volume of 450 μL. The reaction was carried out at 37 °C for 30 min and terminated using ethanol precipitation. The DNA sample was washed once with 70% ethanol, dried, dissolved in 10 mM Tris-HCl (pH 7.4), and heated at 100 °C for 3 min. DNA was digested to deoxyribonucleosides, and the amounts of dG and 8-OHdG were analyzed using HPLC with UV absorbance detection and ECD (BAS amperometric electrochemical detector, West Lafayette, IN) as previously described (*40*). The protection of Cr(VI)/ascorbate-induced 8-OHdG formation by mannitol, catalase, or heat-denatured catalase was determined with the equation $1 - [(X_{m+a} - X_c)/(X_m - X_c)]$, where X_m is the amount of 8-OHdG adducts remaining after treatment with Cr(VI)/ascorbate, X_{m+a} is the amount of 8-OHdG adducts remaining after treatment with Cr(VI)/ascorbate and a ROS modulator, and X_c is the amount of 8-OHdG adducts remaining in the untreated control.

Results

Effects of Mannitol and Catalase on the Cr(VI)/Ascorbate-Induced EPR Spectrum. EPR spectroscopy was used to investigate reactive intermediates such as Cr(V) and radicals generated in an aqueous solution containing Cr(VI)/ascorbate in the presence of either mannitol, catalase, or heat-denatured catalase in 10 mM Hepes buffer (pH 7.0). The spin trap DMPO was added at a concentration of 100 mM to each reaction mixture. The EPR spectrum obtained upon reaction of Cr(VI) and ascorbate at a 1:1 ratio in a solution containing DMPO showed the presence of Cr(V), DMPO−•OH, DMPO−COO•, DMPO−•C, and DMPO−R• radical adducts (Figure 1A) which confirmed previous reports (*32*, *34*). The signal intensities of Cr(V) and DMPO−radical adducts decreased slowly when the incubation time increased; e.g., the intensities observed 30 min after the reaction were roughly 60% of those obtained at 4 min (data not shown). A similar spectrum for Figure 1A was obtained when the Cr(VI):ascorbate ratio increased to 2:1, whereas only the ascorbate radical anion ($g = 2.006$, $A_H = 1.82$ G) was observed at a Cr(VI):ascorbate ratio of 1:2 (data not shown). DMPO did not affect the signal intensity of the Cr(V) or the ascorbate radical anion (data not shown). The signal intensity of Cr(V) increased 3-fold, and the amounts of DMPO−radical adducts were not significantly altered by adding native or heat-denatured catalase (800 units/mL) to the 2 mM Cr(VI)/2 mM ascorbate solution (Figure 1B,C). By contrast, mannitol (50 mM) markedly increased (~200-fold) the magnitude of an altered Cr(V) signal, and inhibited the generation of DMPO−radical adducts in the Cr(VI)/ascorbate solution (Figure 1D). This

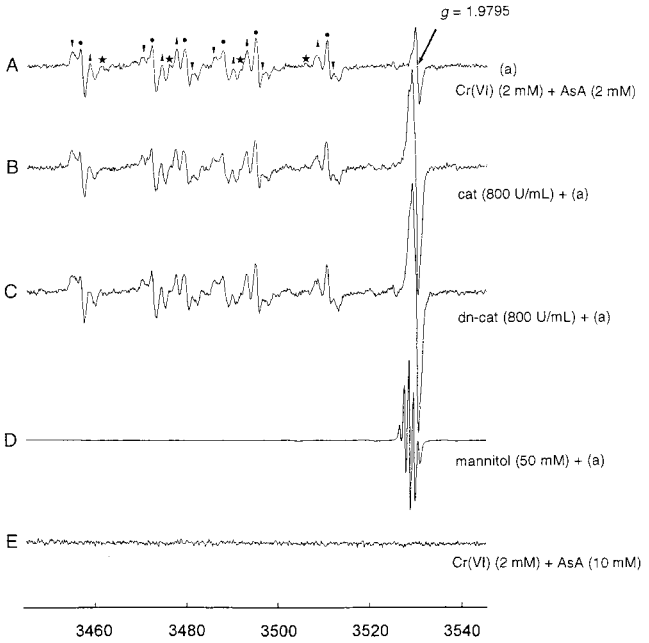

Figure 1. Effects of mannitol and catalase on the EPR spectrum generated in the reaction mixture of Cr(VI), ascorbate (AsA), and DMPO. EPR spectra were generated in an aqueous solution containing 100 mM DMPO and 2 mM Cr(VI)/2 mM AsA (spectrum A) in the presence of either mannitol (spectrum D), catalase (spectrum B), or heat-denatured catalase (spectrum C) in 10 mM of Hepes buffer (pH 7.0). Spectrum E was obtained in similar solutions containing 5-fold higher concentrations of AsA than Cr(VI). The EPR spectrometer settings were as follows: time constant of 0.16 s, modulation amplitude of 0.5 G, scan times of 168 s, and magnetic field of 3495 ± 100 G. The receiver gain for all of the spectra was 2×10^5 except for spectrum D, for which it was 1×10^3. The DMPO adducts in spectrum A were assigned as DMPO−$COO^{\bullet-}$ (▲; $A_H = 18.8$, $A_N = 15.5$) and DMPO−$^{\bullet}OH$ (*; $A_H = 14.9$, $A_N = 14.9$); two sets of other carbon-based DMPO−radical adducts are marked with ▼ ($A_H = 25.7$, $A_N = 15.5$) and ● ($A_H = 22.8$, $A_N = 15.8$) (*32*, *34*). The *g* value for all the DMPO adducts was 2.006. The Cr(V) species in spectrum A is denoted with a *g* value of 1.9795.

altered Cr(V) spectrum was similar to that obtained in a Cr(VI)/fructose system that was assigned to the Cr(V)−diol complex (*41*). The Cr(V)−diol complex observed in the Cr(VI)/ascorbate/mannitol solution was very stable, e.g., the intensity remained constant after incubation for 30 min (data not shown). None of the Cr(V), DMPO−radical adducts, or ascorbate radical anions were observed in the DMPO solution containing 2 mM Cr(VI) and a 5-fold greater concentration of ascorbate (Figure 1E). Also, those EPR signals were not induced by either Cr(VI) or ascorbate under the same conditions (data not shown).

Effects of Mannitol and Catalase on the Electrophoretic Mobility of Cr(VI)/Ascorbate-Treated DNA. Gel electrophoresis analysis was adopted to examine the DNA damage ability of reactive species that generated during Cr(VI) reduction with ascorbate, and its prevention by ROS modulators. Supercoiled plasmids were treated with 150 μM Cr(VI)/ascorbate at a 1:1 ratio in 10 mM Hepes buffer (pH 7.0) and analyzed by electrophoresis in agarose gels. The electrophoretic mobilities of Cr(VI)/ascorbate-treated plasmids were markedly retarded when DNA concentrations were decreased (Figure 2, lanes 1−4). This electrophoretic mobility is different from the pattern of plasmids linearized with *Eco*RI (Figure 2, lanes 5 and 11), indicating that the mobility shift of Cr(VI)/ascorbate-treated plasmids is not attributed to DNA strand breaks. The agarose gel patterns also showed a reduced ethidium bromide fluorescence intensity of supercoiled plasmids treated with Cr(VI)/ascorbate at low DNA concentrations (16 ng/μL). Mannitol (50 mM) markedly recovered the retarded gel mobility of Cr(VI)/ascorbate-treated supercoiled plasmids, whereas native or heat-denatured catalase did not (Figure 2, lanes 10 and 12−14). Figure 2 also shows that treatment with neither 150 μM Cr(VI) nor 150 μM ascorbate alters the electrophoretic mobility of supercoiled plasmids (lanes 8 and 9).

Effects of Mannitol and Catalase on Polymerase-Stop Sites Induced by Cr(VI)/Ascorbate. The electrophoretic mobility of supercoiled plasmids altered by Cr(VI)/ascorbate could be due to unwinding of the negatively supercoiled plasmids, Cr−DNA associations, or DNA interstrand cross-links. DNA interstrand cross-links have been implicated in arresting the DNA polymerase processivity (*37*). We then adopted the polymerase-stop assay to determine how Cr(VI)/ascorbate-treated templates interfered with DNA replication and what specific sites were affected by ROS scavengers. Plasmid pZ189 was digested with *Eco*RI, purified, and treated with Cr(VI)/ascorbate in 10 mM Hepes buffer (pH 7.0) in the presence or absence of mannitol, catalase (500 units/mL), or heat-denatured catalase. Unbound Cr was removed using dialysis before the polymerase-stop assay was carried out. Complete polymerization was verified by the formation of a full-length, 225-base product in the DNA sequencing gels. Figure 3 shows that Cr(VI)/ascorbate-treated (150 μM; 1:1) templates did not generate the full-length DNA products, whereas markedly enhanced radioactivity in the loading well was observed. The enhanced radioactive signals in the loading wells have been considered as primer−template cross-links (*37*). Mannitol (50 mM) inhibited the formation of primer−template cross-links by Cr(VI)/ascorbate. Also, the full-length DNA product was obtained by mannitol cotreatment (Figure 3). Catalase and heat-denatured catalase partially reduced the intensities of the radioactive signals in the loading wells, but they did not increase the yield of the full-length DNA products (Figure 3). The intensities of radioactive signals observed in either the Cr(VI)- or ascorbate-treated templates were the same as those observed in untreated templates (Figure 3).

A lower degree of Cr-mediated cross-links in the templates was generated to determine the specific polymerase-stop sites. Figure 4 shows that most of the polymerase-stop sites found in templates (112 ng/μL) treated with 60 μM Cr(VI)/ascorbate at a 1:1 ratio were located at the 3′-flanking bases of guanines on the template strand. No significant polymerase-stop site was observed in Cr(VI)- or ascorbate-treated templates. Figure 4 also shows that templates treated with 60 μM $CrCl_3$ generated polymerase-stop sites similar to those induced by Cr(VI)/ascorbate. Mannitol (50 mM) markedly inhibited polymerase-stop sites induced by Cr(VI)/ascorbate (Figure 4). In contrast, catalase (500 units/mL) and heat-denatured catalase did not significantly affect the patterns of polymerase-stop sites caused by Cr(VI)/ascorbate (Figure 4). Strong polymerase-stop sites were also observed in an A/T-rich region (positions 132−138) of the *supF* templates treated with 60 μM Cr(VI)/ascorbate or 60 μM Cr(III). These A/T-rich polymerase-stop sites were not significant when a similar reaction was performed using a higher concentration (267 ng/μL) of Cr(VI)/

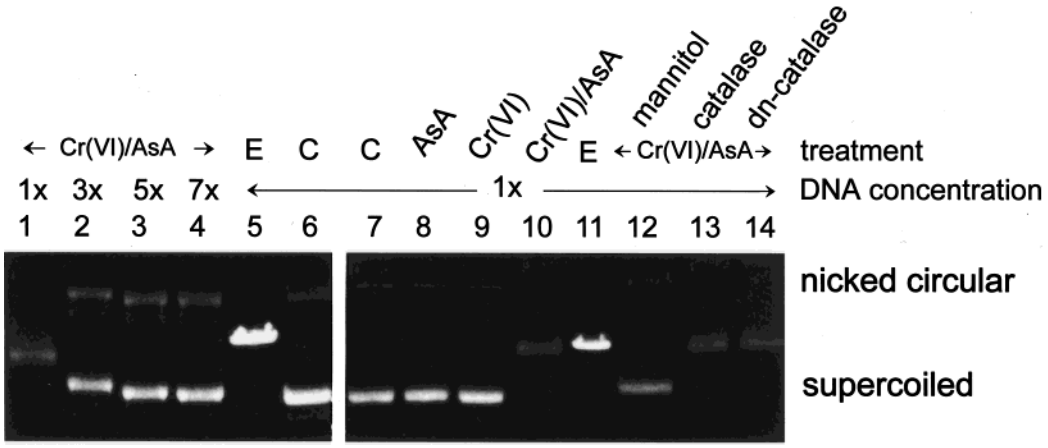

Figure 2. Effects of mannitol and catalase on the electrophoretic mobility of Cr(VI)/AsA-treated supercoiled plasmids. Supercoiled plasmids pGEM3Zf(+) were allowed to react with 150 μM Cr(VI)/150 μM AsA and analyzed as described in Experimental Procedures (lanes 1−4 and 10). The concentration of plasmids in all of the reaction samples was 16 ng/μL, except for the concentrations of the samples in lanes 2−4 which were 48, 80, and 112 ng/μL, respectively. The amount of plasmids per lane was 400 ng. Lanes 6 and 7 contained untreated plasmids. Lanes 8 and 9 contained plasmids treated with Cr(VI) (150 μM) and AsA (150 μM), respectively. Lanes 5 and 11 contained plasmids linearized using *Eco*RI (one cut). Lanes 12−14 contained plasmids treated with 150 μM Cr(VI)/150 μM AsA in the presence of either mannitol (50 mM), catalase (40 units/mL), or heat-denatured catalase.

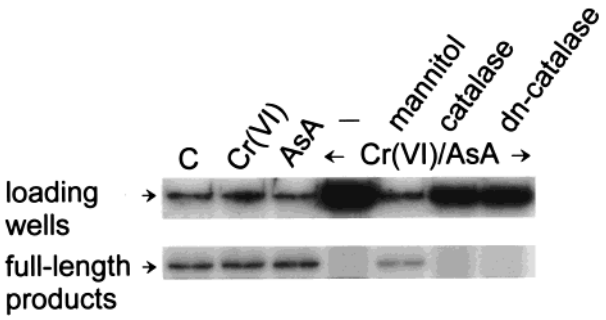

Figure 3. Effects of mannitol and catalase on the primer−template cross-link formation and inhibition of DNA synthesis by Cr(VI)/AsA. Linear double-stranded DNA (112 ng/μL) was treated with 150 μM Cr(VI)/150 μM AsA in the presence of either mannitol, catalase, or heat-denatured catalase, and subjected to the polymerase-stop assay as described in Experimental Procedures. Complete DNA synthesis was verified by the observation of a full-length, 225-base product in DNA sequencing gels. The enhanced radioactive signals in the loading wells obtained in Cr(VI)/AsA-treated DNA represent primer−template cross-links. C represents data obtained from the untreated control plasmids.

ascorbate-treated (data not shown) or Cr(III)-treated templates (*39*), although the majority of polymerase-stop sites still occurred at guanine residues in DNA templates.

Effects of Mannitol and Catalase on the Formation of 8-OHdG Adducts Induced by Cr(VI)/Ascorbate. 8-OHdG is an important oxidative DNA adduct leading to mutagenesis and carcinogenesis. The ability of Cr(VI)/ascorbate to induce 8-OHdG formation in calf thymus DNA in 10 mM Hepes buffer (pH 7.0) was determined using HPLC/UV/ECD. Figure 5A shows that the amount of 8-OHdG adducts in the DNA markedly increased when an equal ratio of the Cr(VI) and ascorbate concentrations was increased (150 μM to 2 mM, in a 1:1 ratio). The amounts of 8-OHdG adducts generated in Cr(VI)/ascorbate-treated DNA at 150 μM and 2 mM (1:1 ratio) were $18/10^5$ dG bases and $146/10^5$ dG bases, respectively. Control experiments with 2 mM Cr(VI) or ascorbate alone did not enhance 8-OHdG formation in the DNA at the background level ($6.5/10^5$ dG bases).

Coadministrating 50 mM mannitol protected approximately 55 and 28% of the 8-OHdG adducts induced by 150 μM Cr(VI) and 500 μM ascorbate (1:1 ratio), respectively (Figure 5B). Mannitol (50−200 mM) also exhibited a dose-dependent protection on the 8-OHdG adducts induced by 1 mM Cr(VI)/ascorbate (Figure 5B). Similarly, catalase (500−5000 units/mL) and heat-denatured catalase protected 32−54% of the 8-OHdG adducts induced by 1 mM Cr(VI)/ascorbate (Figure 5B), suggesting that the prevention of 8-OHdG formation may be due to the proteins' effect but not due to the catalase enzymatic activity.

Effects of Mannitol and Catalase on the Formation of Cr Adducts Induced by Cr(VI)/Ascorbate. The effects of mannitol and catalase on the formation of Cr adducts induced by Cr(VI)/ascorbate were determined using the ICP-MS. Plasmids pSP189 were treated with various concentrations of Cr(VI) and/or ascorbate in 10 mM Hepes buffer (pH 7.0). Following removal of unbound Cr ions by Sephadex G-50 gel filtration, the amounts of Cr bound to plasmids were assayed by the ICP-MS, and calculated as described in Experimental Procedures. As shown in Figure 6, the amount of Cr adducts in plasmid increased linearly when Cr(VI)/ascorbate concentrations at a 1:1 ratio were increased. Cr adducts formed by 150 μM Cr(VI)/ascorbate were decreased approximately 32% by adding 50 mM mannitol. Catalase (500 units/ml) reduced 25% of the Cr adducts formed by 150 μM Cr(VI)/ascorbate, whereas heat-denatured catalase did not affect the amounts of Cr(VI)/ascorbate-induced Cr adducts (Figure 6), suggesting that hydrogen peroxide may be generated in the reaction mixture and contributes to the formation of Cr adducts.

Discussion

EPR spectroscopic results have confirmed that Cr(VI) reduction by ascorbate at stoichiometric ratios can induce reactive Cr(V) and radicals, including carbon-based, $COO^{\bullet-}$, $^{\bullet}OH$, and ascorbate radicals (*32*, *34*). In the study presented here, we also demonstrated that interaction of Cr(VI) with ascorbate generates ROS-related 8-OHdG adducts in DNA. The highly reactive $^{\bullet}OH$ may be the primary species produced in the Cr(VI)/ascorbate solution and then attacks ascorbate to generate ascorbate-derived $COO^{\bullet-}$ and carbon-based radicals. This could account for

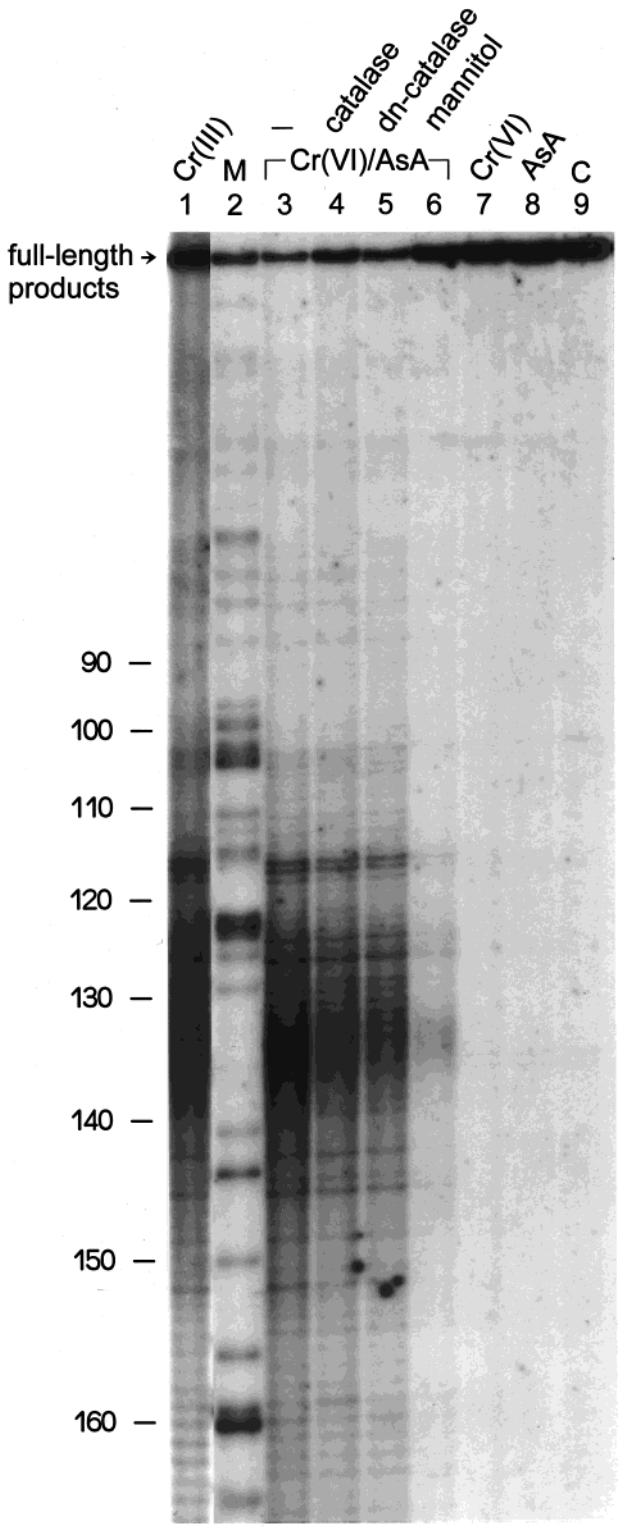

Figure 4. Effects of mannitol and catalase on the polymerase-stop sites generated in Cr(VI)/AsA-treated templates. Linear double-stranded DNA (112 ng/μL) was treated with 60 μM Cr(VI)/60 μM AsA in the presence of either mannitol, catalase, or heat-denatured catalase, and subjected to the polymerase-stop assay as described in Experimental Procedures. The polymerase-stop assay was also performed using a DNA template treated with 60 μM $CrCl_3$. The numbers shown on the left side of lane 1 are the positions of the *supF* gene coding region. M represents markers obtained from the DNA sequencing reaction of untreated plasmids using dideoxycytosine triphosphate and four deoxyribonucleotides (lane 2). C represents data obtained from the untreated control plasmids (lane 9).

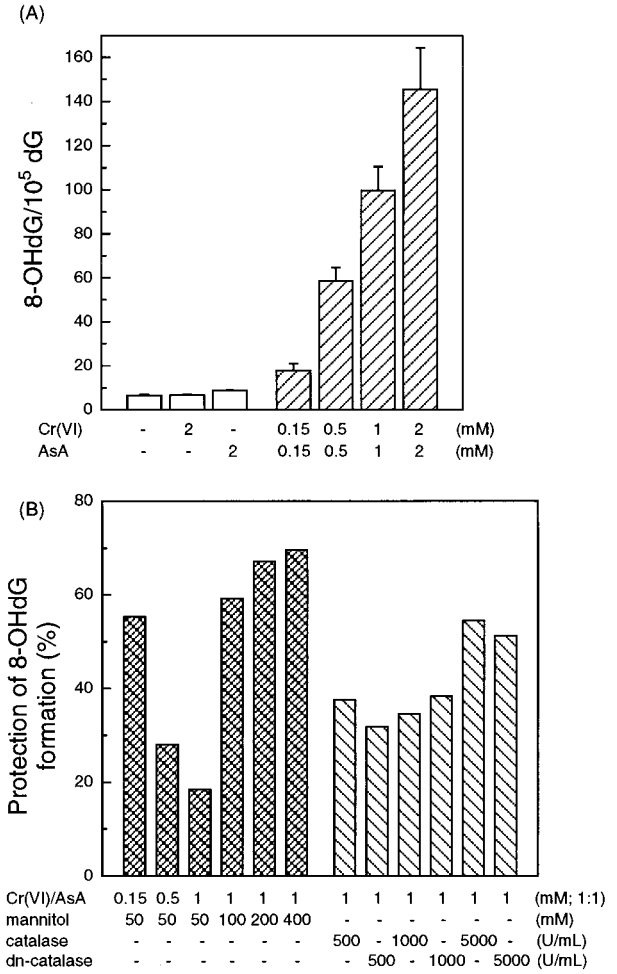

Figure 5. Effects of mannitol and catalase on the 8-OHdG adducts induced by Cr(VI)/AsA. (A) Calf thymus DNA (222 ng/μL) was allowed to react with Cr(VI) and AsA at a 1:1 ratio in 10 mM Hepes buffer (pH 7.0) at 37 °C for 30 min. (B) Mannitol, catalase, or heat-denatured catalase was added to the DNA solutions containing Cr(VI) before the addition of AsA. The DNA sample was precipitated by ethanol and digested to the nucleoside level for the 8-OHdG analysis. The degree of protection was calculated as described in Experimental Procedures. Results were obtained from two to eight experiments, and the bars represent the SEM.

the fact that the intensity of DMPO−•OH observed in Cr(VI)/ascorbate is much lower than that of carbon-based DMPO−radical adducts. We further demonstrated that mannitol induces a stable Cr(V)−diol signal at a marked high intensity and inhibits the DMPO−radical adducts generated during Cr(VI) reduction by ascorbate in the Hepes buffer. Here, mannitol may act as a Cr(V) trapper more efficiently than as a ROS scavenger, and prevents Cr(V) from participating in the subsequent redox cycle. Mannitol inhibits only 32% of Cr adducts formed by Cr(VI)/ascorbate, whereas it inhibits markedly the formation of DNA cross-links and polymerase-stop sites, suggesting that Cr monoadducts are the major DNA lesions generated in the Cr(VI)/ascorbate/mannitol/DNA solution. During Cr(VI) reduction by ascorbate, the reactive Cr(V) may associate with DNA and mannitol, forming DNA−Cr(V)−mannitol monoadducts that do not generate further DNA cross-links. The speculation also agrees with the notion that Cr monoadducts do not induce polymerase-stop sites (*39*). Mannitol exhibits dose-dependent inhibition of the 8-OHdG adducts generated in Cr(VI)/ascorbate-treated DNA; however, ~30−40% of

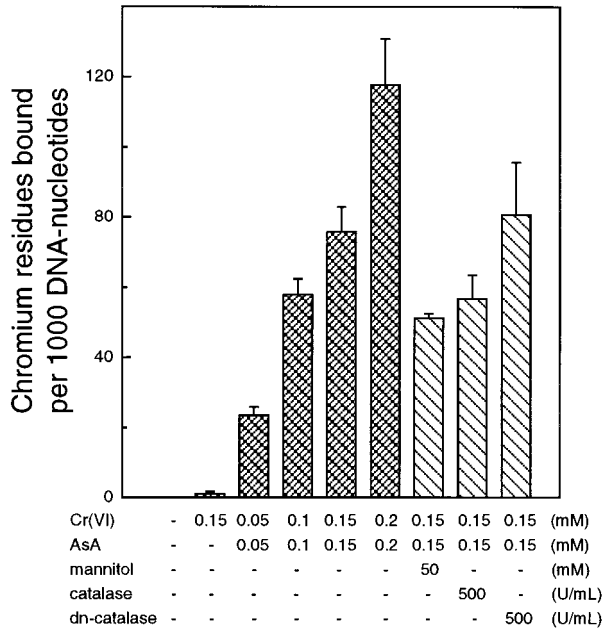

Figure 6. Effects of mannitol and catalase on the Cr adducts formed in Cr(VI)/AsA-treated plasmids. Plasmids pSP189 (112 ng/μL) were treated with various concentrations of Cr(VI) and/or AsA in the presence of either mannitol, catalase, or heat-denatured catalase. The reaction was performed at 37 °C for 30 min in 10 mM Hepes buffer (pH 7.0). The samples were purified, and the amount of Cr bound to the plasmid was determined using the ICP-MS as described in Experimental Procedures. Results were obtained from two or three experiments, and the bars represent the SEM.

these adducts still existed even when very high amounts of mannitol were used. This finding suggests that 8-OHdG adducts may be generated before the action of mannitol to trap Cr(V) and reactive oxygen species induced by Cr(VI)/ascorbate. Alternatively, Cr−peroxide intermediates may also lead to 8-OHdG formation to account for the incomplete prevention by mannitol.

On the other hand, catalase or heat-denatured catalase increases the intensity of the reactive Cr(V) signal without altering the intensities of DMPO−radical adducts induced by Cr(VI)/ascorbate, suggesting that hydrogen peroxide is not involved in the generation of radicals in this solution. Catalase or heat-denatured catalase protects 32−54% of the 8-OHdG adducts formed by Cr(VI)/ascorbate, suggesting that proteins may partially inhibit the formation of this ROS adduct. Cr is known to induce DNA−protein complexes (*26*, *28*, *30*). Native or heat-denatured catalase may bind to the reactive Cr species, forming Cr-mediated DNA−protein complexes that could decrease the accessibility of •OH generated during Cr(VI) reduction by ascorbate, thereby partially preventing the formation of 8-OHdG. However, native or heat-denatured catalase does not decrease the level of formation of DNA polymerase-stop sites, suggesting that Cr-mediated DNA−protein complexes may also arrest DNA replication and generate DNA polymerase-stop sites. The native form of catalase can reduce 25% of Cr−DNA associations induced by Cr(VI)/ascorbate, but heat-denatured catalase does not. These results suggest that hydrogen peroxide may be partially involved in the formation of Cr monoadducts.

The formation of DNA polymerase-stop sites by Cr(VI)/ascorbate in a Hepes buffer agrees with a previous report by Bridgewater et al. (*37*), indicating that Cr-mediated DNA cross-links occur at guanine residues in DNA templates. This specificity is consistent with the fact that Cr has a high affinity for guanine (*13*, *14*). The polymerase-stop sites are possibly not derived from the 8-OHdG lesions because these nonbulky adducts permit efficient bypass synthesis both in vitro (*42*) and in vivo (*43*). Additionally, high ratios of ascorbate to Cr(VI) significantly blocked the formation of reactive Cr(V) and radicals, and decreased the level of DNA cross-links, whereas the total number of Cr residues in DNA remained unchanged (*37*) or slightly increased.[2] This observation may be attributed to excess ascorbate that causes more of the Cr(VI) to be reduced by a two-electron process (*33*), and suggests that Cr(V) may be the primary species inducing polymerase-stop sites in the Cr(VI)/ascorbate solution. Excess ascorbate could act as a radical scavenger and may coordinate with Cr monoadducts, and thereby suppress the formation of DNA cross-links. A scenario is proposed in which the reactive Cr(V) may associate with DNA particularly at guanines during Cr(VI) reduction by ascorbate, and then redox centers and radicals are generated in situ, resulting in base damage, such as 8-OHdG. Also, the different oxidative states of Cr, i.e., Cr(V), Cr(IV), and Cr(III), associated with DNA guanines are produced through redox cycles. This model may also account for the spectrum of DNA polymerase-stop sites generated by Cr(VI)/ascorbate that is similar to that induced by Cr(III) (Figure 4). Although Cr(III) induces DNA polymerase-stop sites, it is a stable species in the presence of ascorbate because the amounts of •OH radicals and 8-OHdG adducts induced by Cr(III)/hydrogen peroxide are significantly reduced by ascorbate (*40*, *44*).

Interestingly, in a phosphate buffer, Cr(VI)/ascorbate does not significantly alter the electrophoretic mobility of plasmids,[2] although the EPR signals of reactive Cr(V) and radicals are the same as those observed in a Hepes buffer (*32*). Previously, we have shown that buffer environments affect the types of Cr(III)−DNA associations; i.e., DNA polymerase-stop sites are observed in templates treated with Cr(III) in Tris but not phosphate buffers (*39*). Cr monoadducts are able to form DNA cross-links in Hepes or Tris buffers. However, the formation of DNA cross-links may be suppressed when Cr monoadducts are coordinated with phosphate ions present in the buffers because Cr also has a high affinity for phosphate (*13*−*15*). Conversely, single-strand DNA breaks were not significantly induced by Cr(VI)/ascorbate in a Hepes buffer (Figure 2), although these lesions can be generated in plasmids treated with Cr(VI)/ascorbate in a phosphate buffer[2] (*35*). Hepes may scavenge the reactive species, leading to the formation of DNA strand breaks.

In conclusion, we have demonstrated that mannitol forms a stable complex with Cr(V) that could inhibit the generation of radicals through the Cr(V) redox center, and thereby significantly reduces the level of formation of DNA cross-links, and 8-OHdG adducts in DNA induced by Cr(VI)/ascorbate. The results suggest that the primary species induced by Cr(VI) reduction with ascorbate are •OH and Cr(V) that lead to the formation of 8-OHdG adducts and DNA cross-links, respectively. Results of catalase studies suggest that hydrogen peroxide may not participate in the formation of radicals, Cr(V), DNA cross-links, and 8-OHdG adducts induced by Cr(VI)/ascorbate,

[2] Unpublished results.

whereas it may be involved in the formation of Cr monoadducts. Native or heat-denatured catalase partially prevents the formation of 8-OHdG adducts, but they do not decrease the levels of radicals generated by Cr(VI)/ascorbate, suggesting that Cr-mediated DNA−protein complexes are less accessible for ROS. However, these Cr-mediated DNA−protein complexes may generate polymerase-stop sites. Together, this study suggests that 8-OHdG adducts and DNA cross-links could be the major adducts induced by Cr(VI)/ascorbate, leading to mutagenesis and carcinogenesis.

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Combined ursodeoxycholic acid and glycyrrhizin therapy for chronic hepatitis C virus infection: a randomized controlled trial in 170 patients

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Objective and design **To assess the efficacy and safety of combination therapy using ursodeoxycholic acid with glycyrrhizin for chronic hepatitis C virus infection, we conducted a prospective randomized controlled trial of glycyrrhizin (group G) compared with glycyrrhizin plus ursodeoxycholic acid (group G+U) in 170 patients.**

Methods **All patients had elevated serum aminotransferase levels over 6 months before entry into the trial. Glycyrrhizin was administered to both groups for 24 weeks, and in group G+U, ursodeoxycholic acid (600 mg/day) was administered orally as well.**

Results **Serum aspartate transaminase and alanine transaminase concentrations significantly decreased during treatment in both groups, but serum gamma-glutamyl transpeptidase concentrations fell significantly only in group G+U. Concentrations of all three enzymes fell significantly more in group G+U than in group G, and had normalized in more cases when the trial ended at 24 weeks. However, levels of HCV viraemia did not change during the trial in either group. Multiple regression analysis linked only the treatment regimen, not HCV-related factors or liver histology, to the degree of serum enzyme reduction. No adverse effects were noted in either group.**

Conclusions **The combined therapy with ursodeoxycholic acid and glycyrrhizin is safe and effective in improving liver-specific enzyme abnormalities, and may be an alternative to interferon in chronic hepatitis C virus infection, especially for interferon-resistant or unstable patients.** ***Eur J Gastroenterol Hepatol*** **11:1077–1083 © 1999 Lippincott Williams & Wilkins**

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Keywords: chronic hepatitis C virus infection, combination therapy, glycyrrhizin, prospective randomized controlled trial, ursodeoxycholic acid

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Introduction

Although interferon (IFN)-α is used widely and considered an effective antiviral agent in chronic hepatitis C virus (HCV) infection [1,2], only about half of patients treated with high IFN doses (528 to 660 million units for 24–60 weeks) sustain long-term responses resulting in viral eradication [3–5]. Specifically, response rates in patients who are infected with HCV genotype 1b, or who show a high pretreatment viraemia level, are significantly lower than those in patients with genotype 2a or 2b or a low viraemia level [6,7]. Furthermore, genotype 1b patients with wild-type amino acid sequences in the NS5A region of the HCV genome tend to have a poor response to IFN irrespective of viraemia level [8,9]. Such patients with IFN resistance arising from HCV-related factors have unfavourable outcomes and may develop progressive liver disease and hepatocellular carcinoma [10]. Moreover, not all patients are suitable candidates for IFN therapy. IFN is costly and can give rise to serious adverse effects. Therefore, alternative therapeutic agents are needed to prevent evolution of liver disease and carcinogenesis in at least some patients with chronic HCV infection.

In the Far East and Southeast Asia, an intravenous preparation of glycyrrhizin (GL) is administered to patients who do not respond to IFN therapy, or who experience relapse after IFN therapy, or who are ineligible for IFN therapy for chronic HCV infection. GL, a saponin component in an aqueous extract from the liquorice root (*Glycyrrhiza radix*), has been used widely in China from ancient times as an anti-inflammatory drug [11]. GL preparations have proven effective in improving liver function indices in chronic viral hepatitis according to a multicentre randomized, double-blind, placebo-controlled trial [12]. A subsequent study has demonstrated that GL significantly lowered serum aspartate transaminase (AST), alanine transaminase (ALT), and gamma-glutamyl transpeptidase (GGT) concentrations and ameliorated histo-

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logically evident necrotic and inflammatory lesions in the liver [13,14]. Accordingly, GL has been used extensively as an anti-inflammatory agent in chronic viral hepatitis in the Far East and Southeast Asia prior to the introduction of IFN therapy. A recent study has reported that long-term GL administration for chronic HCV infection is effective in preventing liver carcinogenesis, by reducing serum ALT levels [15]. Currently, several clinical trials are proceeding, or are planned, to evaluate the effect of GL treatment in some European countries. In this study, aiming to enhance the beneficial effect of the GL treatment, we concurrently administered ursodeoxycholic acid (UDCA). Several studies have reported efficacy of UDCA, a hydrophilic bile acid, in lowering serum liver enzyme concentrations in patients with chronic HCV infection [16,17], as well as in patients with chronic hepatitis of various aetiologies [18–23], primary biliary cirrhosis [24,25], primary sclerosing cholangitis [26,27], liver disease associated with cystic fibrosis [28] and intrahepatic cholestasis of pregnancy [29]. Proposed mechanisms of UDCA action in these liver diseases have included direct cytoprotection, detergent effects upon toxic hydrophobic bile acids, immunomodulatory effects, and induction of hypercholeresis [30–39]. Although several studies of the addition of UDCA to IFN therapy have been reported [40–42], therapy combining UDCA with other putative hepatotropic medicines has not been investigated.

In this study we evaluated the efficacy and safety of the combination of a GL preparation plus UDCA compared with GL preparation alone in a large group of patients with chronic HCV infection in a randomized controlled trial.

Patients and methods

Patients

During the period from January to June 1998, a total of 170 patients with chronic hepatitis C were consecutively enrolled into a randomized controlled trial of GL vs GL plus UDCA at the Toranomon Hospital. Criteria for inclusion were persistent anti-HCV antibodies and HCV RNA in pretreatment sera, as measured by a second-generation RIA (Ortho Diagnostic Systems, Raritan, NJ, USA) and a conventional reverse transcription-nested polymerase chain reaction assay, respectively; persistently elevated serum aminotransferase concentrations ≥ 1.5 times the upper limit of normal for at least 6 months before inclusion in the trial; a diagnosis of chronic hepatitis made from examination of liver biopsy specimens obtained in the preceding 6 months; and an age between 20 and 70 years. Fully informed written consent for participation was obtained from all patients before they entered the trial. All liver biopsy specimens were assessed independently by two investigators (AT and HK) blinded to the clinical information, using the ranking system for grading of necroinflammation activity and staging of fibrosis [43].

Exclusion criteria included occurrence of liver cancer or severe liver failure, as described elsewhere [6], evidence of any other form of liver disease, coexistence of any other serious medical illness, a previous course of IFN therapy or any other antiviral or immunomodulant therapy administered within the previous year, the presence of HBsAg as determined by RIA (Abbott Laboratories, North Chicago, IL, USA), and pregnancy or lactation.

Clinical and laboratory assessments were performed every 4 weeks prior to randomization and during the trial. Adverse effects were monitored clinically by a careful interview and a medical examination every 4 weeks. Patient compliance with treatment was evaluated by a questionnaire and by counting returned tablets.

Study protocol

After fully informed written consent was obtained from eligible patients, randomization was performed by means of sealed, opaque, numbered envelopes, each containing a sheet of paper assigning the patient to receive either GL preparation (group G) or GL preparation plus UDCA (group G+U). Patients in both groups were treated with intravenous injections of 100 ml of GL preparation (Stronger Neo-Minophagen C, or SNMC, Minophagen Pharmaceutical, Tokyo, Japan) three times weekly for 24 weeks. The preparation consists of GL, 0.2%; L-cysteine, 0.1%; and L-glycine, 2.0%, dissolved in physiological saline solution. In addition, group G+U patients were given a daily oral dose of 600 mg of UDCA (Urso, Tokyo Tanabe, Tokyo, Japan) in three portions following meals for 24 weeks. The daily dose was set at 600 mg/day according to results of a previous clinical study [16]. Close supervision of randomization was maintained throughout the period of patient entry. The study protocol was approved by the Local Ethics Committee of Toranomon Hospital.

Quantitation and typing of serum HCV RNA

Serum HCV RNA was quantified at randomization and at the end of treatment by a branched-DNA signal-amplification assay, according to the manufacturer's protocol (bDNA Quantiplex HCV RNA 2.0 Assay; Chiron, Emeryville, CA, USA) [7]. The HCV genome was serologically typed by ELISA using type-specific recombinant proteins according to the manufacturer's instructions (International Reagents, Kobe, Japan) [44].

Statistical methods

The chi-squared test, Fisher's exact two-tail test, or a two-sample Wilcoxon signed-rank test was used for

comparisons between group frequencies where appropriate. Intention-to-treat analysis was used for evaluation of UDCA efficacy. Changes in laboratory tests measured every 4 weeks were calculated as percent changes from the pretreatment baseline in each patient. Differences between pretreatment baseline and percent change at each time point in each group were compared by a paired *t*-test. Comparisons for each laboratory parameter during treatment between the two groups or between time points were performed by a two-way analysis of variance. To assess which potential variables (Table 1) could have contributed to reductions from the baseline of serum AST, ALT, or GGT, we used multiple regression analysis. The criterion for statistical significance was a P value of less than 0.05. All calculations were performed using the SAS program version 6.12 (SAS Institute, Cary, NC, USA).

Results

Comparability of the two groups

We evaluated efficacy data from 84 of the 85 group G patients (GL preparation alone) and 83 of the 85 group G+U patients (GL preparation plus UDCA). These 167 patients had fully completed the assigned treatment and were followed closely as scheduled. Patient compliance with UDCA treatment was good. Three randomized patients (one in group G, two in group G+U) were excluded from analysis because treatment was discontinued. (The reasons were detection of diffuse hepatocellular carcinoma by magnetic resonance imaging during the third study month in one group G patient, superinfection with HBV in one group G+U patient, and a traffic injury during treatment in one group G+U patient). The study population at entry into the study is descriptively profiled in Table 1. No significant differences were evident between the two groups with respect to demographic, laboratory, or virological characteristics.

Biochemical response to therapy

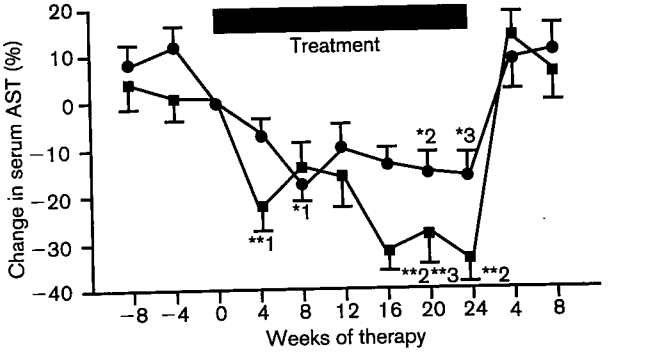
Changes in serum AST concentrations over time were compared between the two groups (Fig. 1). In group G, comparison with the baseline value (0 weeks) disclosed a significant decrease at 8, 20, and 24 weeks after the start of treatment ($P = 0.0022$, 0.026, and 0.030, respectively). In group G+U, a significant reduction was found at 4, 16, 20, and 24 weeks ($P = 0.0010$, < 0.0001, 0.0031, and < 0.0001, respectively). The overall AST level of group G+U during the treatment period (4 to 24 weeks), expressed as a percentage of the pretreatment baseline value, was $-23.4 \pm 35.5\%$ (mean $\pm$ SD), and was $-8.8 \pm 28.1\%$ in group G. Thus, overall AST decreases during treatment were significantly more marked in group G+U than in group G ($P = 0.025$). AST values reached normal levels by the end of the study (24 weeks) in 12 of 53 group G patients (22.6%) who had elevated values at the beginning of the trial.

Table 1 Baseline profiles of 167 patients studied for treatment efficacy

Variable	Group G ($n = 84$)	Group G + U ($n = 83$)
Demographic feature		
Age (years)*	58.7 ± 8.1	56.3 ± 10.8
Gender (F/M)	37/47	26/57
Source of hepatitis		
Transfusion-related	39	44
Sporadic	45	39
Laboratory result		
AST (IU/l)*	63.9 ± 25.1	64.3 ± 21.2
ALT (IU/l)*	77.7 ± 37.6	86.2 ± 36.6
GGT (IU/l)*	96.3 ± 77.2	98.7 ± 67.9
Total protein (g/dl)*	7.8 ± 0.8	7.9 ± 0.6
Albumin (mg/dl)*	4.1 ± 0.4	4.1 ± 0.5
Bilirubin (mg/dl)*	0.8 ± 0.7	0.8 ± 0.4
Potassium*	4.2 ± 0.4	4.3 ± 0.4
Platelet count ($\times 10^3/\mu l$)*	129.1 ± 41.9	135.9 ± 51.6
Histological findings		
Stage (1/2/3/4)	23/34/5/22	28/27/5/23
Grade (mild/moderate)	61/23	57/26
HCV-related marker		
Viraemia level (MEq/ml)**	negative–25.0	negative–27.0
bDNA-negative patients (n)	15	17
bDNA-positive patients (n)	63	61
Viraemia level among positive patients (MEq/ml)*	6.8 ± 6.0	6.2 ± 6.1
Serological type 1/2/ND	70/12/2	67/15/1

AST, aspartate transaminase; ALT, alanine transaminase; GGT, gamma-glutamyl transpeptidase; MEq, megaequivalents; bDNA, branched DNA probe assay; ND, not determined.
Normal reference ranges: 11–50 IU/l for AST; 6–50 IU/l for ALT; 9–75 IU/l for GGT.
Group G received a glycyrrhizin preparation alone; group G+U received glycyrrhizin preparation plus ursodeoxycholic acid.
*Data are expressed as the means ± SD.
**Data are expressed as a range. No significant differences in baseline features were detected between the two groups.

Fig. 1



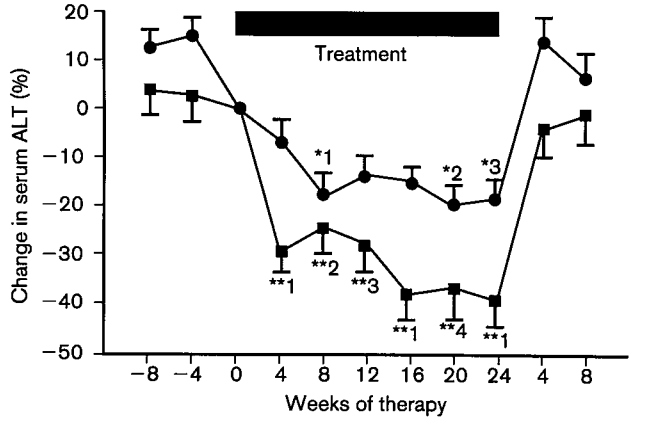
Changes over time in serum aspartate transaminase (AST) concentrations compared with pretreatment values in chronic hepatitis C patients receiving a glycyrrhizin preparation alone (group G) vs the glycyrrhizin preparation plus ursodeoxycholic acid (group G+U). Changes are expressed as mean percentages of pretreatment baseline values for group G (filled circles) vs group G+U (filled squares). The standard error is indicated by vertical bars. Overall, changes in group G+U were significantly greater than those in group G ($P = 0.025$). $^{*1}P = 0.0022$; $^{*2}P = 0.026$; $^{*3}P = 0.030$; $^{**1}P = 0.0010$; $^{**2}P < 0.0001$; $^{**3}P = 0.0031$.

In contrast, 34 of 63 group G+U patients (54.0%) with baseline ALT abnormalities showed normal concentrations by completion of the trial ($P = 0.0006$).

Changes in serum ALT during treatment compared with the baseline value at 0 weeks are depicted in Fig. 2. In group G, serum ALT values were significantly lower than at baseline at 8, 20 and 24 weeks ($P = 0.011$, 0.024, and 0.045, respectively). In group G+U, ALT declined significantly from pretreatment baseline levels at all time points ($P < 0.0001$, 0.022, 0.0008, < 0.0001, 0.0015, and < 0.0001, respectively). Overall percent changes in ALT during treatment were significantly greater in group G+U ($-32.8 \pm 39.5\%$) than in group G ($-10.2 \pm 39.7\%$; $P = 0.0003$). At the end of the trial (24 weeks), 11 of 62 group G patients with increased ALT levels (17.7%) had attained normalization. In group G+U, ALT concentrations had normalized in 23 of 72 patients (31.9%). Normalization by the end of the study was marginally more frequent in group G+U than in group G ($P = 0.074$).

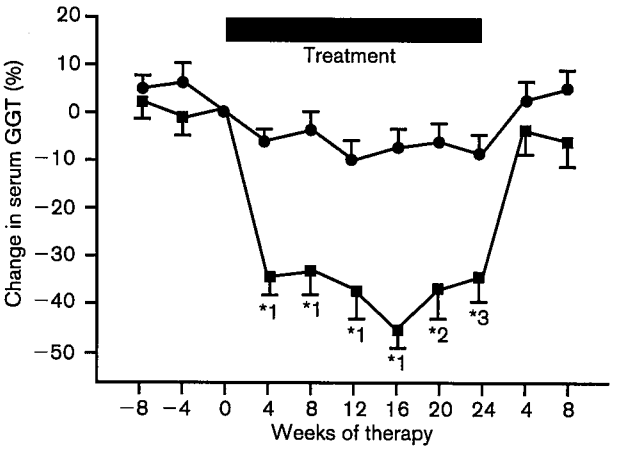
As for changes in serum GGT (Fig. 3), no significant changes from baseline were seen at any time points in group G. In group G+U, a significant percentage reduction from baseline occurred at all time points ($P < 0.0001$, < 0.0001, < 0.0001, < 0.0001, 0.0002, and 0.0003, respectively). Overall percent changes in GGT during treatment were significantly greater in group G+U ($-37.1 \pm 39.3\%$) than in group G ($-5.7 \pm 27.3\%$; $P = 0.0001$). Normalization by the end of the study was significantly more frequent in group G+U (26 of 42, 61.9%) than in group G (eight of 35, 22.9%; $P = 0.001$).

Fig. 2



Changes over time in serum alanine transaminase (ALT) concentrations compared with pretreatment values in chronic hepatitis C patients receiving a glycyrrhizin preparation alone (group G) vs the glycyrrhizin preparation plus ursodeoxycholic acid (group G+U). Changes are expressed as mean percentages of pretreatment baseline values for group G (filled circles) vs group G+U (filled squares). The standard error is indicated by vertical bars. Overall, changes in the group G+U were significantly greater than those in group G ($P = 0.0003$). $^{*1}P = 0.011$; $^{*2}P = 0.024$; $^{*3}P = 0.045$; $^{**1}P < 0.0001$; $^{**2}P = 0.022$; $^{**3}P = 0.0008$; $^{**4}P = 0.0015$.

Fig. 3



Changes over time in serum gamma-glutamyl transpeptidase (GGT) concentrations compared with pretreatment values in chronic hepatitis C patients receiving a glycyrrhizin preparation alone (group G) vs the glycyrrhizin preparation plus ursodeoxycholic acid (group G+U). Changes are expressed as mean percentages of pretreatment baseline values for group G (filled circles) vs group G+U (filled squares). The standard error is indicated by vertical bars. Overall, changes in group G+U were significantly greater than those in group G ($P = 0.0001$). $^{*1}P < 0.0001$; $^{*2}P = 0.0002$; $^{*3}P = 0.0003$.

Within each group, no significant variation occurred in the percentage reduction of AST, ALT, and GGT among time points during treatment (from 4 to 24 weeks), indicating that improvements in serum liver enzyme concentration were steadily sustained.

Platelet count and serum total protein, albumin, bilirubin, and potassium concentrations did not significantly change from baseline during treatment in either group.

Changes in HCV viraemia level

HCV viraemia levels at initiation of the trial did not differ significantly between the two groups (Table 1). At the end of treatment they were 6.0 ± 6.2 and 5.9 ± 6.0 MEq/ml in groups G and G+U, respectively. No significant differences were seen between pre- and post-treatment viraemia levels in either group, so neither treatment had any significant effect on HCV replication.

Biochemical responses and HCV type

Overall, percent reductions in AST, ALT, and GGT did not differ significantly between patients infected with HCV types 1 and 2, nor were there any significant

differences between HCV types 1 and 2 patients according to treatment regimen.

Biochemical responses and liver histology

No significant differences were observed in AST, ALT, or GGT changes with treatment according to grade or stage of liver histology, nor were significant differences evident in grade or stage according to treatment group.

Factors influencing outcome

In multiple regression analyses, the treatment regimen was the only significant or marginally significant factor contributing to the percent reduction rate from baseline in AST, ALT, and GGT concentrations ($P = 0.082$, 0.052 and 0.037, respectively); no other factors were identified. In neither group did another factor significantly or marginally influence the response to treatment.

Side effects

In neither group did any patient complain of drug-related symptoms or develop signs of an adverse reaction during the treatment period. All patients fully completed the treatment protocol as scheduled without reporting difficulties in activities of daily living.

Discussion

In the Far East and Southeast Asia, intravenous GL preparations have been widely administered to patients with chronic viral hepatitis, and the efficacy of GL in improving liver enzyme parameters has been confirmed in a multicentre randomized, double-blind, placebo-controlled trial [12]. The present study also confirmed the effect in chronic HCV infection of GL administered alone on serum AST and ALT concentrations, but not those of GGT. Hepatic necrosis and inflammation have been shown histologically to be lessened by GL treatment [13,14]. Although no definitive explanation exists for the beneficial effect of GL in improving liver functions, biochemical studies have suggested that the mode of action of GL involves stabilization of cell membranes [45–48]. In an *in vitro* study, GL was found to inhibit production of prostaglandin E_2 arising from macrophage activation and the activity of phospholipase A_2 in arachidonic acid cascades [49]. The GL compound, representing a kind of triterpenoid sapogenin noted to be an IFN inducer, has been shown to induce IFN [50] while increasing natural killer cell activity in sera from humans or mice [51]. However, the present study indicated that GL is unlikely to suppress HCV replication despite its ability to stabilize liver function and induce IFN. A preliminary study has shown that intraperitoneal injection of GL inhibited growth of Meth A tumours in BALB/c mice and metastasis of Yoshida sarcomas in rats [52]. In addition, a recent clinical study has demonstrated that long-term GL treatment contributes to prophylaxis against liver carcinogenesis in chronic hepatitis C patients [15]. Based on these findings, we now attempt to evaluate the ability of UDCA to augment prevention of progression of the disease and to ultimately promote the efficacy of GL as a future strategy for protection against liver carcinogenesis.

Although the mechanism by which UDCA decreases serum liver enzyme concentrations in treatment of chronic liver disease of varying aetiology has not been clearly established, several *in vitro* and *in vivo* studies have demonstrated direct cytoprotective effects of UDCA upon the hepatocyte [30–33,35,37–39]. Alternatively, because HCV-induced chronic liver injury may be aggravated by exposure to relatively hydrophobic endogenous or exogenous bile acids such as chenodeoxycholic acid or deoxycholic acid [31,32,53], UDCA could directly counteract hepatotoxicity of cytotoxic bile acids, promote their excretion, or bind to hepatocytes or bile duct cell membranes to minimize bile acid damage [24,32–34,37,38]. UDCA administration also may modify the hydrophobic–hydrophilic bile acid balance [34,39]. UDCA has been suggested to act via an immunoregulatory mechanism involving suppression of expression of aberrant HLA class I molecules, ICAM-1 molecules, and Bcl-2 and nuclear DNA fragmentation on hepatocytes or bile duct epithelia [36,54,55]. Significant further reductions in liver enzyme concentrations in serum, especially those of GGT, were observed with addition of UDCA to GL in this study. UDCA is likely to lower serum liver enzymes in a manner differing from the action of GL. Specifically, a marked reduction in serum GGT concentrations is likely to result from marked choleretic properties of UDCA [24,56,57]. Biliary tract histology has shown significant improvement with UDCA administration, as have certain cholestatic parameters, and these changes were significantly associated with changes in GGT [23].

Among HCV-related factors, genotype, pretreatment viraemia load, and interferon sensitivity-determining region in the NS5A of the genotype 1b genome are strong predictors of a sustained response to IFN therapy in chronic HCV infection [5–9]. In contrast, no significant correlation was noted between viral factors and biochemical response to GL treatment alone or GL plus UDCA. The difference may be related to the absence of action of GL or UDCA involving suppression of HCV replication. In addition, the difference suggests that UDCA plus GL given in combination may be particularly well suited to patients with virologic factors that are unfavourable for IFN therapy. As for host factors, the absence of cirrhosis has been associated with a better response to IFN therapy [6,58]. In the presently evaluated combination therapy, transaminase reductions appeared unrelated to

liver histology. Accordingly, patients with advanced liver disease can effectively and safely undergo combination therapy.

The steroid-like chemical structure of GL is known to provoke pseudoaldosteronism (hypertension, aldosteronopenia, hypopotassaemia, and suppressed plasma renin activity) when high-dose GL alone is given to humans [59]. However, the GL preparation used for our study regimen has been shown to be very safe; ingredients other than GL in the preparation may prevent toxicity [60]. In addition, we confirmed that addition of UDCA to the GL preparation is well tolerated without any adverse pharmacological interactions; in itself, UDCA is also safe and well tolerated. From an economic standpoint, costs of the GL preparation (for 100 ml, ¥725 or $6.0) and UDCA (for 600 mg, ¥108 or $0.9) are relatively low. The absence of adverse effects and low cost are both important for long-term combination therapy.

UDCA-plus-GL combined therapy may be an alternative to IFN for patients who fail to respond to IFN, experience relapse after completing IFN treatment, or encounter severe side effects from IFN. Further studies are needed to evaluate the potential benefits of long-term combination therapy for preventing progression of chronic HCV infection and related liver carcinogenesis.

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5 U.S.C. § 552(b)(4)

Article Link: http://www.webmd.com/digestive-disorders/understanding-cirrhosis-basic-information?page=2

Digestive Disorders Health Center
Understanding Cirrhosis of the Liver

In this article

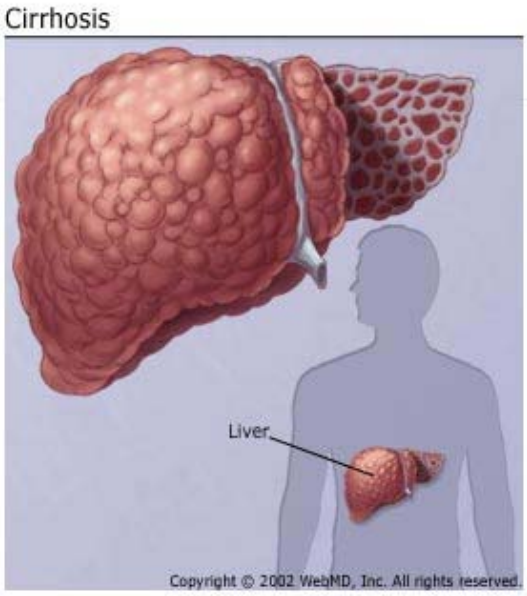
What Is Cirrhosis of the Liver?

Cirrhosis is a serious degenerative disease that occurs when healthy cells in the liver are damaged and replaced by scar tissue, usually as a result of alcohol abuse or chronic hepatitis. As liver cells give way to tough scar tissue, the organ loses its ability to function properly. Severe damage can lead to liver failure ar possibly death.

Cirrhosis poses another danger as well: Dense scarring slows the normal flow of blood through the liver, causing blood to find alternate pathways to return to the heart. This includes veins along the stomach and esophagus. The added pressure in these blood vessels, called varices, can cause them to enlarge and, in some cases, rupture. This is especially a problem for the blood vessels in the esophagus.

Every year, about 31,000 people in the U.S. die from cirrhosis, mainly due to alcoholic liver disease and chronic hepatitis C. The disease cannot be reversed or cured except, in some cases, through a liver transplant. It can often be slowed or halted, however, especially if the disease is detected in the early stage of development. Patients who think they might have cirrhosis should see a doctor without delay.

Cirrhosis is serious because of the importance of the organ it affects. The liver, weighing about three pounds and roughly the size of a football, is the largest of the body's internal organs. Among its many functions, the liver serves as an essential part of the digestive system by producing bile, which is stored in the gallbladder, then released into the small intestine, where it helps break down fatty food. The liver also helps maintain the proper composition of the blood by regulating the amounts of fat, protein, and sugar th enter the bloodstream.



As the body's primary blood filter, the liver works to detoxify alcohol, drugs, and other potentially harmful chemicals. Along with the spleen, the liver traps and disposes of worn-out red blood cells. And because it aids in the removal of bacteria and viruses from the blood, the liver is a vital component of the immune system. If your liver is not functioning properly, you are more susceptible to infection.

What Is Cirrhosis of the Liver? continued...

The liver is remarkably tolerant of disease and injury. Even after 70% of its mass has been destroyed or removed, the organ can still function, albeit with decreased effectiveness. If the conditions that caused the destruction have been removed or corrected, the liver usually can bounce back.

Although function can never be restored to parts of your liver that have turned to scar tissue, you can live healthy life with the remaining portion if the disease is caught in time. However, there is a point of no retu with cirrhosis. As more cells are replaced by scar tissue, fewer healthy cells are left to handle the liver's many tasks. Eventually, function problems arise and may remain. This is why it's important to identify the underlying causes as soon as possible and begin taking steps to eliminate them.

What Causes Cirrhosis of the Liver?

Cirrhosis occurs as the result of long-term injury to the liver. Possible causes include viruses, genetic deficiencies, prolonged obstruction of bile flow, and long periods of exposure to drugs and other toxic substances. In the majority of cases, however, the culprit is excessive consumption of alcohol.

The link between alcohol and cirrhosis is well documented. Studies show that while moderate drinking ma actually help prevent strokes and heart disease, heavy drinking has a clearly harmful effect on the liver. Fo example, the French -- famous for their wine consumption -- have a relatively low incidence of heart disease, but the rate of cirrhosis in France is very high. Many doctors believe that more drinkers die from cirrhosis than are protected from heart disease.

Simply put, the more alcohol you drink -- and the greater the frequency of drinks -- the more likely you are to develop cirrhosis. Because the bodies of men and women process alcohol differently, the amount that you can safely imbibe depends largely on your sex. Women are more susceptible to alcohol-induced liver damage than men.

It's important to note that alcohol tolerance may vary from one person to the next. For some people, one drink per day is enough to leave permanent scars in the liver. If you drink, especially if you do so heavily an often, have a doctor examine you for signs of cirrhosis. This is necessary even if you feel healthy, since the symptoms of cirrhosis often do not appear until it is too late to stop the disease or slow its progress.

What Causes Cirrhosis of the Liver? continued...

Excessive drinking almost inevitably causes some liver damage, but it does not always lead to cirrhosis. Some people who drink heavily develop alcoholic hepatitis, an inflammation of the liver that can last a wee or two, producing symptoms of nausea, fever, loss of appetite, jaundice, and confusion. Over time, the condition can also lead to cirrhosis. Even light drinkers who go on a bender for several days can develop a condition known as fatty liver, caused when cells of the liver become swollen with accumulated fat and water. This condition can cause pain or tenderness in the liver and abnormalities in other liver functions. Fatty liver can also result from diabetes, elevated cholesterol, obesity, and severe malnutrition.

Another frequent cause of cirrhosis is viral hepatitis, a general term meaning inflammation of the liver because of a viral infection. Of the various forms of this disease, only two, hepatitis B and hepatitis C, are likely to cause chronic infection which can lead to scarring and cirrhosis. Scarring usually occurs after hepatitis has become chronic (lasting six months or more). The symptoms may be so mild at first that patients with chronic hepatitis do not even realize their livers are scarring. Meanwhile, the damage continues, perhaps resulting in a serious case of cirrhosis later in life. Therefore, it is important for people with hepatitis to have regular medical checkups, especially since hepatitis can be treated and, in some cas cured. And because hepatitis is contagious, family members of an infected person should also be tested.

Cirrhosis sometimes, though rarely, occurs because of an inherited liver disorder. In Wilson's disease, for example, a genetic deficiency inhibits the body's ability to metabolize copper. As a result, excessive amoun of the metal accumulate in various body organs, particularly the liver, where it destroys tissue. Similarly, ir hemochromatosis the body absorbs excess amounts of iron, which damages the liver and causes scarring. This disorder mostly strikes men between the ages of 40 and 60; women who have not gone through menopause are usually not affected because their bodies lose iron during menstruation. Alpha-1-antitrypsin deficiency is an enzyme deficiency that results in the accumulation of products in the liver causing destruction of liver tissue.

What Causes Cirrhosis of the Liver? continued...

Children born with galactosemia lack an enzyme needed to digest a component of milk sugar. Milk sugar, also known as lactose, is composed of two sugars, glucose and galactose. The body needs to convert the galactose to glucose. In people with galactosemia, the enzyme to do this conversion is missing or not functioning adequately. Galactose accumulates in the liver at levels that become toxic and potentially fata

without proper treatment. Infants with this disorder should be taken off milk and given a substitute formu that does not contain galactose.

Some babies are born with no bile ducts, or with ducts that are malformed. Because bile is unable to drain out of the body, it accumulates in the liver and eventually poisons it. Although the problem can sometimes be corrected through surgery, most children with this disorder die from cirrhosis before they reach the ag of 2.

Cirrhosis can result when strictures or scarring block the flow of bile in the bile ducts and cause it to back into the liver for long periods of time. This occurs in conditions such as primary sclerosing cholangitis or primary biliary cirrhosis. The disease may also come as a consequence of long-term exposure to certain drugs, including methotrexate and isoniazid, and to toxic substances in the environment, such as pesticide and arsenic-based compounds. Lastly, autoimmune hepatitis is an inflammatory process in the liver that ca result in scarring and cirrhosis due to antibodies produced by the body that attack the liver. The cause is unknown.

WebMD Medical Reference
SOURCES:
American Liver Foundation.
Life Extension Foundation.
CDC.
National Center for Health Statistics.

Reviewed by Jennifer Robinson, MD on March 01, 2015

My Notes:

Further Reading:

Cirrhosis of the Liver

Ascites and Cirrhosis

Primary Biliary Cirrhosis (PBC)-Topic Overview

Cirrhosis: Sodium and Ascites-Topic Overview

Cirrhosis: Surgical Shunts for Portal Hypertension-Topic Overview

Cirrhosis Complications: Encephalopathy-Topic Overview

Cirrhosis Complications: Spontaneous Bacterial Peritonitis-Topic Overview

See All Cirrhosis of the Liver Topics

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HEPATITIS C VIRAL INFECTION

Intravenous glycyrrhizin for the treatment of chronic hepatitis C: A double-blind, randomized, placebo-controlled phase I/II trial

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Abstract

Background: In Japan, glycyrrhizin therapy is widely used for chronic hepatitis C and reportedly reduces the progression of liver disease to hepatocellular carcinoma. The aims of this study were to evaluate the effect of glycyrrhizin on serum alanine aminotransferase (ALT), hepatitis C virus (HCV)-RNA and its safety in European patients.

Methods: Fifty-seven patients with chronic hepatitis C, non-responders or unlikely to respond (genotype 1/cirrhosis) to interferon therapy, were randomized to one of the four dose groups: 240, 160 or 80 mg glycyrrhizin or placebo (0 mg glycyrrhizin). Medication was administered intravenously thrice weekly for 4 weeks; follow up also lasted for 4 weeks.

Results: Within 2 days of start of therapy, serum ALT had dropped 15% below baseline in the three dosage groups ($P < 0.02$). The mean ALT decrease at the end of active treatment was 26%, significantly higher than the placebo group (6%). A clear dose–response effect was not observed (29, 26, 23% ALT decrease for 240, 160 and 80 mg, respectively). Normalization of ALT at the end of treatment occurred in 10% (four of 41). The effect on ALT disappeared after cessation of therapy. During treatment, viral clearance was not observed: the mean decrease in plasma HCV-RNA after active treatment was 4.1×10^6 genome equivalents/mL (95% confidence interval, $0–8.2 \times 10^6$; $P > 0.1$). No major side-effects were noted. None of the patients withdrew from the study because of intolerance.

Conclusions: Glycyrrhizin up to 240 mg, thrice weekly, lowers serum ALT during treatment, but has no effect on HCV-RNA levels. The drug appears to be safe and is well tolerated. In view of the reported long-term effect of glycyrrhizin, further controlled investigation of the Japanese mode of administration (six times weekly) for induction appears of interest.

Key words: alanine aminotransferase normalization, glycyrrhizin, hepatitis C.

INTRODUCTION

Chronic hepatitis C infection can be associated with progressive liver disease that may evolve insidiously to cirrhosis and carries an increased risk of hepatocellular carcinoma.[1] Alpha-interferon monotherapy leads to hepatitis C virus (HCV)-RNA clearance in a minority of patients. Although combination therapy with Ribavirin increases efficacy, the sustained response rate is still below 50%.[2]

For patients who do not respond with viral clearance, different treatment strategies have to be sought. These approaches include virus suppressive or hepatoprotective medication. In long-term studies on chronic hepatitis B and C, persistent normalization of alanine aminotransferase (ALT) appears to be a key prognostic factor for reduction of complications, regardless of the presence of viral markers in the serum.[3–6]

In Japan, glycyrrhizin extracted from the roots of the plant *Glycyrrhiza glabra* (liquorice) has been used for

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the treatment of chronic hepatitis for more than 20 years.[7] Glycyrrhizin is a conjugate of a molecule of glycyrrhetinic acid and two molecules of glucuronic acid (Fig. 1). Suzuki *et al.* found that treatment with glycyrrhizin, given as Stronger Neo-Minophagen C (SNMC, an aqueous solution for intravenous administration), lowered the serum transaminases significantly.[8] However, after discontinuation of the medication, the serum transaminases rebounded. Glycyrrhizin has no influence on the viral load.[9] Despite this finding, Arase *et al.* recently reported that long-term treatment with glycyrrhizin of Japanese patients with chronic hepatitis C, prevented the development of hepatocellular carcinoma when ALT normalized.[4]

At present, millions of ampoules of SNMC, each containing 40 mg glycyrrhizin, are used each year in Japan for the treatment of chronic hepatitis. It is remarkable that a drug that is used in Japan and other Asian countries on such a large scale is virtually unknown in Europe and the USA. Can this discrepancy be explained by a difference in efficacy of glycyrrhizin between Asian people and white people resulting from genetic polymorphisms in drug metabolism?[10]

We performed a randomized, double-blind, placebo-controlled trial to evaluate whether the effect of glycyrrhizin on serum ALT is also demonstrable in European patients.

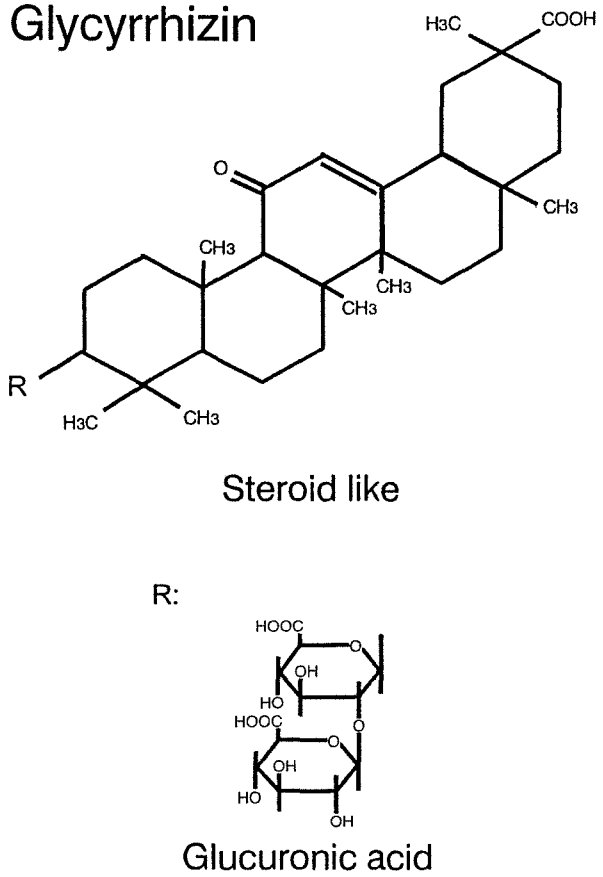

Figure 1 Molecular structure of glycyrrhizin.

METHODS

Patients

Patients were recruited from the University Hospital Rotterdam, between February 1997 and March 1998. Patients between 18 and 65 years of age were eligible if they met all inclusion criteria: serum antibodies against HCV; HCV-RNA positive; serum ALT at least 1.5-fold the upper limit of normal in the 8 weeks prior to treatment and at entry to the trial; liver biopsy consistent with mild to moderate fibrosis or cirrhosis with mild to moderately active hepatitis; relapse after, non-response to, or unlikely to respond to, interferon (cirrhosis, genotype 1); or contraindication for, or refusal to take, interferon.

Patients were not eligible if they met one or more exclusion criteria: other causes of liver disease: chronic hepatitis B, alcohol abuse, autoimmune hepatitis, haemochromatosis, Wilson's disease, α-l-antitrypsin deficiency; decompensated cirrhosis, hepatocellular carcinoma; significant cardiovascular or pulmonary dysfunction in the past 6 months; malignancy in the previous 5 years; human immunodeficiency virus 1/2 infection; immunosuppressive therapy; antiviral treatment in the preceding 3 months; pregnancy, breast-feeding; hypokalemia and liquorice addiction.

The study was conducted according to the Declaration of Helsinki and Good Clinical Practice. The Protocol was approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam and all patients gave their written, informed consent.

Study design

Patients were stratified for the presence or absence of cirrhosis and were randomized to receive a high (240 mg), standard (160 mg), low (80 mg) dose glycyrrhizin or placebo (0 mg glycyrrhizin). Randomization was performed by means of a computer-generated randomization schedule made by the statistician. The randomization code was known by the statistician and the Department of Pharmacy where the medication was packaged and labelled with consecutive randomization numbers. During the study neither the patients nor the physicians were aware of the dose of the trial drug. After completion of the clinical part of the study and closure of data collection, the randomization code was broken and the physicians and patients were notified of the actual treatment.

Glycyrrhizin was given as Stronger Neo-Minophagen C (SNMC), a clear solution for intravenous use, consisting of 2 mg/mL glycyrrhizin, 1 mg/mL cysteine and 20 mg/mL glycine diluted with physiological saline. The placebo consisted of 1 mg/mL cysteine and 20 mg/mL glycine diluted with physiological saline.

Both SNMC and placebo ampoules were supplied by Minophagen Pharmaceutical Co. Ltd, Tokyo, Japan.

Medication per visit consisted of six ampoules of 20 mL each (placebo or verum). The six ampoules (120 mL) were added to a 250 mL infusion bag

containing 100 mL of a 5% glucose solution. The time between preparation and administration was less than 4 h. The 220 mL solution was administered intravenously within 15 min via an indwelling venous catheter. The system was flushed with 25 mL NaCl to ensure administration of all of the medication.

Because glycyrrhizin has a saponin-like structure it may foam, so the placebo and verum ampoules were not fully indistinguishable. After mixing with 100 mL 5% glucose, both verum and placebo show foaming and became indistinguishable. To keep administration double-blind, the infusion bag was prepared by an independent third person.

Patients were treated as out-patients. Medication was administered intravenously thrice weekly for 4 weeks (12 infusions). The duration of follow up after the end of treatment was also 4 weeks. Routine haematological and biochemical assessments were performed weekly during the treatment period and every 2 weeks during follow up. Virological assessments were performed on day 0 and day 28 (pretreatment and post-treatment).

Side-effects were checked every visit by asking: 'Have you had any (other) medical problem since your last visit?' Blood pressure and weight were checked every visit: a complete physical examination was carried out before treatment, at the end of treatment and at the end of follow up. Electrolytes (Na and K) were assessed weekly during treatment and every 2 weeks during the follow-up period. Because administration took place in a hospital, patient compliance could be noted accurately by checking scheduled visits.

Virological assessments

Antibodies to hepatitis C virus were determined by screening for antibodies by a third-generation enzyme-linked immunosorbent assay (IMX; Abbott, Chicago, IL, USA.). The EDTA samples taken for HCV-RNA determination were processed into plasma within 2 h and stored at −70°C. Hepatitis C virus-RNA was determined qualitatively by a modified version of the Amplicor HCV assay (Roche Molecular Systems, Alameda, CA, USA). The extracted RNA was resuspended in 150 μL instead of 1 mL. The level of HCV-RNA was assessed quantitatively by means of the branched DNA assay (Quantiplex 2.0, Chiron Corporation, Emeryville, CA, USA), which has a lower detection limit of 200 000 HCV genome equivalents/mL.

For genotyping, the polymerase chain reaction product generated with the Amplicor HCV assay was used. Sequence analysis was performed on an automated sequencer (Perkin Elmer, Nieuwerkerk, The Netherlands). The genotype was determined according to the rules described by the International HCV Collaborative Study Group.[11]

Assessments of outcome

The primary (biochemical) response parameters were the percentage decrease in ALT from baseline and the number of patients with ALT normalization at the end of treatment and at the end of follow up. The secondary response parameter was the virological response, defined as the decrease in plasma HCV-RNA. Tolerability and occurrence of side-effects were the third outcome measures.

Statistical analysis

Before the beginning of the study, a power analysis was performed to determine the size of the study population. A linear relationship between the decrease in ALT and dose was assumed. The analysis was based on a two-tailed comparison with an α of 0.05 and a power of 0.80 ($\beta = 0.20$). It was calculated that to detect a correlation of $r = 0.40$ or more at least 12 patients were required per dosage group.

The baseline ALT was calculated as the mean ALT level at screening (in the 8 weeks before start of the study) and the ALT level on day 0. Statistical analysis was performed by using Stata 5.0 software (Stata Corporation, College Station, TX, USA). Comparisons between the four groups were evaluated using the Kruskal–Wallis test. If P was < 0.05, the Mann–Whitney U-test was used to perform further comparisons between the groups. Changes within groups were assessed by using the signed-rank test. Fisher's exact test was applied to compare percentages.

RESULTS

Characteristics of patients

Figure 2 shows the trial profile. There were 77 potential participants: 13 patients were not willing to participate and seven were not eligible. Therefore, 57 patients were randomized to receive treatment. One patient dropped out because of social problems. Two patients were mistakenly randomized: one patient had a normal ALT value on day 0; the other had several protocol violations: ALT on day 0 was 1.3 × upper limit of normal, liver biopsy showed no fibrosis and the time between antiviral (interferon) treatment and start of the glycyrrhizin

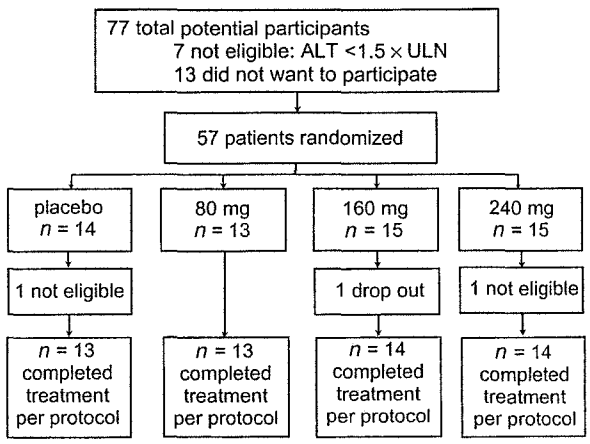

Figure 2 Trial profile.

study was less than 3 months. These two patients were included in the safety and tolerability analyses. Statistical analysis of all randomized patients led to the same conclusion as analysis of the 54 patients who completed treatment according to protocol. The efficacy data on 54 patients and the safety data on 56 patients are presented.

The baseline characteristics of the 54 patients are shown in Table 1. The groups appear well balanced. Each group consisted of approximately 40% of patients with liver cirrhosis. Most patients were white people. Approximately 80% of the patients per group were male. Although the average baseline level of ALT in the 240 mg group was lower than in the other groups, there was no significant difference between the four groups (Kruskal–Wallis, $P=0.13$). Median HCV-RNA levels were around 10^7 genome equivalents/ml.

Biochemical response

Figure 3 shows the effects of the placebo and the three doses of glycyrrhizin on the ALT level during the study. There was no significant difference within any of the four groups between ALT at screening (in the 8 weeks before start) and ALT on day 0 ($P>0.09$). During the study period there was no significant change in ALT in the placebo group.

Within 2 days of start of therapy, serum ALT dropped 15% below baseline in the three dosage groups ($P<0.02$) and remained significantly lower, except in the 80 mg group on day 28.

During follow up after the end of treatment ALT levels tended to increase again: only in the 240 mg group were ALT levels at the end of follow up significantly higher than at the end of treatment ($P=0.02$). At the end of follow up, the ALT values in the 80 and 240 mg groups were significantly lower than the baseline value ($P<0.01$ and 0.05, respectively).

During treatment it was found that on all assessment days the percentage decrease in ALT was significantly higher in the treatment groups compared with the placebo group (all $P<0.03$), while there was no difference between the three active groups. Figure 4 shows the mean percentage ALT decrease from baseline at the end of treatment per group. The mean percentage

Table 1 General characteristics of the 54 patients at baseline analysed by treatment group

	Glycyrrhizin treatment (mg)			
	0	80	160	240
Number	13	13	14	14
White patients/black patients/others	8/0/5	8/0/5	5/2/6	10/0/4
Males/females	13/0	11/2	10/4	11/3
Median age (range)	47 (37–60)	45 (32–66)	52 (35–69)	44 (34–61)
Non-cirrhotic/cirrhotic	7/6	8/5	8/6	8/6
Previous interferon (yes/no)	12/1	10/3	11/3	11/3
ALT baseline (×ULN) median (range)	3.1 (1.5–6.8)	3.4 (1.4–11.8)	3.0 (1.8–6.9)	2.2 (1.6–5.2)
γ-GTP baseline (×ULN) median (range)	1.9 (0.5–10.0)	1.4 (0.4–10.4)	2.1 (0.7–8.1)	1.2 (0.4–3.7)
HCV-RNA × 10^6 genome eq/mL, median (range)	4.5 (1.4–39.2)	21.7 (0.2–104)	11.1 (1.4–66.9)	14.4 (1.1–51.4)
Genotype 1a/1b/2/3/4a	1/6/1/3/2	3/3/2/5/0	2/6/4/1/1	2/4/2/6/0

ALT, alanine aminotransferase; γ-GTP, gamma glutamyltranspeptidase. ALT and γ-GTP baselines were calculated as the mean value at screening (in the 8 weeks before start) and at day 0. ULN, upper limit of normal.

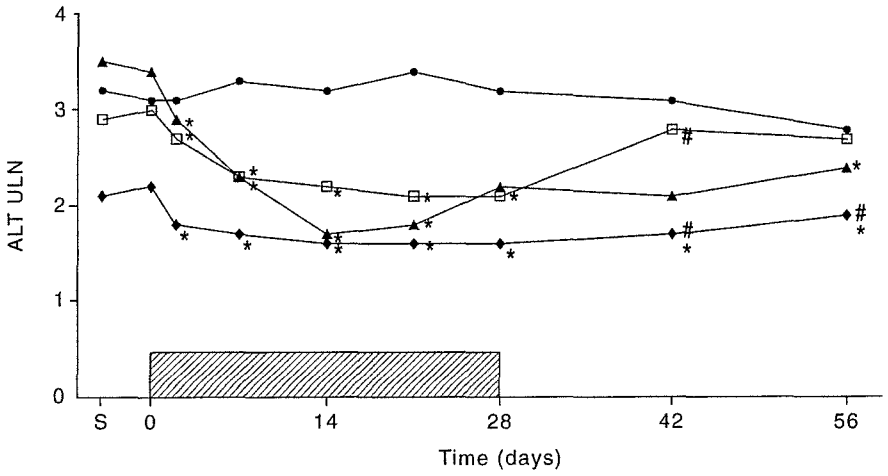

Figure 3 Median alanine aminotransferase (ALT) × upper limit of normal (ULN) according to dosage group. * Significantly lower than baseline, $P<0.05$ by the signed rank test. # Significantly higher than ALT at the end of treatment (day 28), $P<0.05$ by the signed rank test. (●) Placebo, (▲) 80 mg glycyrrhizin, (□) 160 mg glycyrrhizin, (◆) 240 mg glycyrrhizin, (▨) Stronger Neo-Minophagen C (SNMC), t.i.w, i.v.

decrease for the three glycyrrhizin groups at that time was 23, 26 and 29%, respectively, all being significantly larger than the 6% decrease found for the placebo group.

The percentage of patients exhibiting ALT normalization at the end of treatment was 0 (zero of 13), 15 (two of 13), 0 (zero of 14) and 14 (two of 14) %, in the 0, 80, 160 and 240 mg glycyrrhizin groups, respectively. Only one patient in the 240 mg group had normal ALT levels at the end of follow up. There was no significant difference in ALT normalization between the four groups at the end of treatment and at the end of follow up. There was no significant difference in gamma glutamyltranspeptidase (γ-GTP) at baseline and γ-GTP at the end of treatment (all $P > 0.08$) within any of the four groups (Fig. 5).

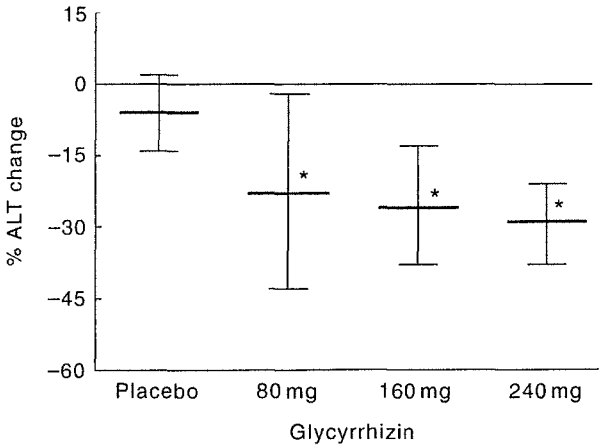

Figure 4 Mean percentage change in alanine aminotransferase (ALT) at the end of treatment with respect to baseline ±95% confidence interval per dosage group. * Significantly greater than placebo group, $P < 0.03$ by the rank-sum test.

Virological response

The HCV-RNA levels at baseline were high (range $0.2–104 \times 10^6$ genome equivalents/mL) as a result of selection of the patients (the majority were non-responders to interferon).

After treatment HCV-RNA was still detectable in all patients. In each of the four groups, the HCV-RNA levels at the end of treatment did not differ significantly from those at baseline (all $P > 0.11$; Table 2). The mean decrease in plasma HCV-RNA after active treatment was -4.1×10^6 genome equivalents/mL (95% confidence interval $-8.2–0.0 \times 10^6$).

Safety and tolerability

The expected side-effects of glycyrrhizin are hypokalemia, sodium retention, increase in bodyweight, elevated blood pressure and retention of body fluids (i.e. oedema).[12] None of these expected side-effects were observed during the study.

Table 3 shows the adverse events mentioned spontaneously or after the question 'Have you had any problems since your last visit?' by more than two patients in the course of the study. Headache, increased fatigue since treatment, pain or haematoma at the puncture site, strange feeling during administration, stabs of pain in the region of the liver and dizziness were mentioned by patients of the placebo as well as the three dosage groups. No significant differences between the four groups were observed. Cold or 'flu-like' symptoms, diarrhoea, rash or itching and nausea were not mentioned by patients in the placebo group, only by patients of one of the three dosage groups. The only statistically significant difference was six patients of the 160 mg group with cold or 'flu-like' symptoms; this was significantly higher than the zero patients of the placebo group (Fisher's exact test, $P = 0.016$). The number of patients reporting an adverse event did not differ significantly between the four groups (Fisher's exact test, $P = 0{:}63$).

Treatment was generally well tolerated, no patients were lost due to intolerance.

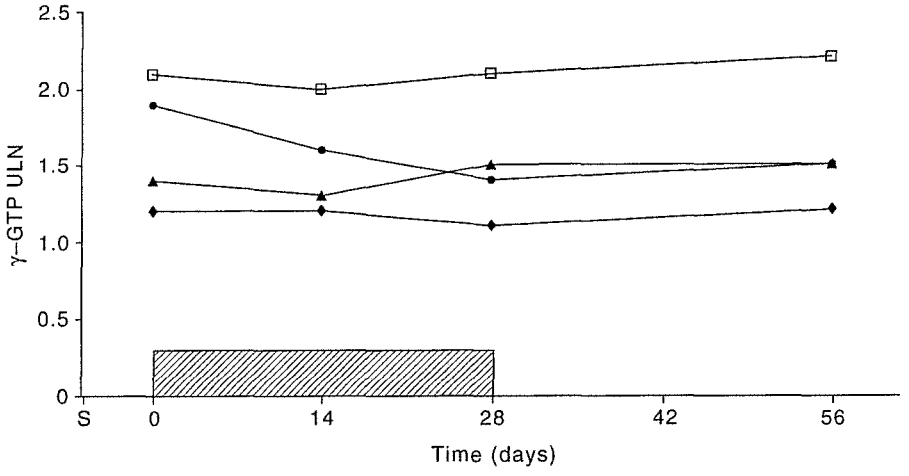

Figure 5 Median gamma-glutamyltranspeptidase × upper limit of normal (ULN) according to dosage group. (●) Placebo, (▲) 80 mg glycyrrhizin, (□) 160 mg glycyrrhizin, (◆) 240 mg glycyrrhizin, (▨) Stronger Neo-Minophagen C (SNMC), t.i.w, i.v.

Table 2 Median HCV-RNA levels before and at the end of treatment and mean HCV-RNA decline per group

HCV-RNA (10^6 geq/mL)	Glycyrrhizin treatment (mg) 0	80	160	240
Before treatment	4.5 (1.4–39.2)	21.7 (0.2–104)	11.1 (1.4–66.9)	14.4 (1.1–51.4)
At the end of treatment	6.9 (1.4–38.3)	17.8 (0.5–73.2)	11.5 (1.9–61.6)	8.9 (4.4–43.8)
Mean decline (95% CI)	−0.3 (1.7–1.1)	−3.6 (−10.7–3.5)	−1.6 (−7.2–4.0)	−7.3 (−17.2–2.7)

HCV, hepatitis C virus; geq, genome equivalents. Figures in parentheses represent the range of data.

Table 3 Number of patients who mentioned an adverse event spontaneously or after the question: 'Have you had any (other) medical problem since your last visit?'

Adverse event/Group	Glycyrrhizin treatment (mg) 0 ($n=14$)	80 ($n=13$)	160 ($n=14$)	240 ($n=15$)	Total	P*
Headache	5	2	1	5	13	0.21
Having a cold/'flu-like' symptoms	0	1	6†	3	10	0.01
Increased fatigue since treatment	2	2	3	1	8	0.73
Pain/haematoma at puncture site	1	1	3	1	6	0.67
Strange feeling during administration	2	2	0	1	5	0.48
Diarrhoea	0	1	1	3	5	0.35
Stitches in region of liver	1	0	1	1	3	1.00
Rash/itching	0	0	1	2	3	0.61
Dizziness	1	1	0	1	3	0.89
Nausea	0	1	1	1	3	0.89
Total with at least one side effect	10	10	13	10	43	0.63

Fifty-six patients were analysed. * Overall P value determined by using Fisher's exact test. † Significantly higher than placebo ($P=0.016$ by Fisher's exact test).

DISCUSSION

This European phase I/II study confirms the effect of glycyrrhizin in lowering serum ALT, as already observed in Japan. The efficacy of glycyrrhizin in normalizing serum ALT in the present study was less than that reported in Japan. In the present study, 10% (four of 41) of the patients treated with glycyrrhizin achieved ALT levels within the normal range at the end of treatment, while in Japan 36% of patients exhibited this important outcome measure.[4] It is possible that Asian people benefit more from glycyrrhizin treatment due to genetic polymorphisms in drug metabolism. Another possible explanation might be the difference in treatment schedules. In Japan, daily administration of glycyrrhizin is standard practice. In Europe, however, it is unusual to treat patients daily with intravenous medication for which they have to visit the hospital. Therefore, in the present study glycyrrhizin was administered three times per week. We expected at least a 15% dropout rate because of intolerance. However, we did not have any dropouts due to intravenous drug administration. In view of our observation that the thrice weekly schedule was well tolerated, it seems logical to conduct an additional study in which European patients are treated according to the Japanese schedule to find out whether the different treatment schedule was the cause of the difference in efficacy.

Treatment with SNMC rapidly induces a significant decrease in ALT. Within the four groups there was no significant difference between ALT at screening and ALT on day 0, but within 2 days of the first dose of glycyrrhizin, ALT became significantly lower than baseline ($P<0.02$ signed-rank test), while no such effect was seen in the placebo group. We may, therefore, conclude that the observed ALT decrease during active treatment is not caused by a natural fluctuation of ALT in chronic hepatitis C patients. After cessation of therapy the ALT-decreasing effect of glycyrrhizin disappeared.

The mechanism by which glycyrrhizin decreases the transaminases is not known. One of the proposed mechanisms is that glycyrrhizin induces an ALT decrease by stabilizing the cell membrane of the hepatocyte.[13]

In the present study, glycyrrhizin was given as SNMC, which contains glycyrrhizin and a 10-fold higher concentration of glycine. Both molecules are said to be hepatoprotective *in vitro*.[13,14] The placebo used in this trial consisted of all the ingredients of SNMC except for glycyrrhizin. Because serum ALT did not drop in the placebo group, whereas it was decreased significantly in the three SNMC groups, we can conclude that the ALT-decreasing effect of SNMC in this study is caused by glycyrrhizin and not by glycine.

None of the 57 randomized patients left the study because of drug intolerance. Pseudoaldosteronism with sodium retention, hypokalemia and hypertension, is a

well-known side-effect of glycyrrhizin.[12] This phenomenon is caused by the metabolite glycyrrhetinic acid, which inhibits the conversion of cortisol to cortisone by the enzyme 11β-hydroxy steroid dehydrogenase in the kidney. This inhibition leads to increased cortisol levels in the kidney. Because cortisol and aldosterone bind with the same affinity to the mineralocorticoid receptor, a rise in renal cortisol will result in a hypermineralocorticoid effect.[15] In the current study we did not observe sodium retention, hypokalemia, hypertension or an increase in bodyweight. The addition of glycine possibly prevented the occurrence of these side-effects, as reported by Suzuki *et al.*[8] Alternatively, the administered dose of glycyrrhizin, a maximum of 720 mg glycyrrhizin/week (= 130 mg/day), is too low to cause side-effects. Side-effects are more likely to occur when the daily dose of glycyrrhizin is several-fold more than 130 mg.[16]

Unexpected side-effects, such as headache, increased fatigue since treatment, a strange feeling during administration and pain or haematoma at the puncture site, were encountered as often in the placebo group as in the glycyrrhizin groups; so these symptoms are, in all likelihood, not caused by glycyrrhizin itself but by the total treatment (e.g., visiting the hospital three times a week; receiving intravenous medication). Therefore, treatment with SNMC up to 240 mg, thrice weekly for 4 weeks, appears to be safe and well tolerated.

In conclusion, we confirmed that a minority of the European patients normalized ALT during glycyrrhizin therapy.

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Glycyrrhizin-Induced Reduction of ALT in European Patients With Chronic Hepatitis C

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OBJECTIVE: In Japan, ALT normalization induced by long-term *i.v.* glycyrrhizin treatment reportedly reduces the progression of liver disease to hepatocellular carcinoma in chronic hepatitis C patients. The aim of this study was to evaluate the short-term (4-wk) feasibility and efficacy on serum ALT of three or six times per week *i.v.* glycyrrhizin therapy in European patients.

METHODS: Patients with chronic hepatitis C, nonresponders, or unlikely to respond (genotype 1/cirrhosis) to interferon therapy were included in this study. Medication was administered *i.v.* three or six times per week for 4 wk; follow-up also lasted 4 wk.

RESULTS: Sixty-nine out of 72 treatment courses were completed according to protocol. There were no significant changes in ALT levels within the placebo group (n = 13). The mean percentage ALT decrease from baseline at the end of treatment was 26% and 47% for the three times per week and six times per week treatment group, respectively (both $p < 0.001$ *vs* placebo). At the end of active treatment, 10% (four of 41) and 20% (three of 15) of the patients reached normal ALT levels for the three times per week and six times per week treatment group, respectively. The ALT lowering effect disappeared after cessation of treatment. No major side effects were observed.

CONCLUSION: It appeared feasible to treat European outpatients with chronic hepatitis C three or six times per week with *i.v.* glycyrrhizin. Glycyrrhizin treatment induces a significant ALT decrease in patients with chronic hepatitis C. Six times per week treatment appears more effective than three times per week. (Am J Gastroenterol 2001;96: 2432–2437. © 2001 by Am. Coll. of Gastroenterology)

INTRODUCTION

Treatment of chronic hepatitis C infection with α-interferon and ribavirin combination therapy results in a sustained virological response in <50% of the treated patients (1–3). For those who do not respond with viral clearance, different treatment strategies have to be sought. In long-term studies on chronic hepatitis B and C, persistent normalization of ALT appears to be a key prognostic factor for reduction of long-term complications, regardless of the presence of viral markers in the serum (4–8).

In Japan, glycyrrhizin—extracted from the roots of the plant *Glycyrrhiza glabra* (liquorice)—has been used for the treatment of chronic hepatitis for >20 yr (9). Glycyrrhizin is a conjugate of a molecule of glycyrrhetinic acid and two molecules of glucuronic acid.

In 1997, Arase *et al.* reported that in Japanese patients with chronic hepatitis C, long-term treatment (median 10.1 yr) with two to seven times per week *i.v.* glycyrrhizin prevented the development of hepatocellular carcinoma when ALT normalized (5). Ito reported that glycyrrhizin treatment does not influence the viral load (10).

At present, nearly 100 million of ampules of Stronger Neo-Minophagen C (SNMC)—each containing 40 mg of glycyrrhizin—are used each year in Japan for the treatment of chronic hepatitis. It is remarkable that a drug, which is used in Japan and other Asian countries on such a large scale, is virtually unknown in Europe. In Europe, it is unusual to treat patients with daily *i.v.* medication in the outpatient clinic. Therefore, the feasibility and the efficacy of glycyrrhizin treatment in European patients should be evaluated. We performed a double-blind, randomized, placebo-controlled study in which medication was administered three times per week (11). In this study, only 10% of our patients treated with glycyrrhizin reached a normal ALT value at the end of treatment, whereas in Japan, 36% of the patients reached ALT normalization albeit with daily administration (5). To evaluate whether we could increase the efficacy and whether it is feasible in Europe to administer six times per week *i.v.* medication, we extended the study with a second part in which glycyrrhizin was given six times per week. Here we report the combined results of the two treatment modalities for *i.v.* glycyrrhizin in an European setting.

PATIENTS AND METHODS

Patients

Patients between 18 and 70 yr of age were eligible if they met all inclusion criteria: serum antibodies against hepatitis C virus (HCV), HCV RNA positive; serum ALT at least 1.5 times the upper limit of normal (ULN) in the 8 wk before

treatment and at start of treatment; liver biopsy consistent with mild-to-moderate fibrosis or cirrhosis with mild-to-moderately active hepatitis; relapse after, nonresponse to, or unlikely to respond to interferon-based therapy (cirrhosis, genotype 1); or contraindication for or refusal to take interferon-based therapy. Patients were not eligible if they met one or more exclusion criteria: other causes of liver disease (chronic hepatitis B, alcohol abuse, autoimmune hepatitis, hemochromatosis, Wilson's disease, α-1-antitrypsin deficiency); decompensated cirrhosis, hepatocellular carcinoma; significant cardiovascular or pulmonary dysfunction in the past 6 months; malignancy in the previous 5 yr; HIV infection; immunosuppressive therapy; antiviral treatment in the preceding 3 months; pregnancy, breast-feeding; hypokalemia and liquorice addiction.

The studies were conducted according to the Declaration of Helsinki and Good Clinical Practice. The Medical Ethical Committee of the Erasmus Medical Center Rotterdam approved both protocols, and all patients gave their written informed consent.

Study Design

Glycyrrhizin was given as Stronger Neo-Minophagen C (SNMC, Minophagen Pharmaceutical, Tokyo, Japan), a clear solution for *i.v.* use, containing 2 mg of glycyrrhizin, 1 mg of cysteine, and 20 mg of glycine per ml in saline.

Part I (double-blind, randomized, placebo-controlled trial): placebo (SNMC without glycyrrhizin) or glycyrrhizin (80, 160, or 240 mg) was given *i.v.* three times per week. Medication was diluted with 100 ml of glucose 5% and administered by drip infusion in 15–20 min.

Part II (open study): 200 mg of glycyrrhizin (100 ml of SNMC) was administered six times per week. Undiluted medication was directly injected into a peripheral vein via a butterfly needle (Neofly 21G, Ohmeda, Japan) in 3–5 min. Eight of the 15 patients in this study received placebo, and five of the 15 received active treatment in part I; the time between the two treatments was at least 6 months.

Treatment duration for both parts was 4 wk; follow-up thereafter was also 4 wk. Routine hematological and biochemical assessments were performed weekly during the treatment period and every 2 wk during follow-up. Virological assessments were performed on day 0 and day 28 (pretreatment and at the end of treatment). Side effects were checked every visit by asking: "Have you had any (other) medical problem since your last visit?." Patients were treated as outpatients. Because administration took place in a hospital, patient compliance was directly linked with scheduled visits.

Virological Assessments

Anti-HCV was determined by third-generation ELISA (IMX, Abbott, Chicago, IL). Ethylenediaminetetra-acetic acid blood samples were centrifuged within 2 h, plasma was stored at −70°C. HCV-RNA was determined qualitatively by a modified version of the Amplicor HCV assay (Roche Molecular Systems, Alameda, CA); the extracted RNA was resuspended in 150 μl instead of 1 ml. HCV-RNA was quantified by means of the branched DNA assay (Quantiplex 2.0, Chiron, Emeryville, CA), which has a lower detection limit of 200,000 HCV genome equivalents per ml.

For genotyping, the polymerase chain reaction product generated with the Amplicor HCV assay was used. Sequence analysis was performed on an automated sequencer (Perkin Elmer, Nieuwerkerk, The Netherlands). The genotype was determined according to the rules described by the International HCV Collaborative Study Group (12).

Assessments of Outcome

The primary (biochemical) response parameters were ALT normalization at the end of treatment and the mean percentage decrease of ALT from baseline at the end of treatment. The secondary response parameter was the virological response, defined as >1 ^{10}log decrease in plasma HCV-RNA. Tolerability and side effects were also monitored.

Statistical Analysis

Statistical analysis was performed by using Stata 5.0 software (Stata Corporation, College Station, TX). The baseline ALT was calculated as the mean of two values: ALT level at screening (in the 8 wk before start of the study) and ALT level on day 0. A linear relationship between the mean decrease in ALT and treatment dose was assumed in the power analysis of the randomized part of the study. This analysis, based on a two-tailed comparison with an α of 0.05 and a power of 0.80, showed that to detect a correlation of $r \geq 0.40$, at least 12 patients per dosage group were required.

Changes within the group were assessed with the Wilcoxon signed-rank test. Comparisons between groups were evaluated with the Mann-Whitney *U* test. Fisher's exact test was applied to compare percentages; $p = 0.05$ (two-sided) was considered the limit of significance.

RESULTS

Characteristics of Patients

In total, 59 patients were treated in our studies. Thirteen of the 15 patients included in part II participated also in part I: eight received placebo, and five received active treatment. Two patients were found not to have met the inclusion criteria; these two patients were included in the safety and tolerability analysis. One patient discontinued after one administration because of social circumstances and was excluded from analysis. Figure 1 shows the flow of patients.

As no significant differences regarding ALT normalization and ALT decrease were found between the three groups receiving active treatment three times per week (80 mg [n = 13], 160 mg [n = 14], or 240 mg [n = 14]), these 41 actively treated patients were combined into one group (the three times per week frequency group).

The baseline characteristics of the patients are shown in

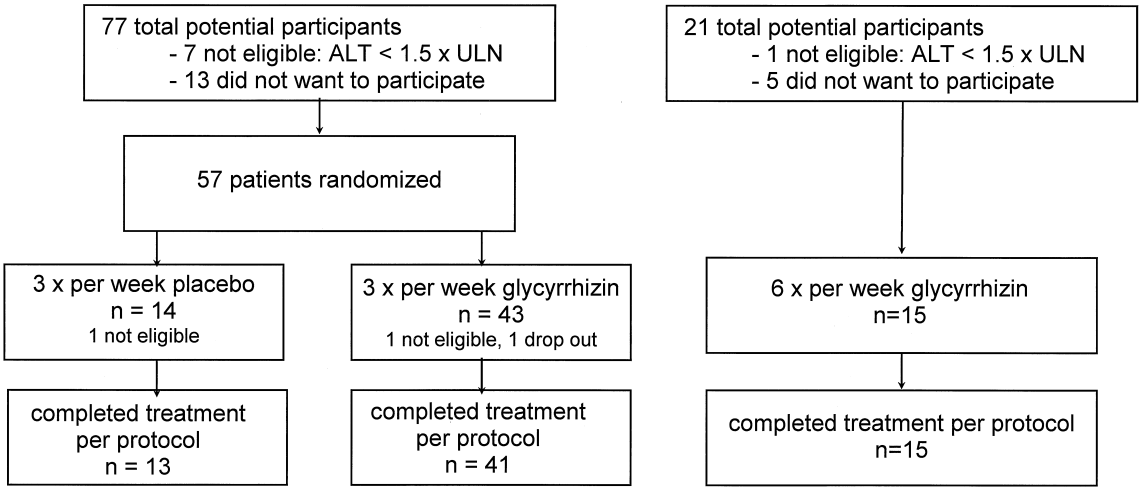

Figure 1. Patient flow chart. *(Left)* Part I: double-blind, randomized, placebo-controlled trial. *(Right)* Part II: additional open study.

Table 1. About 80% of the patients were men; >80% of the patients were interferon or interferon/ribavirin nonresponders. ALT levels were 2.5–3 times the ULN (31 IU/L for women and 41 IU/L for men). The median HCV-RNA levels were around 10^7 genome equivalents per milliliter.

Biochemical Response

For none of the groups, there was a significant difference between ALT at screening and ALT at day 0.

None of the patients (zero of 13) receiving placebo showed ALT normalization at the end of treatment. For 10% of the patients (four of 41) after three times per week and for 20% (three of 15) of the patients after six times per week, glycyrrhizin treatment reached normal ALT levels at the end of treatment. ALT normalization at the end of treatment was not significantly different between the three study groups ($p_{trend} = 0.12$). Figure 2 shows the distribution of ALT at the end of treatment.

No significant changes were observed during study period in the placebo group. After 1 wk of treatment, the mean percentage ALT decrease from baseline was 21% and 41% and at the end of treatment, it was 26% and 47%, for the three times per week and six times per week treatment group, respectively (Fig. 3). The mean percentage ALT decrease during active treatment (three times and six times per week) was significantly greater compared with placebo at all time points ($p < 0.001$). Also, the differences between both active treatment groups were significant ($p < 0.002$). After cessation of active treatment, ALT levels increased again; there was no significant difference between ALT at the end of follow-up and ALT at baseline for any of the groups.

Virological Response

The HCV-RNA levels at baseline were high: range 0.2–104×10^6 genome equivalents per ml. HCV-RNA levels at the end of treatment did not differ significantly from those at baseline. None of the patients cleared HCV-RNA.

Safety and Tolerability

Expected side effects of *i.v.* glycyrrhizin treatment are hematoma at the injection place and pseudoaldosteronism with hypokalemia, sodium retention, elevated blood pressure, and retention of bodily fluids (13, 14). Only within the six times per week treatment group, minor reversible symptoms of pseudoaldosteronism occurred. The mean serum potassium decreased from 4.2 ± 0.3 before treatment to 4.0 ± 0.3 at the end of treatment ($p = 0.05$), and the mean systolic blood pressure rose from 132 ± 15 to 142 ± 14 ($p = 0.01$). There was no significant difference between cirrhotic and

Table 1. Baseline Characteristics of the 69 Patients Evaluable for Efficacy per Group

Treatment Group	Placebo	Three Times per Wk	Six Times per Wk
Number of patients	13	41	15
Male/female	13/0	32/9	12/3
White/other	8/5	23/18	11/4
Median age* (yr) (range)	47 (37–60)	46 (32 – 69)	49 (39 – 70)
Noncirrhosis/cirrhosis	7/6	24/17	7/8
Previous interferon (ribavirin) yes/no	12/1	32/9	13/2
Median ALT ULN† (range)	3.1 (1.5–6.8)	2.6 (1.4–11.8)	3.0 (1.6–12.5)
Median HCV-RNA Mgeneq†/ml (range)	4.5 (1.4–39.2)	14.9 (0.2–104)	14.1 (0.7–76.3)
Genotype-1/genotype non-1	7/6	20/21	7/8

* At start of treatment.
† Mega genome equivalent.

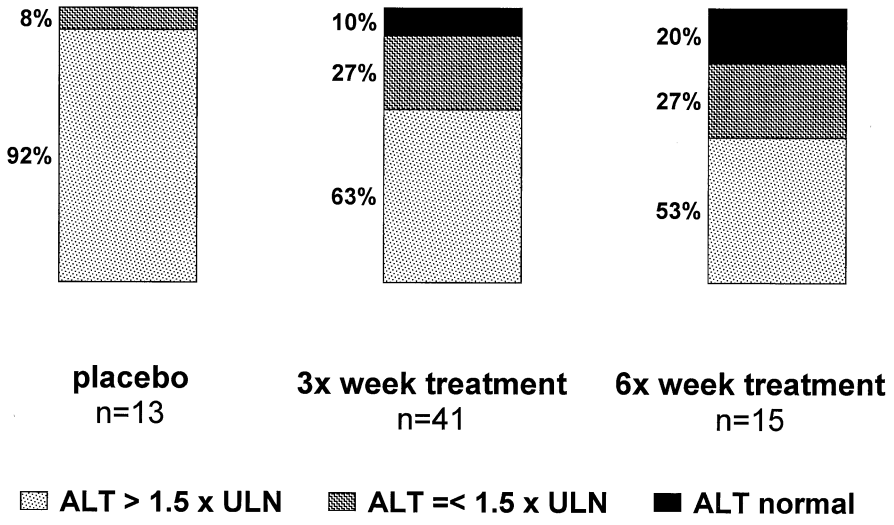

Figure 2. Distribution of ALT at the end of treatment. ALT is expressed as the ULN (men, 41 IU/L, < women, 31 IU/L). Patients were treated with three times per week *i.v.* placebo or three or six times per week with *i.v.* glycyrrhizin for 4 wk. Before treatment, all patients had ALT levels at least 1.5 × ULN.

noncirrhotic patients with regard to decrease of potassium or rise of systolic blood pressure during glycyrrhizin treatment. Ten patients suffered hematoma at the injection place. Headache, common cold, more tired than before treatment, and strange feeling during administration of the medication were unexpected adverse events mentioned spontaneously by more than five patients (Table 2). There were no clinically significant differences between the three frequency groups.

DISCUSSION

For most herbal remedies, there is a need for well-conducted clinical trials (Complementary and Alternative Medicine in Chronic Liver Disease, NIH, Bethesda, MD, August, 1999). In Japan, glycyrrhizin has been in use for the treatment of chronic hepatitis for >20 yr (9); it is now used extensively for chronic hepatitis C, in particular for patients without a response to α-interferon. We performed this clinical study, according to Good Clinical Practice, to evaluate the short-term efficacy of glycyrrhizin treatment on serum ALT levels and the feasibility of three to six times per week *i.v.* glycyrrhizin administration in a European setting.

No patient left the study because of treatment intolerance. Clinically significant adverse events did not occur more often in the six times per week group compared with three times per week or placebo; thus, short-term treatment with three to six times per week *i.v.* glycyrrhizin appeared fea-

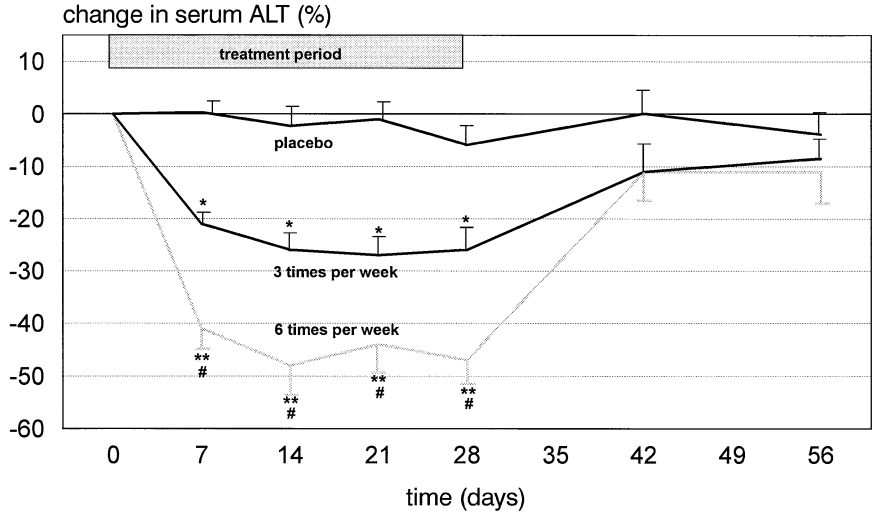

Figure 3. The mean percentages ALT change from baseline. Vertical bars indicate the SE of the means. After active treatment, the mean percentage ALT decrease was significantly lower compared with placebo (*$p < 0.001$, **$p < 0.0001$). The mean percentage ALT decrease at the end of treatment from baseline was 26% and 47% for three times and six times per week treatment, respectively. Six times per week treatment was significantly better than three times per week (#$p < 0.002$).

Table 2. Number of Patients Who Mentioned an Adverse Event Spontaneously or After the Question: "Have You Had Any (Other) Medical Problem Since Your Last Visit?"

Adverse Event/Frequency Group	Zero Times per Wk (n = 14)	Three Times per Wk (n = 42)	Six Times per Wk (n = 15)	Total	*p**
Headache	5	8	0†	13	0.03
Common cold	0	10	3	13	0.1
Hematoma at injection place	1	5	4	10	0.4
Strange feeling during administration	2	3	5‡	10	0.04
More tired than before treatment	2	6	0	8	0.3

* Overall *p* value (Fisher's exact test).
† Significantly lower than placebo ($p = 0.01$).
‡ Significantly higher than three times per week group ($p = 0.03$).

sible and safe for patients with chronic hepatitis C including cirrhosis. Most patients were interferon nonresponders, so they were well aware of the difference in patient burden associated with the administration of drug. Still, a fair number of patients asked for prolongation of glycyrrhizin therapy instead of maintenance therapy with interferon because they felt better during glycyrrhizin therapy. Glycyrrhizin may improve symptoms in chronic hepatitis C; therefore, the quality of life during glycyrrhizin treatment should be assessed objectively in future long-term studies.

The proportion of patients with ALT normalization at the end of treatment was higher in actively treated patients than in placebo, and higher in six times per week than three times per week glycyrrhizin administration; however, the differences failed to reach statistical significance. Because of the relatively small numbers of patients studied, real differences cannot be excluded. Comparing our data with the results reported by Arase *et al.* (5), no significant difference was found between our data on six times per week *versus* their data (20% [three of 15] *vs* 36% [30 of 84], $p = 0.4$). However, the outcome differed significantly for our data on three times per week *versus* their data (10% [four of 41] *vs* 36% [30 of 84], $p = 0.002$). This comparison further supports the view that six times per week glycyrrhizin administration is more effective than three times per week or placebo.

The mean percentage ALT decrease at the end of treatment after six times per week treatment was significantly greater than after three times per week treatment (47% [95% CI = 38–57] *vs* 26% [95% CI = 18–34], $p = 0.002$). Within the three times per week treatment group, the mean percentage ALT decrease did not show a linear dose-response relationship, the mean percentage ALT was 23% (95% CI = 2–43), 26% (23–38), and 29% (21–38) after 80, 160, and 240 mg of glycyrrhizin, respectively. This suggests that not a total week dosage of 1200 mg of glycyrrhizin, but six times per week administration are necessary to reach a mean percentage ALT decrease of 47%. This hypothesis is corroborated by the half-life of glycyrrhizin, which is 8 h in patients with chronic hepatitis C (15). Administration of the medication every 24 h results in a continuously detectable level of glycyrrhizin, whereas with three times per week glycyrrhizin administration, glycyrrhizin is not detectable after 48 h (15).

Is treatment with glycyrrhizin only beneficial when ALT normalization occurs? Tarao *et al.* (16) recently reported that persistently high ALT levels (>80 IU/L) in patients with chronic hepatitis C were associated with a more rapid development of hepatocellular carcinoma than persistently low ALT levels (<80 IU/L). They followed 69 consecutive HCV patients with cirrhosis for >5 yr; the 5-yr rate incidence of hepatocellular carcinoma was 53.6% *versus* 7.1%, for the high ALT and low ALT group, respectively (16). Therefore, there is some evidence that not only patients with ALT normalization but also patients with an ALT decrease to <80 IU/L might benefit from long-term glycyrrhizin treatment.

For the subset of patients showing significant biochemical improvement during 4 wk of therapy, long-term treatment with glycyrrhizin might retard the progression of liver disease. A prospective 6-month treatment trial in Europe should be performed to study feasibility, tolerance, quality of life, and histological improvement in these patients with an ALT response. If this 6-months study shows tolerance and histological improvement, it appears justified to explore further glycyrrhizin as a treatment for chronic hepatitis C.

We observed that the decrease of ALT, induced by glycyrrhizin treatment, occurs within the first 2 wk of treatment, and thereafter ALT levels remain stable. This is in agreement with the finding of Tsubota *et al.* that ALT levels did not significantly change during three times per week 200-mg glycyrrhizin therapy from 4 to 24 wk (17). Based on our current results and experience, we suggest starting with an induction period in which glycyrrhizin is given six times per week for at least 2 wk followed by a maintenance regimen with three times per week glycyrrhizin administration.

Recently, an oral form of glycyrrhizin has been developed; if its efficacy is similar to that of the intravenous preparation, oral maintenance therapy would be a more convenient option for long-term treatment.

The mechanism by which glycyrrhizin reduces the progression of liver disease without clearing the virus is unknown. A few *in vitro* and animal (rat) studies suggest that glycyrrhizin or its metabolite glycyrrhetinic acid inhibits lipid peroxidation, thereby protecting the hepatocytes (18–21). Shiota *et al.* recently reported the first animal model in which they described that treatment with glycyrrhizin sig-

nificantly reduced the occurrence of hepatocellular carcinoma in diethylnitrosamine-treated mice (22). The mechanism in this mouse model is still unclear, but this model might help to unravel a possible mechanism of action.

In conclusion, it is feasible to treat European patients with three to six times per week *i.v.* glycyrrhizin for 4 wk. Glycyrrhizin treatment induces a significant ALT decrease in patients with chronic hepatitis C. Six times per week treatment is more effective than three times per week treatment. To justify further exploration of long-term treatment with glycyrrhizin for chronic hepatitis C, a 6–12 month treatment study in which the benefit of treatment on liver histology is evaluated should be performed.

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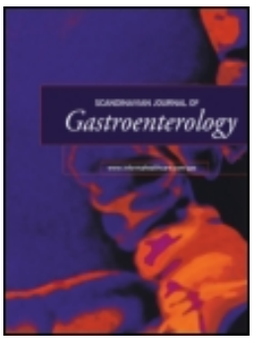

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Long-term clinical outcome and effect of glycyrrhizin in 1093 chronic hepatitis C patients with non-response or relapse to interferon

Bart J. Veldt, Bettina E. Hansen, Kenji Ikeda, Elke Verhey, Hiroshi Suzuki & Solko W. Schalm

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ORIGINAL ARTICLE

Long-term clinical outcome and effect of glycyrrhizin in 1093 chronic hepatitis C patients with non-response or relapse to interferon

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Abstract

Objective. Patients with chronic hepatitis C who do not respond to interferon can be treated with glycyrrhizin to reduce disease activity. The objective of this study was to evaluate the effect of glycyrrhizin on the incidence of hepatocellular carcinoma (HCC) during long-term follow-up after non-response to interferon. ***Material and methods.*** We analyzed individual patient data of all consecutive patients treated with interferon in 12 major Japanese hospitals between 1990 and 1995 who showed no sustained response. ***Results.*** The study comprised 1093 patients. During a mean follow-up of 6.1 ± 1.8 years, 107 patients developed HCC. The Cox regression analysis with time-dependent variables showed that older age, male gender, higher alanine aminotransferase (ALAT) and higher fibrosis stage were significantly associated with a higher risk of developing HCC. Response to glycyrrhizin, defined as ALAT $< 1.5 \times$ upper limit of normal, was significantly associated with a decreased incidence of HCC: hazard ratio 0.39 (95% CI 0.21–0.72; $p < 0.01$). G-estimation, used to correct for ALAT as the confounder, showed no significant benefit of glycyrrhizin in the overall study population. ***Conclusions.*** This study provides some evidence to show that interferon non-responder patients with chronic hepatitis C and fibrosis stage 3 or 4 may have a reduced incidence of HCC if glycyrrhizin therapy leads to normalization of ALAT levels.

Key Words: *Chronic hepatitis C, hepatocellular carcinoma, G-estimation, interferon, non-responder, SNMC*

Introduction

Chronic hepatitis C is a major cause of liver disease world-wide. Infection with the hepatitis C virus may lead to chronic inflammation of the liver, which is manifested in elevated liver enzymes such as alanine aminotransferase (ALAT). This chronic inflammation may lead to fibrosis and subsequent cirrhosis. It has been estimated that the delay for developing cirrhosis is about 30 years, but the individual prognosis may vary substantially depending on factors such as age at infection, gender, alcohol abuse and co-infection with hepatitis B or the human immunodeficiency virus (HIV) [1].

Over the past 15 years, treatment regimens based on the administration of interferon have proven to be increasingly effective against hepatitis C. Combination treatment with pegylated interferon and ribavirin will lead to disappearance of the virus from the blood in 50% to 80% of patients [2,3]. If the virus remains undetectable in the blood at 6 months after the end of treatment, we speak of a sustained virological response. Sustained virological response is almost always associated with normalization of serum ALAT and a survival similar to that for the overall population [4]. There still remains a considerable proportion of patients who do not achieve a sustained virological response. These patients require other therapeutic approaches, and various long-term interferon-based regimens are being investigated [5,6].

In Japan, glycyrrhizin has been propagated as an anti-inflammatory drug, capable of minimizing disease activity in the chronically infected liver. Glycyrrhizin is extracted from the roots of the plant

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Glycyrrhiza glabra (liquorice) and is a conjugate of a molecule of glycyrrhetinic acid and two molecules of glucuronic acid. Although the mechanism of action remains to be elucidated, it has been suggested that glycyrrhizin acts in a cytoprotective manner by its ability to inhibit tumor necrosis factor (TNF) alpha-mediated apoptosis and/or via inhibition of anti-Fas antibody-induced hepatitis [7–9]. Glycyrrhizin is hydrolyzed to a pharmacologically active metabolite, glycyrrhetic acid, which inhibits 11-β-hydroxysteroid dehydrogenase and other enzymes involved in the metabolism of corticosteroids. Although this may lead to increased cortisol levels in the kidneys and other mineralocorticoid-selective tissues, van Rossum et al. showed that patients with chronic hepatitis or compensated cirrhosis only show minor reversible symptoms of pseudo-aldosteronism after treatment with 1200 mg glycyrrhizin weekly for 4 weeks [10].

Placebo-controlled trials have proven that the administration of glycyrrhizin leads to a significant reduction of ALAT levels in chronic hepatitis C patients [11]. The question remains whether this reduction in ALAT levels leads to a reduced risk of liver-related morbidity and mortality. Ideally, a randomized, controlled trial with a prolonged follow-up of at least several years should be designed, in order to investigate the effect of glycyrrhizin on these clinical end-points. However, even if such a study were restricted to cirrhotics, based on the incidence of hepatocellular carcinoma (HCC), decompensation and mortality [12], it would take at least 5 years before we had an answer to whether glycyrrhizin is a beneficial drug or not. Therefore we performed a large retrospective multicenter study, in which data collected in Japan were analyzed independently, Japan being the only country, so far, where hepatologists have extensively used this compound. We were especially careful to minimize the various biases associated with retrospective studies and to apply the most sophisticated statistics designed for such studies.

The aim of this study was to evaluate the effect of glycyrrhizin treatment on the incidence of HCC among patients with chronic hepatitis C who did not respond to interferon monotherapy.

Material and methods

Study design

Japanese academic hospitals and major general hospitals were invited to participate in this retrospective cohort study. Hospitals could participate if they had searchable databases with available data on previous treatment with interferon and on clinical outcomes.

All consecutive chronic hepatitis C patients who received interferon alpha treatment between 1 January 1990 and 31 December 1995 and who did not show a sustained virological response were included. Thus, the study population consisted of non-responders and relapsers to interferon monotherapy. Sustained virological response was defined as a normal ALAT level and negative HCV-RNA at the end of treatment and 6 months thereafter.

The ethics committees of all the participating centers approved the protocol. In order to safeguard the privacy of the patients, the treating physician replaced patients' names with a code before entry in the database.

Patient selection

Data on all consecutive patients with chronic hepatitis C with a non-response to previous interferon treatment were collected. Data were collected on separate case record forms, one per patient, by the local investigator. The case record forms were sent to the co-ordination center in Rotterdam, where the data were entered in a central database. Before the data were entered, they were checked and if there was any doubt, the local investigator was contacted.

Data recorded

Information was obtained on demographics (age, gender) and on details of the interferon treatment (starting date, duration, total dose) as well as the glycyrrhizin treatment (starting date, duration, total dose). Virological data (genotype, viremia), hematological (platelet count) and biochemical data (aminotransferase levels, bilirubin and gamma glutamyltransferase) were measured in the certified laboratories of the participating hospitals and added to the case record form by the local investigator. Centrally, the results were corrected for local normal values. Liver biopsies were scored by local pathologists using the METAVIR fibrosis score.

Follow-up data were recorded every four weeks if available and included ALAT levels, start of glycyrrhizin treatment and the occurrence of HCC. Patients were considered to have HCC if proven by biopsy or if ultrasound or computed tomography showed a focal lesion in the presence of a serum alpha-fetoprotein of >400.

Statistics

A data analysis plan was developed before closure of the database. The Kaplan-Meier method was used to estimate the occurrence of HCC over time, according to baseline ALAT levels. Entry into the study started at 24 weeks after interferon treatment.

The Cox regression analysis was applied to determine which factors were independently associated with development of HCC. The following baseline factors were considered: age, gender, fibrosis stage, ALAT levels, anti-HBc positivity, genotype, viral load and route of transmission. All variables were checked for interactions.

In order to analyze the hypothesis of an association between glycyrrhizin therapy and a reduced occurrence of HCC over time, glycyrrhizin therapy was considered as a time-dependent factor, since glycyrrhizin treatment was started at various follow-up times. This means that all patients enter at time 0 as untreated. At the time of glycyrrhizin treatment the patient is censored in the untreated group and the patient enters the glycyrrhizin group. In this way, the period that a patient lived as untreated is calculated as the "event-free survival period" in the Cox analysis. In order to avoid bias, cases were censored at the time of a second interferon-based treatment. According to the data analysis plan, a second analysis was done to assess the effect of glycyrrhizin according to response. Response to glycyrrhizin was defined as ALAT levels $< 1.5 \times$ upper limit of normal (ULN) at the first measurement 3 months after initiation of treatment.

Statistical analyses were performed using SPSS Windows version 11 (SPSS Inc., Chicago, Ill., USA). The findings showed a strong influence of fibrosis and ALAT elevation on the development of HCC. Therefore, an additional analysis was done in a more homogeneous group of patients with advanced fibrosis.

The outcome of simply adjusting for ALAT as a time-dependent covariate in a Cox model may be a biased estimate of the treatment effect, since higher ALAT levels were associated with a higher probability of developing HCC and also of starting glycyrrhizin therapy (Figure 1). In order to estimate the causal effect of time-dependent glycyrrhizin treatment in the presence of a time-dependent covariate, ALAT, we used the G-estimation described by Robins et al. [13]. This method is designed to gain an unbiased estimate of a treatment effect in the presence of a confounding variable, which is also intermediate. The G-estimation estimates the factor ψ. We use the exponent of $-\psi$, further referred to as E, as the factor by which the time towards development of HCC would be expanded (or contracted should E be smaller than 1.0) if the treatment with glycyrrhizin were not given (Appendix 1). This G-estimation was carried out with a macro written in SAS (SAS Institute Inc., Cary, N.C., USA).

Results

Descriptives

A total of 1093 chronic hepatitis C patients with non-response to previous interferon therapy were included in the study. Follow-up started at 6 months after the end of treatment. During a mean follow-up of 6.1 years (SD 1.8) 26,450 visits were recorded. The mean duration of follow-up was 6.3 years (SD 1.8) for patients who were treated with glycyrrhizin and 6.0 years (SD 1.8) for patients who were not. Fifty-eight percent of the patients were males and the median age at time of inclusion was 52 years (range 17–81 years). Forty percent of the patients had acquired hepatitis C through blood transfusions. Further patient characteristics are presented in Table I.

Four hundred and sixty-five patients received intravenous glycyrrhizin therapy, given as Stronger Neo Minophagen C (SNMC), which was started at

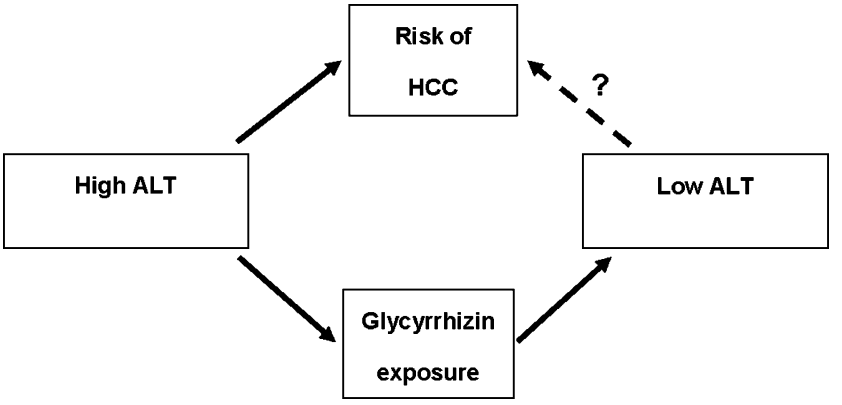

Figure 1. Elevated alanine aminotransferase (ALAT) levels during follow-up were associated with a higher probability of receiving glycyrrhizin, but also lead to a higher probability of developing hepatocellular carcinoma (HCC). As ALAT levels are lowered by glycyrrhizin treatment, ALAT is regarded as a time-dependent covariate which is both a confounder and an intermediate. In order to investigate whether glycyrrhizin reduces the risk of developing HCC by lowering ALAT levels (*dotted arrow*), sophisticated statistical analyses were required and a G-estimation was performed.

Table I. Descriptives.

	Overall	Glycyrrhizin	No glycyrrhizin	p-value* (Chi-square/Mann- Whitney)
Number	1093	465	628	
M/F (%)	628/455 (58/42)	262/198 (56/43)	366/257 (58/41)	0.67
Age, mean (range)	52.2 (17–81)	53.9 (29–80)	50.9 (17–81)	<0.01
Genotype				<0.01
1 (%)	750 (69)	338 (73)	412 (66)	
2 (%)	214 (20)	90 (19)	124 (20)	
3 (%)	9 (0.8)	6 (1.3)	3 (0.5)	
4 (%)	4 (0.4)	0 (0)	4 (0.6)	
Fibrosis stage				<0.01
1 (%)	451 (41)	117 (25)	334 (53)	
2 (%)	372 (34)	181 (39)	191 (30)	
3 (%)	203 (19)	135 (29)	68 (11)	
4 (%)	54 (5)	29 (6)	25 (4)	
ALAT at t = 0				<0.01
$<1 \times$ULN (%)	319 (29)	81 (17)	238 (38)	
$1-1.5 \times$ULN (%)	225 (21)	68 (15)	157 (25)	
$1.5-2 \times$ULN (%)	161 (15)	65 (14)	96 (15)	
$2-3 \times$ULN (%)	159 (15)	82 (18)	77 (12)	
$>3 \times$ULN(%)	222 (20)	167 (36)	55 (9)	

Abbreviations: ALAT = alanine aminotransferase; ULN = upper limit of normal.
*p-value of the difference between patients treated or not treated with glycyrrhizin.

various follow-up times; 164 of these patients had advanced fibrosis. The mean treatment duration with glycyrrhizin was 4.1 years (SD 2.6); 79% of the patients received treatment for 3 years or longer. The patients received a mean dose of 506 mg glycyrrhizin (191 ml SNMC) per week (range 106–1855 mg). Six patients stopped treatment because of side effects. Other treatments given to the interferon non-responders were interferon plus ribavirin ($n = 23$), ursodeoxycholic acid ($n = 310$ in the glycyrrhizin-treated group and $n = 347$ in the untreated group) and herbal medicines ($n = 48$ in the glycyrrhizin-treated group and $n = 46$ in the untreated group). The patients receiving interferon plus ribavirin were censored at the start of this treatment.

Events

One hundred and seven patients developed HCC. We performed a Kaplan-Meier analysis in order to investigate the influence of raised ALAT levels on the risk of developing HCC (Figure 2). In patients with normal ALAT levels during the first year of follow-up, the 5-year incidence of HCC was 3.1% (95% CI 0.8–5.5). The incidence of HCC increased to 4.9 (95% CI 2.0–7.8) for ALAT levels between 1 and $1.5 \times$ULN, 8.3% (95% CI 4.1–12.5) for ALAT levels between 1.5 and $2 \times$ULN and 8.3% (95% CI 4.2–12.3) for ALAT levels between 2 and $3 \times$ULN. The highest occurrence of HCC was seen in patients with ALAT levels three times above the ULN during the first year of follow-up: 16.6% (95% CI 9.3–24.0).

The time-dependent Cox regression analysis showed that older age, male gender, higher fibrosis stage and non-response to glycyrrhizin were significantly associated with a higher risk for developing HCC (Table II). The 5-year occurrence of HCC was 1.2% (95% CI 0.1–2.2) for patients with fibrosis stage 1 and 3.8% (95% CI 1.7–5.9) for patients with fibrosis stage 2. The occurrence of HCC was highest among patients with fibrosis stage 3 and 4: 13.7% (95% CI 8.6–18.8) and 26.6% (95% CI 13.9–39.4), respectively.

Subgroup analysis of patients with fibrosis stages 3 and 4 showed a trend towards less development of HCC among patients with a response to glycyrrhizin (hazard ratio = 0.50 (95% CI 0.22–1.12, $p = 0.09$).

Seventy-four percent (343/465) of the patients treated with glycyrrhizin had ALAT levels above $1.5 \times$ULN at the start of therapy and 66% (228/343) of these responded with decreased ALAT levels. In comparison, the rate of spontaneous ALAT normalization in patients with elevated ALAT levels at start of follow-up who were not treated with glycyrrhizin was 33% (114/344) at 3 months after inclusion in the study. In an analysis of all 465 treated patients, those with an ALAT response had a significant, lower risk of developing

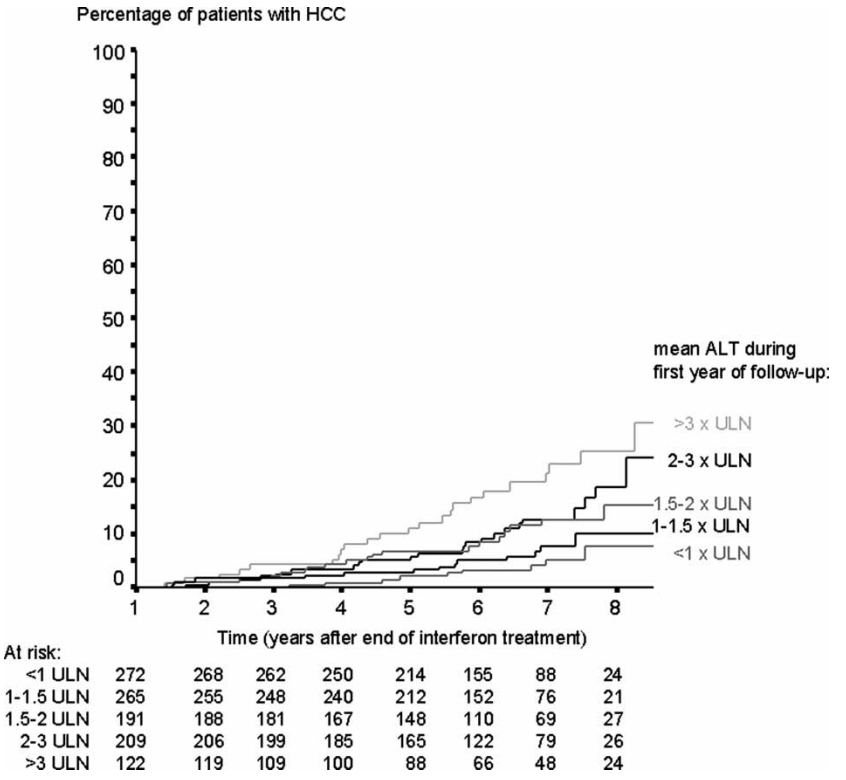

Figure 2. Kaplan-Meier curve showing the development of hepatocellular carcinoma (HCC) over time, according to mean alanine aminotransferase (ALAT) during the first year after interferon therapy. As the mean ALAT was calculated over the first year, the time-scale starts at one year of follow-up. Patients who did not fulfill one year of follow-up ($n=7$) and patients who developed HCC within the first year of follow-up ($n=27$) were excluded from this analysis.

HCC than non-responders; hazard ratio 0.39 (95% CI 0.21–0.72, $p<0.01$) (Table III). The Cox regression analysis for untreated patients (patients censored at start of glycyrrhizin therapy) showed that spontaneous normalization of ALAT levels at 4 months after start of follow-up, though two times less common than normalization after initiation of glycyrrhizin, also tended to be associated with a lower risk of developing HCC (hazard ratio 0.44 (95% CI 0.19–1.02, $p=0.06$).

G-estimation

The G-estimation performed for the overall study population, showed that the time towards development of HCC was not significantly influenced by glycyrrhizin treatment ($E=0.96$ (95% CI 0.76–2.10).

There was a trend towards a prolonged time to development of HCC among patients with fibrosis stage 3 or 4 if they received glycyrrhizin; $E=1.17$ (95% CI 0.65–2.29). Among patients with fibrosis stage 1 or 2, no beneficial effect of glycyrrhizin was seen during the observation period, but the number of events was too small to make a reliable estimate in this subgroup.

Discussion

The aim of this study was to evaluate the effect of glycyrrhizin treatment on the incidence of HCC among patients with chronic hepatitis C who did not respond to interferon monotherapy.

During follow-up 107 patients developed HCC. This is concordant with data published by Yoshida et al., who presented the rates of development of HCC by age, gender and fibrosis stage in their population of non-sustained responders. Applying these rates to our data set would lead to an expected number of 117 HCCs (95% CI 99–139) during 6.1 years of follow-up [14]. The incidence of HCC in our cohort is high, the 5-year incidence of HCC among patients with F1 and F2 fibrosis being 1.2% and 3.8%, respectively. However, we investigated a selected group of interferon non-responders and the incidence of HCC is much higher in Japan than in Europe. In our cohort the overall yearly incidence of HCC was 1.6%. Previous large cohort studies found a yearly incidence of 0.3% to 2.7% per year in Japanese non-sustained responders to interferon treatment [14–16]. In the literature, lower rates of HCC development are described for patients who relapsed after an initial response and for patients with persistently low ALAT levels [17]. Similarly, in

Table II. Time-dependent Cox regression analysis assessing risk factors for HCC ($n=1093$).

	Hazard ratio	95% CI	p-value
Gender			
Male	1		
Female	0.31	0.19–0.51	<0.01
Age	1.08	1.05–1.11	<0.01
ALAT levels at t = 0			
<1.5 × ULN	1		
>1.5 × ULN	1.58	0.92–2.70	0.10
Alcohol			
<50 g/day	1		
>50 g/day	1.15	0.64–2.04	0.65
Fibrosis stage			
Fibrosis stage 1	1		
Fibrosis stage 2	4.04	1.66–9.83	<0.01
Fibrosis stage 3	8.75	3.56–21.5	<0.01
Fibrosis stage 4	15.2	5.82–39.7	<0.01
Glycyrrhizin			
No glycyrrhizin	1		
Glycyrrhizin, no ALAT response	2.03	1.21–3.42	0.01
Glycyrrhizin, ALAT response	0.81	0.41–1.60	0.54

Abbreviations: HCC = hepatocellular carcinoma; ALAT = alanine aminotransferase; ULN = upper limit of normal.
The hazard ratios with their 95% CIs and p-values associated with these factors are given. Hazard ratio <1.0 indicates a decreased risk for HCC. Older age, male gender, higher fibrosis stage and non-response to glycyrrhizin treatment were significantly associated with a higher risk of developing HCC. Gender, ALAT, alcohol intake, fibrosis stage and glycyrrhizin treatment were entered as categorial values. A hazard ratio of 1 indicates the reference value. Age was entered as a continuous value.

Table III. Time-dependent Cox regression analysis assessing risk factors for HCC in patients who received glycyrrhizin treatment ($n=465$).

	Hazard ratio	95% CI	p-value
Gender			
Male	1		
Female	0.23	0.12–0.42	<0.01
Age	1.09	1.05–1.13	<0.01
ALAT levels at start of treatment			
<1.5 × ULN	1		
>1.5 × ULN	0.44	0.17–1.14	0.09
Fibrosis stage			
1	1		
2	2.41	0.89–6.50	0.08
3	3.35	1.26–8.92	0.02
4	7.95	2.71–23.3	<0.01
Response to glycyrrhizin			
No	1		
Yes	0.39	0.21–0.72	<0.01

Abbreviations: HCC = hepatocellular carcinoma; ULN = upper limit of normal; ALAT = alanine aminotransferase.
The hazard ratios with their 95% CIs and p-values associated with these factors are given. Hazard ratio <1.0 indicates a decreased risk for HCC. Older age, male gender and advanced fibrosis were significantly associated with a higher risk of developing HCC. Patients with an ALAT response to glycyrrhizin had a significantly decreased chance of developing HCC, compared with non-responders. Gender, ALAT, fibrosis stage and glycyrrhizin treatment were entered as categorial values. A hazard ratio of 1 indicates the reference value. Age was entered as a continuous value.

our cohort, patients with lower baseline ALAT levels had a smaller probability of developing HCC.

As chronic hepatitis C progresses slowly, it is difficult to evaluate the efficacy of treatment on clinical outcomes such as mortality and development of HCC in randomized controlled trials. Therefore, "best" information should be derived from cohort studies. However, cohort studies are only reliable if the drop-out rate is low compared to the events. In retrospective cohort studies the risk of introducing bias is even greater. Incomplete capture of early clinical events, confounding bias and compliance bias have been described as possible confounders in retrospective studies [18]. In large randomized trials this problem is usually avoided, as unmeasured confounders are likely to be equally divided over the groups by randomization.

We executed this retrospective cohort analysis with great care to preclude these biases. Incomplete capture of clinical events could not play a role in our analysis as the development of HCC was monitored during the whole follow-up period. Secondly, confounding bias may have played a role, as raised ALAT levels increased both the chance of receiving glycyrrhizin treatment and the risk of developing HCC. Sophisticated statistical analyses were used to correct for this confounder [13,19,20].

Finally, compliance bias may have played a role in this study, as patients who are willing to attend the hospital several times a week for intravenous injections of glycyrrhizin are possibly also more likely to adhere to other protective types of behavior. However, the fact that the follow-up of patients who did not receive glycyrrhizin was similar to those who did, suggests that they were equally compliant in their hospital visits.

A previous study on the effect of glycyrrhizin on clinical outcome showed a significant protective effect on development of HCC [21]. In our study we refined the methodology by using an intention-to-treat approach. All patients who received glycyrrhizin were included, even those who were treated for a short time. In this way we sought to avoid the exclusion of patients who stopped their glycyrrhizin early because they had died of HCC.

In the present study, there were significant differences in baseline demographics between treated and non-treated patients concerning genotype

distribution, mean age and fibrosis stage. Therefore, we first of all used a multivariate Cox regression analysis to assess the effect of glycyrrhizin. Overall, there was no significant effect, but in patients with fibrosis stages 3 and 4 there was a trend towards a protective effect on development of HCC. An intention-to-treat analysis of all patients treated with glycyrrhizin showed that patients responding by decreased ALAT levels had a significantly lower probability of developing HCC. A G-estimation was performed to address the problem of confounding by ALAT levels. The latter analysis failed to show an overall beneficial effect of glycyrrhizin, but in patients with fibrosis stage 3 or 4 at the start of follow-up, there was a trend towards a protective effect.

In conclusion, this study provides some evidence to suggest that interferon non-responder patients with chronic hepatitis C and fibrosis stage 3 or 4 may have a reduced incidence of HCC if glycyrrhizin therapy leads to normalization of ALAT levels.

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Appendix 1

The method of G-estimation by J. M. Robins offers a solution to estimate the causal effect of time-dependent glycyrrhizin treatment on the development of HCC, in the presence of a time-dependent covariate, ALAT, that is both a confounder and an intermediate variable.

G-estimation of the parameter of a nested structural model estimates the expansion or contraction parameter ψ of the time to event (HCC) due to the exposure to glycyrrhizin treatment. If, for instance, the exponent of $-\psi$ (referred to as E in the text) = 1.20, the time to HCC is expanded by 20%, corresponding with a beneficial effect.

Fundamental to this approach is the assumption of no unmeasured confounders. This means that all covariates influencing both the decision to use glycyrrhizin and the HCC-free survival time should be measured. This means that, given the covariates, the decision to start treatment is independent of the patient's (possibly counterfactual) HCC-free survival time under any treatment regimen.

A pooled logistic regression analysis over all visits was applied, with glycyrrhizin therapy at visit κ as outcome. This means that each subject contributed with multiple observations, one for each visit, until development of HCC or censoring. Covariates considered for inclusion in the model are baseline factors (age, gender, fibrosis stage, ALAT and gamma glutamyltransferase at the start of the study) and the covariate history before visit κ (ALAT, glycyrrhizin treatment and concomitant medication at the two visits prior to visit κ). Furthermore, the number of weeks since the prior visit and the number of weeks since the start of the study were included in the model.

The parameter ψ is G-estimated by extending the logistic model with sets of imaginary (counterfactual) HCC-free survival times, had glycyrrhizin treatment never been given. Weights have been calculated to adjust for patients who are lost to follow-up or who are censored at a second interferon-based treatment.

Data description and annotation

T_i = Observed failure time for subject i.
U_i = Time to failure (HCC) for subject i if never exposed to glycyrrhizin (= counterfactual failure time).
$\text{Glycyrrhizin}_i(t)$ = The treatment status of subject i at time-point t.

The model that relates the observed data T_i and $\text{glycyrrhizin}_i(Ti)$ to the counterfactual failure time U_i is assumed to be:

$$\text{Ui}\,(\psi) = \int_0^{T_i} \exp\,(\psi\ \text{Glycyrrhizin}_i(t))\ dt$$

The model of U as a function of ψ describes the relation between the counterfactual failure time, the observed failure time and the use of glycyrrhizin over time.

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甘草甜素对急性乙醇中毒大鼠脂质代谢的影响

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摘要 本文研究急性乙醇中毒的大鼠脂质代谢的改变及甘草甜素的保护作用。与对照组比较，给 5 周龄 SD 大鼠 50%乙醇按 5 g/kg 灌胃，可使血清总脂、胆固醇及甘油三酯分别增加 31%、50%和 42%；肝脂质过氧化产物(丙二醛)增加 184%；而还原型谷胱甘肽则下降 58%。预先给予甘草甜素，按 150 mg/kg 灌胃 7 天，则可使乙醇引起的上述变化基本恢复正常，提示甘草甜素对乙醇引起的大鼠脂质代谢改变有保护作用。

主题词 甘草/药效学；乙醇；脂质/代谢；丙二醛/代谢；谷胱甘肽/代谢

临床上高血压、动脉粥样硬化及高脂血症患者口服甘草甜素可改善血清脂质和肝功能，并可防止动脉粥样硬化的发展[1]。但甘草甜素对实验性脂质代谢异常的作用如何尚未见报道。本文研究甘草甜素对大剂量乙醇诱导大鼠脂质代谢异常的模型，进一步说明甘草甜素的药理作用。

1 材料与方法

1.1 药品 甘草甜素由杭州胡庆余堂制药厂提供。硫代巴比妥酸(TBA)为 Sigma 产品，其余试剂为国产分析纯试剂。

1.2 动物处理及分组 5 周龄雄性 SD 大鼠 30 只由本院动物室提供，体重为 80～100 g。动物分组如下：①甘草甜素＋乙醇组 甘草甜素剂量 150 mg/kg 每日灌胃一次共 7 天，禁食过夜，第 8 天以 50%乙醇按 5 g/kg 灌胃；②乙醇组 以等量生理盐水灌胃 7 天，禁食过夜，第 8 天给予乙醇，剂量同上；③对照组 以生理盐水灌胃 7 天，禁食过夜，第 8 天以 50%葡萄糖液按 5 g/kg 灌胃。维持 25℃，6 小时后处死动物，取血，留取肝脏。

1.3 实验材料制备 凝固大鼠血 3500 r/min 离心 15 min，制备血清，置－30℃冰箱备用。取肝组织，滤纸吸干，称重，剪碎，用生理盐水制备 25%肝匀浆。

1.4 生化分析 血脂分析按文献[2]方法进行。采用 TBA 比色法测定肝脂质过氧化产物(MDA)含量[3]。依文献[4]测定肝匀浆还原型谷胱甘肽(GSH)含量。

2 结 果

2.1 血清总脂、胆固醇及甘油三酯含量 结果见表 1。乙醇可使大鼠血清总脂、胆固醇及甘油三酯分别增高 31%、50%及 42%；而且甘草甜素则可使此改变基本恢复接近正常，其中以甘油三酯最为明显。

表 1 大鼠血清总脂、胆固醇及甘油三酯含量($\bar{X}\pm S$, $n=10$)

组别	总脂(g/L)	胆固醇(mmol/L)	甘油三酯(mmol/L)
对照组	3.58＋0.27	1.53＋0.21	1.05＋0.09
乙醇组①	5.22＋0.62**	3.07＋0.21**	1.79＋0.24**
甘草甜素＋乙醇组②	3.81＋0.35**	2.13＋0.23**	1.16＋0.21**

①与对照组比较 **$P<0.01$ ②与乙醇组比较 **$P<0.01$

2.2 肝匀浆脂质过氧化产物(MDA)及还原型谷胱甘肽(GSH)含量 结果见表 2。乙醇使脂质过氧化产物(MDA)增加 184%；而还原型谷胱甘肽(GSH)含量则减少 58%。甘

草甜素可使这些变化基本恢复正常（$P<0.01$）。

表 2　大鼠肝匀浆脂质过氧化物（MDA）及还原型谷胱甘肽（GSH）含量（$\bar{X}\pm S$，$n=10$）

组别	MDA (nmol/g)	GSH (μg/g)
对照组	4.4+1.8	132+50
乙醇组①	12.5+7.8**	56+23**
甘草甜素+乙醇组②	5.8+2.1**	113+36**

①与对照组比较　**$P<0.01$　②与乙醇组比较　**$P<0.01$

3　讨　论

在临床上过量饮酒是脑血管意外、心绞痛及心肌梗塞的诱因，而这些疾病与体内脂质代谢有密切关系。故对乙醇引起脂质代谢异常的研究具有重要意义。本文研究说明，一次性大量摄取乙醇即可使大鼠体内脂质代谢发生明显变化，即使血清总脂、胆固醇及甘油三酯较对照组增加 30%～50%，说明短期内大量摄取乙醇可致大鼠高脂血症，其机理可能与乙醇干扰脂质代谢即增加脂质吸收、合成、减少脂质氧化、排泄有关[5]。

观察乙醇对大鼠肝 GSH 和 MDA 含量的影响可发现，与对照组比较乙醇使 MDA 增加 184%；而 GSH 含量则降低 58%。大量乙醇进入体内可诱导脂质过氧化物产生，脂质过氧物的增加则可能是乙醇产生毒性效应如脂肪肝、酒精性肝炎、酒精性肝硬化的原因之一[6]。机体对乙醇的代谢和对脂质过氧化物的清除均要消耗 GSH 使体内 GSH 量减少。

甘草甜素可通过维持肝脏药物代谢 I 相酶含量和活性及微粒体膜流动性而具有保护四氯化碳和醋氨酚引起的肝损害（待发表资料）。在大鼠急性酒精中毒的模型中，预先给予甘草甜素可明显改善乙醇所致的高脂血症，也降低乙醇所致脂质过氧化物（MDA）的增加，使 GSH 水平恢复。提示甘草甜素可能通过对机体代谢发生改变而保护机体以防御外源性化合物的毒性，其机理有待于进一步研究。

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Effects of Glycyrrhizin on the Metabolism of Lipids in Rats of Acute Ethanol Intoxication

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Abstract

The metabolism of lipids and the effects of Glycyrrhizin in rats of acute ethanol intoxication have been investigated. Compared with those of the control group, the concentrations of total serum lipids, cholesterol and triglyceride increased by 31%, 50%and 42% respectively after the treatment of rats aged 5 weeks with 50% ethanol alcohol (5 g/kg, i.g). The hepatic lipid peroxidation product, malondialdehyde (MDA), increased by 184%, in contrast to the content of reduced glutathione (GSH) which decreased by 58%. Preteatment of rats with Glycyrrhizin (150 mg/kg, i.g) for 7 days reversed the abmormal metaboliam induced by ethanol alcohol.

MeSH　glycyrrhiza/PD; alcohol ethyl; lipids/ME; malondialdehyde/ME; glutathione/ME

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·基础研究·

甘草甜素对肝硬化动物模型肝脏内NF-κB结合活性的抑制作用

王吉耀　郭津生　刘淑玲　Mark A. Zern

【摘要】 **目的**　从分子水平探讨强力宁生物活性的发生机理。**方法**　大鼠随机分为正常对照组、模型对照组、强力宁组，后两组给予四氯化碳(CCl_4)和乙醇造模处理以诱导慢性肝损伤，强力宁组在造模处理同时予强力宁治疗。各组大鼠在CCl_4等处理后第9周处死，收集血清和肝脏标本，测定血清ALT活性并进行组织学观察。部分肝组织提取细胞核蛋白进行凝胶迟滞实验以观察NF-κB活性。**结果**　CCl_4等处理后第9周模型对照组血清ALT水平显著高于强力宁组。模型大鼠肝脏脂肪变性和纤维化程度较强力宁治疗组更为严重。模型对照组肝脏内NF-κB活性较正常对照组显著增加，而强力宁组大鼠肝脏内NF-κB结合活性与正常组相接近。**结论**　强力宁能够抑制CCl_4联合乙醇诱导的慢性肝损伤大鼠肝脏内NF-κB的结合活性的增加，可能是强力宁具有保护肝毒素性肝损伤和纤维化作用的分子机制之一。

【关键词】 肝硬化　甘草甜素　核因子κB

Inhibitory effect of glycyrrhizin on NF-κB binding activity in CCl_4 plus ethanol induced liver cirrhosis in rats　*WANG Jiyao, GUO Jinsheng, LIU Shuling, et al. Department of Gastraenterology, ZhongShan Hospital, Shanghai Medical University, Shanghai* 200032

【Abstract】 **Objective**　To investigate the effects of Potenlini on nuclear factor-κB(NF-κB) binding activity in the livers of animals models with liver cirrhosis, and to delineate the molecular mechanism of the bioactivities of Potenlini. **Methods**　Male SD rats were randomly allocated into a normal control group, a model control group, and a Potenlini group. Rats in the latter two groups were treated with CCl_4 and Ethanol solution in order to induce chronic liver injury. Rats in Potenlini group were given Potenlini treatment at the same time. All rats were killed at thd 9th week after CCl_4 administration. Serum and liver specimens were collected, serum ALT activities and histological findings were assessed. Nuclear extracts from liver tissues were prepared and gel retardation assays were performed for the evaluation of NF-κB activity. **Results**　(1)Serum ALT levels were significantly reduced in rats treated with Potenlini compared with those in rats of the model control group, which had dramatically increased ALT levels. (2) Histologically, liver steatosis and fibrosis were severe in the rats of the model group, but were significantly improved in rats of the Potenlini group. (3)NF-κB binding activity was markedly increased in the liver specimens taken from the rats of the model control group in comparison with the binding of normal livers, but the binding levels were nearly normal in the livers of the Potenlini group. **Conclusion**　Potenlini can inhibit the NF-κB binding activity in CCl_4 and ethanol induced chronic liver injury, and that may partially be the mechanism by which Potenlini protects liver from hepatotoxin-induced liver injury and cirrhosis.

【Key words】 Liver cirrhosis　Glycyrrhizin　NF-κB

本研究探讨甘草甜素(Glycyrrhizin, GL)对肝硬化动物模型肝脏内核因子κB(NF-κB)结合活性的影响，以助阐明该药物的分子机理。

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王吉耀　女，54岁，内科教研室主任、消化科副主任、博士生导师。长期从事胃肠及肝病的研究，在国内外杂志上发表论文70余篇。

材料与方法

1. GL的剂型是强力宁针剂，由浙江海宁制药厂生产并惠赠。它含有体积分数为0.2%的甘草酸单铵、体

积分数为0.1%的L-半胱氨酸、体积分数为2%的甘氨酸，能够经静脉或腹腔内给药。

2. 肝硬化动物模型的建立：49只雄性SD大鼠(购于中科院实验动物中心)，250 g左右，随机分成三组：正常对照组、模型对照组、强力宁组。正常对照组皮下注射豆油；另两组大鼠予体积分数为40%CCl_4豆油溶液0.3 ml/100 g体重每周两次皮下注射，首剂加倍，并饮用体积分数为10%乙醇水(为唯一饮料)。强力宁组在CCl_4处理前一天给予强力宁针剂1.5 ml/100 g腹腔内注射，以后每周三次。

3. 血清和肝脏标本的采集：在CCl_4和乙醇处理9周时处死大鼠收集血清和肝脏标本。

4. 肝组织细胞核抽提：鼠肝组织细胞核抽提基本上采用Gorski的方法[1]经过部分改进。蛋白提取物以少量水溶解快速冻存于液氮并贮存于-150℃，蛋白质浓度以放免法检测。

5. 凝胶迟滞法检测NF-κB结合活性：凝胶迟滞法检测简单操作步骤如下：总反应体积为15 μl，含有10 μg核蛋白抽提物，1μg或2 μg多聚dI/dC，和0.1 ng双链寡核苷酸探针。经过结合，DNA蛋白复合物在8%非变性聚丙烯酰胺凝胶中进行电泳，电泳凝胶干燥后在-70℃下用增感屏和X光片曝光。

结　果

9周时正常对照组收集3只标本，模型对照组5只，强力宁组6只。大体观察模型对照组肝脏大部分已形成肉眼可见的硬化结节，而强力宁组肝脏外观几乎正常。

模型对照组血清ALT显著增高，而强力宁治疗能降低CCl_4和乙醇引起的血清ALT的增高水平。

模型对照组肝脏第9周时见纤维间隔和假小叶结节已清晰形成，而强力宁组肝脏变性和纤维化程度较轻(肝纤维化分级见表1，分级标准主要参照朱家旋等[2]报道)。

表1 CCl_4处理9周时各组大鼠血清ALT及肝纤维化分期的比较

组别	动物数	ALT(U/L) ($\bar{x}\pm s$)	肝纤维化分期					
			正常 Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ	Ⅵ
正常对照组*	3	48 ± 7	3	0	0	0	0	0
模型对照组	5	164 ± 46	0	0	0	0	1	1	3
强力宁治疗组*	6	72 ± 44	0	2	1	1	1	1	0

*：$P<0.05$，与模型对照组相比具有显著意义

肝纤维化分期：Ⅰ：少量胶原纤维自汇管区或中央静脉向外延伸；Ⅱ：纤维延伸明显但未包绕整个肝小叶；Ⅲ纤维互相连接包绕肝小叶；Ⅳ：纤维分割肝小叶形成假小叶但以大方型假小叶为主；Ⅴ：大方型假小叶与小圆形假小叶各占50%；Ⅵ：肝内布满小圆型假小叶，其间充满粗大增生的纤维组织

9周各组标本中随机选取三只大鼠肝脏进行核抽提和凝胶迟滞实验，结果发现CCl_4和乙醇损伤后NF-κB与相应序列的特异结合显著增加。强力宁治疗能够降低NF-κB的结合活性而使之接近正常水平。

讨　论

NF-κB是一个多功能的转录激活剂，它由Rel家族的同二聚体或异二聚体组成，在胞浆中以无活性的形式与NF-κB抑制剂(i κB)结合。NF-κB在许多细胞刺激信号如丝裂原、细胞因子、细菌脂多糖、病毒和病毒蛋白、许多环境微扰如氧自由基损伤基至紫外光照射等作用下被激活而导致i κB蛋白降解释放自由的NF-κB，使之能够转移到细胞核中与多种不同组织的不同基因调节区域结合启动转录过程。NF-κB能诱导和调控的基因包括许多重要的基因如编码炎症性细胞因子、趋化因子、干扰素、MHC蛋白、生长因子、细胞粘附分子和病毒。除了在免疫调节和发生中具有功能性作用外，NF-κB还能够参与调节细胞凋亡、抗病毒和微生物反应、多种张力反应、衰老过程；并可能参与胚胎发生[3, 4]。

实验发现在肝脏中NF-κB结合活性在枯否氏细胞和肝细胞中均存在，推测它在纤维化过程和肝再生过程中都直接起到重要作用[5]，而不仅仅是促进肝细胞坏死的因子。通过强力宁来抑制NF-κB结合活性或阻断NF-κB的激活可最终减轻NF-κB途径导致的肝损伤及纤维化过程，NF-κB途径可能是GL发挥其抗损伤和纤维化作用的重要分子途径。

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ORIGINAL RESEARCH

Glycyrrhizic acid nanoparticles inhibit LPS-induced inflammatory mediators in 264.7 mouse macrophages compared with unprocessed glycyrrhizic acid

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Abstract: Glycyrrhizic acid (GA), the main component of radix glycyrrhizae, has a variety of pharmacological activities. In the present study, suspensions of GA nanoparticles with the average particle size about 200nm were prepared by a supercritical antisolvent (SAS) process. Comparative studies were undertaken using lipopolysaccaride(LPS)-stimulated mouse macrophages RAW 264.7 as in vitro inflammatory model. Several important inflammation mediators such as NO, PGE_2, TNF-α and IL-6 were examined. These markers were highly stimulated by LPS and were inhibited both by nano-GA and unprocessed GA in a dose-dependent manner, especially PGE_2 and TNF-α. However nano-GA and unprocessed GA inhibited NO only at a high concentration. In general, we found that GA nanoparticle suspensions exhibited much better anti-inflammatory activities compared to unprocessed GA.

Keywords: glycyrrhizic acid, nanoparticle, mouse macrophages RAW 264.7, inflammatory cytokines

Introduction

Inflammation is a natural biological response to injury or infection in the human body. Various factors, such as microbial infections, chemicals, and immunologic reactions can cause inflammation.[1] Prolonged inflammation can be harmful, contributing to the pathogenesis of many diseases, including rheumatoid arthritis, obesity, cardiovascular diseases, neurodegenerative diseases, diabetes, and cancer.[2,3]

Macrophages are considered to play essential roles in inflammation. If activated by endotoxins, macrophages produce inflammatory cytokines, which in turn activate other macrophages and other nearby cells to promote, for example, inducible nitric oxide (NO) synthase gene expression.[4] Much progress has been made in the delineation of cell signaling pathways, in which the inflammation initiates a cascade of events that result in the overproduction of certain inflammation-associated genes and proinflammatory cytokines.[5]

Inhibition of inflammatory cytokine and mediator production serves as a key mechanism in the control of inflammation. A number of anti-inflammatory molecules have already entered clinical trials for the treatment of inflammatory disorder such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), prostaglandin E_2 (PGE_2) or nitric oxide (NO).[6] Drugs that suppress the expression of these inflammatory mediators have therefore attracted significant interest as potential therapeutics for the treatment of inflammatory diseases.[3]

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Glycyrrhizic acid (GA), a terpenoid compound, is the main component of radix glycyrrhizae. GA has a variety of pharmacological activities such as antioxidative, anti-inflammatory, antiulcerous, antidotal, antiallergic, antiviral, immunomodulating, hepatoprotective, and cardioprotective properties.[7–10] However, GA is slowly absorbed in vivo because of its poor water solubility. Large number of studies have shown that the reduction of the drug particle size to the nanometer scale considerably increases the activity and bioavailability of the drug.[11] Supercritical antisolvent (SAS) process is an environmentally friendly technology developed in recent years. In this process, the drug is firstly dissolved in the solvent and then the drug solution is quickly sprayed into supercritical fluids (the antisolvent). Precipitation occurs immediately by a rapid recrystallization of the drug. In general, the SAS process can control the production of particle sizes within the nanometer range, and the solvent can be fully recovered. It is suitable to prepare nanocrystals of drugs or biologicaly active substance because of low temperature and inertia.

This study explored the anti-inflammatory activity and mechanisms of GA nanoparticles prepared by SAS in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages compared to unprocessed GA particles.

Materials and methods

Materials

GA (≥98%) for nanosuspension preparation was purchased from Xi'an Green-Life Natural Products Co, Ltd (Xi'an, People's Republic of China). GA (99%) for quantitative analysis was purchased from Sigma-Aldrich (St Louis, MO, USA). Compound glycyrrhizin tablets were manufactured by Minophagen Pharmaceutical Co., Ltd (Tokyo, Japan). LPS was purchased from Sigma-Aldrich. Griess reagent kit was obtained from Biyuntian Biological Technology Co., Ltd (Shanghai, People's Republic of China). PGE_2, TNF-α, and IL-6 enzyme-linked immunosorbent assay kits were purchased from 4A Biotech Co., Ltd (Beijing, People's Republic of China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. High-performance liquid chromatography grade methanol was purchased from J&K Chemical Ltd (Beijing, People's Republic of China). Purified water was obtained from a Milli-Q® system from EMD Millipore (Billerica, MA, USA). All other chemicals were of analytical reagent grade.

Preparation of GA nanosuspension

Two processes were performed to obtain the final GA nanoparticle suspension. First, the micronized GA was prepared with an SAS apparatus modified from a carbon dioxide supercritical extraction apparatus by using dimethyl sulfoxide (DMSO) as the solvent and carbon dioxide as the antisolvent. Under the optimum conditions, micronized GA with a particle size about 200 nm was obtained.

Surface morphology determined by scanning electron microscopy (SEM) of nanoparticles and unprocessed GA

The samples were fixed to an SEM stub and sputter coated with gold using SBC-12 ion sputter coater (KYKY Technology Development Ltd, Beijing, People's Republic of China) to form a carbon conductive film. The surface morphology of the particles was then observed by SEM (Quanta™ 200; FEI Company, Hillsboro, OR, USA).

Cell culture

The RAW 264.7 cells, a murine macrophage-like cell line, were obtained from China Cell Line Bank (Beijing, People's Republic of China). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere (5% carbon dioxide). The test compounds were diluted with 2% Dulbecco's modified Eagle's medium to the appropriate concentrations and added 1 hour before LPS treatment.

MTT assay for cell viability

The MTT assay was performed to measure cell viability. RAW 264.7 cells were mechanically scraped, seeded in 96-well plates, and incubated in a 37°C, 5% carbon dioxide incubator overnight. After 24 hours, cells were treated with different concentrations of GA nanoparticles and unprocessed GA or glycyrrhizin tablets for 24 hours. Subsequently, 20 μL of 5 mg/mL MTT in fetal bovine serum-free medium was added to each well, and the cells were incubated for 4 hours. MTT was removed and resolved with 150 μL/well dimethyl sulfoxide. The optical density was measured at 492 nm using a microplate reader (Tecan Infinite® M200, Tecan Group Ltd, Männedorf, Austria). Concentrations were determined for three wells of each sample, and this experiment was done in triplicate.

Analysis of NO production

Nitrite, a stable product of NO, was used to assess NO production based on the Griess reaction. RAW 264.7 cells were incubated with different concentrations of GA nanoparticles and unprocessed GA or glycyrrhizin tablets in the absence or presence of 1 μg/mL LPS. After 24 hours of incubation, the level of NO production was monitored by measuring the

nitrite concentration in the supernatant of cultured medium using the Griess reagent assay. For the NO assay, 50 µL supernatant of cultured medium was mixed with the same volume of Griess reagent. The absorbance was measured at 540 nm on a microplate reader. The concentration of NO in the media of sample-treated cells was calculated using the standard curve obtained for sodium nitrite dissolved in Dulbecco's modified Eagle's medium. Concentrations were determined for three wells in each sample, and this experiment was done in triplicate.

Determination of PGE_2, TNF-α, and IL-6 levels

To investigate the effect of GA nanoparticles and unprocessed GA or glycyrrhizin tablets on cytokine responses from LPS-treated cells, RAW 264.7 cells seeded on 96-well plates were treated with 303, 606, and 909 µM GA 1 hour before treatment with 1 µg/mL LPS for 24 hours in a 37°C, 5% carbon dioxide incubator. Cell-free supernatants were collected and stored at −20°C until assayed for cytokines. The concentrations of PGE_2, TNF-α, and IL-6 in the culture supernatants of RAW 264.7 cell cultures were determined using an enzyme-linked immunosorbent assay kit. Concentrations were determined for three wells in each sample, and this experiment was done in triplicate.

Statistical analysis

The experimental values were represented as arithmetic mean ± standard deviation. The unpaired Student's *t*-test was used to determine the statistical significance. Statistically, a *P*-value less than 0.05 was considered to be significant and a *P*-value less than 0.01 was considered to be very significant.

Results

Surface morphology of GA nanoparticles and unprocessed GA

Figure 1A and B show the particle size of unprocessed GA material and GA nanoparticle suspensions performed by scanning electron microscopy (SEM). The final GA nanosuspension prepared by SAS contained particles of about 200 nm, whereas GA raw materials exhibited an irregular shape and micrograde particle size.

Cell viability and comparative effects of GA nanoparticles and unprocessed GA on LPS-induced NO production

Unprocessed GA, nano-GA and glycyrrhizin tablets up to 909 µM did not show cytotoxic effects (data not shown).

Nitric oxide (NO) is a short-living free radical that is produced from L-arginine by catalytic reactions of NO synthases in mammalian immune, cardiovascular and neural systems, where it functions as signaling molecule.[12] To analyze potential anti-inflammatory properties of GA nanoparticles and unprocessed GA, we used murine RAW 264.7 macrophage cells, which produce NO upon stimulation with LPS. The amount of produced NO was determined by measurement of nitrite, a stable metabolite of NO. Cells were pretreated with GA nanoparticles or unprocessed GA, and then stimulated with 1 µg/mL LPS. The control group was untreated with both LPS and samples. After the cell culture media were collected, nitrite levels were determined. During the incubation time of 24 hours, RAW 264.7 macrophages produced 2.68 ± 0.34 µM nitrite in the resting state, whereas after LPS (1 µg/mL) stimulation, NO production dramatically increased to 20.12 ± 3.69 µM. Nano-GA and unprocessed GA inhibited nitrite production 24 hours after LPS stimulation in a dose-dependent manner (Figure 2). At 303 µM, nano-GA and unprocessed GA reduced NO production by 13.33% and 0.03%, respectively. At concentrations of 606 µM, nano-GA and unprocessed GA reduced NO production by 51.44% and 18.67%, respectively, whereas at 909 µM, nano-GA and unprocessed GA reduced NO production by 87.78% and 23.56%, respectively. Nano-GA inhibited NO generation much better than unprocessed GA, especially at high concentrations. When compared to the positive control (glycyrrhizin tablets), the inhibition rate of NO was: nano-GA > positive control > unprocessed GA.

Comparative effects of GA nanoparticles and unprocessed GA on LPS-induced PGE_2 production

Levels of the pro-inflammatory lipid mediator PGE_2 were analyzed upon LPS stimulation in RAW 264.7 cells. During the incubation for 24 hours, RAW 264.7 macrophages produced 19.75 ± 5.24 pg/mL PGE_2 in the resting state, whereas after LPS stimulation, PEG_2 production dramatically increased to 628.47 ± 33.59 pg/mL. Both GA nanoparticles and unprocessed GA reduced PEG_2 production in a dose-dependent manner (Figure 3). At concentrations of 303 µM, GA nanoparticles and unprocessed GA reduced PGE_2 production by 59.38% and 27.18%, respectively. Nano-GA exhibiting more than two-fold inhibition activity than unprocessed GA. At 606 µM, PGE_2 production was reduced by 73.22% and 47.49%, respectively, whereas at 909 µM, nano-GA and unprocessed GA reduced PGE_2 production by 86.78% and 66.90%, respectively. Nano-GA inhibited PGE_2 generation much better than unprocessed

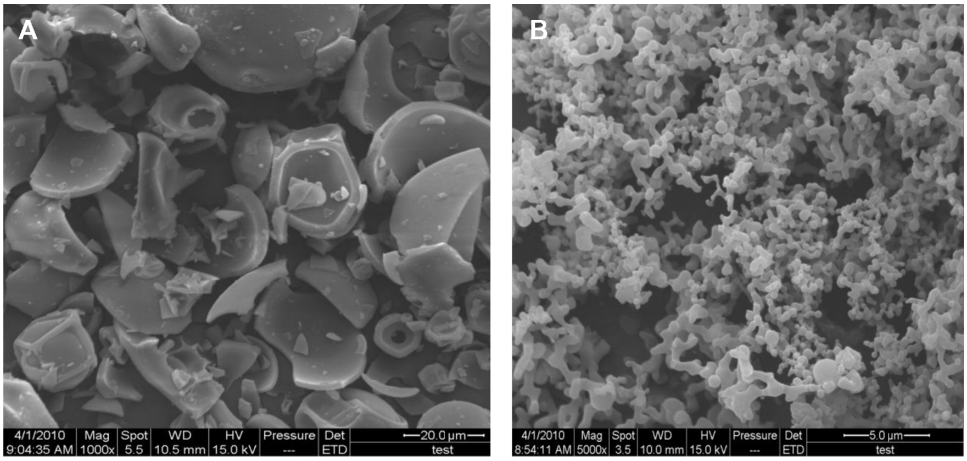

Figure 1 Scanning electron microscopy result of (**A**) unprocessed GA and (**B**) GA nanoparticles.
Abbreviation: GA, glycyrrhizic acid.

GA. When compared to the positive control (glycyrrhizin tablets), the inhibition rate of PGE_2 was: nano-GA > positive control > unprocessed GA.

Comparative effects of GA nanoparticles and unprocessed GA on LPS-induced TNF-α production

TNF-α is one of the most important cytokines and is required for the induction of NO synthesis in LPS-stimulated macrophages. TNF-α elicits a number of physiological effects, such as septic shock, inflammation, cachexia, and cytotoxicity.[13] TNF-α concentrations in the culture supernatants of RAW 264.7 cells were measured by enzyme-linked immunosorbent assay (Figure 4). RAW 264.7 cells treated with LPS produced significant amounts of TNF-α. The concentration of TNF-α increased from 1.47 ± 0.27 ng/mL to 62.99 ± 9.56 ng/mL after LPS stimulation. However, the concentrations of TNF-α in the supernatant of cells treated with GA nanoparticles and unprocessed GA were significantly decreased compared to the LPS control group. At concentrations of 303 µM, GA nanoparticles and unprocessed GA reduced TNF-α production by 76.94% and 57.01%, respectively. At 606 µM, TNF-α production was reduced by 79.61% and 69.02%, respectively, whereas at 909 µM, TNF-α production was reduced by 89.51% and 69.58%, respectively. Both nano-GA and unprocessed GA strongly inhibited TNF-α generation. When compared to the positive control (glycyrrhizin tablets), the reduction

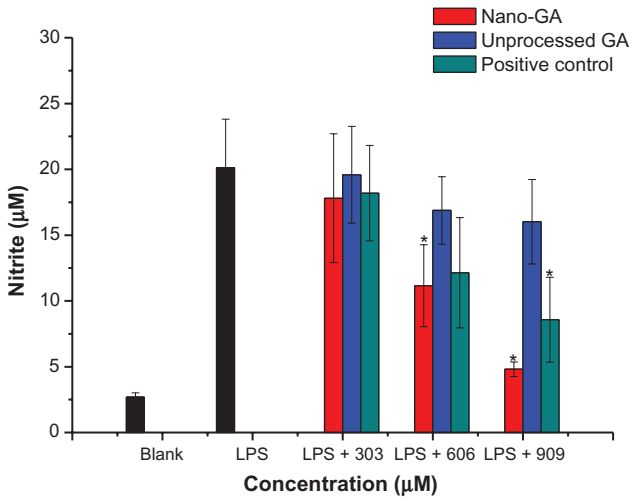

Figure 2 Effect of different concentrations of GA nanoparticles and unprocessed GA on LPS-induced nitric oxide.
Note: *$P < 0.05$, significantly different from the LPS-treated group by unpaired Student's *t*-test.
Abbreviations: GA, glycyrrhizic acid; LPS, lipopolysaccharide.

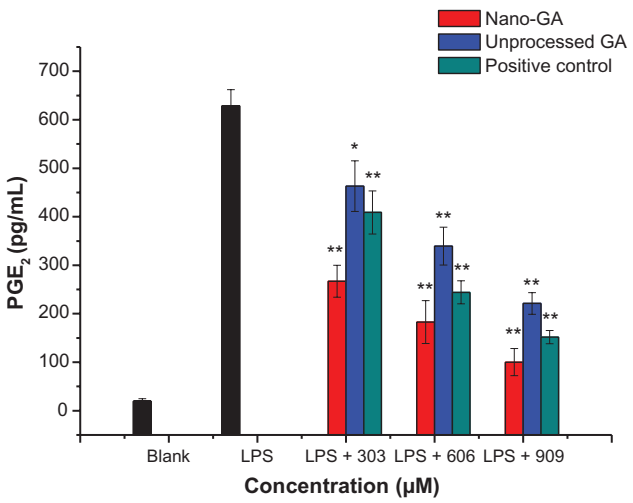

Figure 3 Effect of different concentrations of GA nanoparticles and unprocessed GA on LPS-induced PGE_2.
Notes: *$P < 0.05$; **$P < 0.01$, significantly different from the LPS-treated group by unpaired Student's *t*-test.
Abbreviations: GA, glycyrrhizic acid; LPS, lipopolysaccharide; PGE_2, prostaglandin E_2.

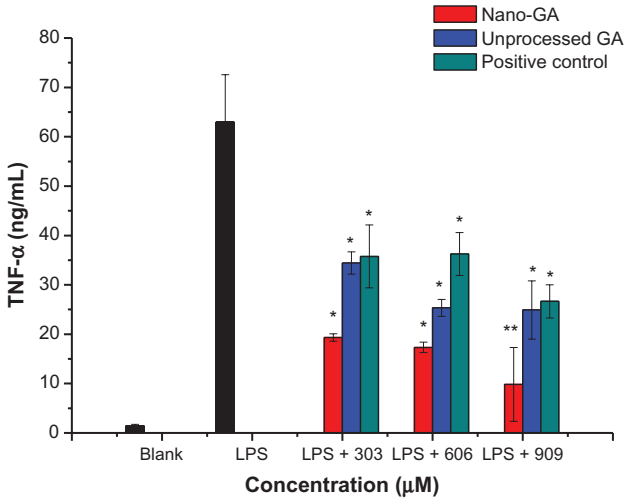

Figure 4 Effect of different concentrations of GA nanoparticles and unprocessed GA on LPS-induced TNF-α.
Notes: *$P < 0.05$; **$P < 0.01$, significantly different from the LPS-treated group by unpaired Student's *t*-test.
Abbreviations: GA, glycyrrhizic acid; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

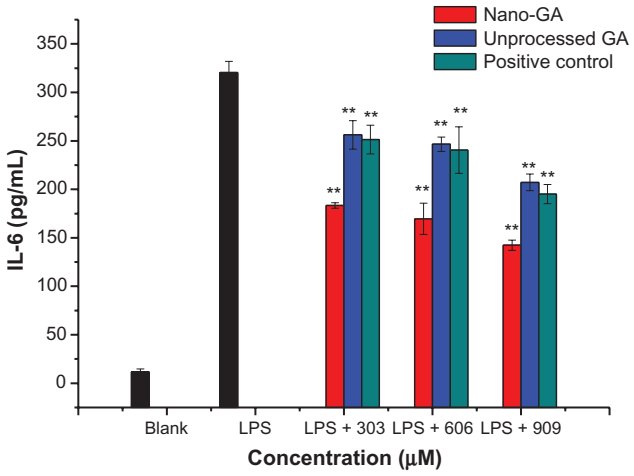

Figure 5 Effect of different concentrations of GA nanoparticles and unprocessed GA on LPS-induced IL-6.
Note: **$P < 0.01$, significantly different from the LPS-treated group by unpaired Student's *t*-test.
Abbreviations: GA, glycyrrhizic acid; LPS, lipopolysaccharide;IL-6, interleukin-6.

rate of TNF-α was: nano-GA > unprocessed GA > positive control.

Comparative effects of GA nanoparticles and unprocessed GA on LPS-induced IL-6 production

IL-6 represents an endogenous mediator of LPS-induced fever. RAW 264.7 cells treated with LPS produced significant amounts of IL-6, increasing from 11.67 ± 3.06 pg/mL to 320.33 ± 11.59 pg/mL after LPS stimulation (Figure 5). IL-6 in the supernatant of cells treated with GA nanoparticles and unprocessed GA did not significantly change compared to the LPS control group. At concentrations of 303 μM, GA nanoparticles and unprocessed GA reduced IL-6 production by 44.39% and 20.79%, respectively. At 606 μM, IL-6 production was reduced by 48.85% and 23.92%, respectively, whereas at 909 μM, IL-6 production was reduced by 57.67% and 36.66%, respectively. When compared to the positive control (glycyrrhizin tablets), the reduction rate of IL-6 was: nano-GA > positive control > unprocessed GA. Nano-GA inhibited IL-6 generation much better than unprocessed GA.

Discussion

Inflammation is the primary response of the immune system against infection or irritation, and macrophages play a crucial role during the inflammatory process.[14] In the presence of stimuli such as LPS, macrophages are activated and produce various cytokines such as TNF-α, IL-1β, IL-6, and IL-10, as well as inflammatory mediators such as NO and PGE_2.[3,15,16] Therefore, LPS-activated macrophages have typically been used to evaluate the anti-inflammatory effects of various materials. The production of these cytokines and mediators may result in the systemic inflammatory response syndrome, severe tissue damage, and septic shock.[17] Therefore, agents that regulate cytokines and inflammatory mediators may have therapeutic effects.

Glycyrrhiza uralensis (Leguminosae) has long been used throughout the world as a sweetener and in folk medicine because of its antioxidative, anti-inflammatory, antibacterial, antiangiogenic, and antiallergenic properties.[1] Its main components are considered to be the triterpene saponins glycyrrhizin and GA.[18–20]

Nanomaterials are important materials due to their unique physical and chemical properties.[21] Due to accumulation of nanoparticles in the cell by enhanced permeability and retention effect, nanoparticles achieve new biological properties.[22,23] In the current report, solid dispersion microparticles of poorly water-soluble GA were prepared by using SAS method. This technique is a feasible and efficient way to enhance the solubility of poorly water-soluble GA with high dissolution rate.

In order to compare the anti-inflammatory activity of GA nanoparticles and unprocessed GA in LPS-stimulated RAW 264.7 macrophages, several important inflammation mediators were examined. We found that the release of NO, PGE_2, TNF-α and IL-6 release, which was highly stimulated by LPS, was inhibited by both nano-GA and unprocessed GA

in a dose-dependent manner. In particular, GA suspensions suspension, compared with the unprocessed GA significantly enhanced the reduction of PGE_2 and TNF-α even at low concentrations, but did not significantly affect NO production at low concentrations. Nano-GA exhibited much better inhibition activities compared to unprocessed GA. This is due to the fact that nanoparticles have a smaller particle sizes compared to unprocessed drug particles, which leads to an increase of the interfacial surface area and consequently improvement of water solubility, allowing for smaller dosages and more rapid and direct usage of otherwise poorly water-soluble drugs.[24] When compared to the positive control (glycyrrhizin tablets) the reduction rate of NO, PGE_2, and IL-6 was: nano-GA > positive control > unprocessed GA, whereas for TNF-α it was: GA nanoparticles > unprocessed GA> positive control.

Conclusion

In summary, the findings presented here suggest that GA nanoparticles prepared by the SAS method performed much better inhibition activity for LPS-induced NO, PGE_2, TNF-α, and IL-6 production in macrophage cells than unprocessed GA. Thus, GA nanoparticles may have therapeutic potential for the modulation and regulation of macrophage activation, and may provide safe and effective treatment options for a variety of inflammation-mediated diseases.

Acknowledgements

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Disclosure

The authors report no conflicts of interest in this work.

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Alcohol / Addiction / Illegal Drugs | Biology / Biochemistry

Young adults 'damage DNA' with weekend alcohol consumption

Written by Honor Whiteman

Published: Thursday 2 January 2014

College students are renowned for partying at the weekends, and this usually involves having a drink or two. But new research has found that this level of alcohol consumption may cause damage to DNA. This is according to a study published in the journal *Alcohol*.

The National Institute on Alcohol Abuse and Alcoholism states that around **four out of five college students in the US drink alcohol** and 1,825 college students between the ages of 18 and 24 die each year as a result of unintentional alcohol-related injuries.

According to the study researchers, including co-author Jesús Velázquez of the Autonomous University of Nayarit in Mexico, previous research studying the effects of alcohol consumption has mainly been carried out in individuals who have been drinking for long periods of time.

These individuals usually have illnesses as a result of their alcohol consumption, such as liver damage, **cancer** or **depression**.

But the investigators say their study is "pioneering," as it analyzes the effects of alcohol consumption on young people who are healthy.

Oxidative damage caused by alcohol consumption

The researchers set out to determine the level of oxidative damage caused by alcohol consumption in two groups of people between the ages of 18 and 23. Oxidative stress can cause damage to proteins, membranes and genes.

One group drank an average of 1.5 liters of alcoholic beverages every weekend, while the other group did not consume any alcohol.

All participants underwent blood tests to ensure they were healthy and were free of any diseases or addictions.

The researchers also measured the activity of dehydrogenase - an enzyme responsible for metabolizing alcohol (ethanol) into acetaldehyde - as well as acetoacetate and acetone activity.

A new study suggests that alcohol consumption for young adults, even if only at weekends, may be damaging to their DNA.

Using a thiobarbituric acid reactive substances (TBARS) test, the researchers were able to assess oxidative damage. The test allowed them to see how ethanol in the blood, and the acetaldehyde produced by dehydrogenase in reaction to ethanol, affects the lipid peroxidation that impacts cell membranes.

Results of the study revealed that the alcohol-consuming group demonstrated twice as much oxidative damage to their cell membranes, compared with the group that did not drink.

Signs of DNA damage through alcohol consumption

An additional experiment, called the comet test, was conducted to see whether the participants' DNA was also affected by alcohol consumption. This involved taking out the nucleus of lymphocytic cells in the blood and putting it through electrophoresis.

The researchers explain that if the cells are faulty and DNA is damaged, it causes a "halo" in the electrophoresis, called "the comet tail."

The experiment revealed that the group who consumed alcohol showed significantly bigger comet tails in the electrophoresis, compared with the group that did not drink alcohol.

In detail, 8% of cells were damaged in the control group, but 44% were damaged in the drinking group. This means the drinking group had 5.3 times more damage to their cells.

However, the investigators say that they were unable to confirm there was extensive damage to the DNA, as the comet tail was less than 20 nanometers. But the investigators say their findings still raise concern.

They explain:

> "The fact is, there should not have been any damage at all because they had not been consuming alcohol for very long, they had not been exposed in a chronic way."

Overall, they conclude that oxidative damage can be found in young adults with only 4-5 years' alcohol drinking history, and that this is the first study to provide evidence of this damage in individuals at the early stages of alcohol abuse.

Other studies have detailed some positive effects of moderate alcohol consumption. *Medical News Today* recently reported on a study suggesting that drinking alcohol in small doses **may boost the immune system**.

Written by Honor Whiteman

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Alcohol leaves its mark on youngsters' DNA, news release from Basque Research, accessed 31 December 2013.

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Young adults 'damage DNA' with weekend alcohol consumption

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Article

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Protective Effects of Glycyrrhizic Acid and 18β-Glycyrrhetinic Acid against Cisplatin-Induced Nephrotoxicity in BALB/c Mice

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Supporting Information

ABSTRACT: The clinical use of antineoplastic drug cisplatin (CP) is commonly complicated by nephrotoxic side effects that limit its application and therapeutic efficiency. This study used a model of CP-induced renal injury in male BALB/c mice to investigate the protective effects of the active components of licorice, glycyrrhizic acid (GA), and 18β-glycyrrhetinic acid (18βGA) against CP-induced nephrotoxicity, and the chemoprotectant, amifostine, was used as a control. Oral administration of GA or 18βGA significantly reduced CP-induced increases in the levels of blood urea nitrogen, creatinine, and lactate dehydrogenase. Hematoxylin and eosin staining revealed that GA and 18βGA delayed the progression of renal injury, including tubular necrosis, hyaline casts, and tubular degeneration in response to CP exposure. Oxidative status and inflammatory responses in CP-treated mice were restored to near-normal levels by treatment with GA or 18βGA. These protective effects might be associated with upregulation of nuclear factor E2-related protein (Nrf2) and downregulation of nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB) in the kidney. Notably, we demonstrated that GA and 18βGA rendered renal cells resistant to CP-induced HMGB1 cytoplasmic translocation and release. These findings suggest that GA and 18βGA might be act as the chemoprotectants against CP-induced nephrotoxicity.

KEYWORDS: *cisplatin, anti-inflammation, glycyrrhizic acid, 18β-glycyrrhetinic acid, nephrotoxicity*

INTRODUCTION

Cisplatin (CP) is a platinum-containing anticancer drug that directly intercalates into DNA double strands to cause cross-linking of intra- or interstrands, twisting of the double helix, interference with nucleic acid replication and DNA synthesis, and eventual occurrence of apoptosis.[1] CP is usually used in clinical settings to control numerous types of solid tumors, including tumors involved in head and neck cancers, esophageal cancer, genital cancer, and non-small cell lung cancer. The treatment effect of CP on testicular cancer has the greatest success rate at 90%. Therefore, it is an indispensable drug for chemotherapy.[2,3] However, nephrotoxicity is a major side effect of CP, and the clinical characteristics of renal function abnormalities, such as an increase of serum blood urea nitrogen (BUN) and creatinine and reduction of creatinine clearance (Ccr), usually occur after 2 weeks of drug administration.[4] Amifostine (AMF) is a thiophosphate preparation currently used to relieve the side effects of chemotherapy and radiotherapy. AMF eliminates free radicals and promotes antioxidant enzyme gene expressions and DNA repair, which reduce the toxicity of platinum-based or alkylating chemotherapeutic drugs. However, studies have shown that AMF may have unintended effects that increase the survival rate of certain tumor cells.[5−7] Therefore, it is necessary to develop adjuvants from natural bioactives that are safe and do not influence treatment effects.

Licorice is a traditional medicinal plant, and its roots and rhizomes, which are used for medicine, contain 4−20% triterpenoid saponins, of which glycyrrhizic acid (GA) is the major active component. GA is the source of the sweetness of licorice, and it is 50 times sweeter than sucrose.[8] Studies have shown that oral administration of GA can be metabolized into 18β-glycyrrhetinic acid (18βGA) by β-D-glucuronidase, which is produced by intestinal bacteria. Intravenously injected GA is first metabolized by β-D-glucuronidase in hepatic lysosomes into 3-monoglucuronide glycyrrhetinic acid; after secretion into the intestines along with bile, it is further metabolized into 18βGA by intestinal bacteria and reabsorbed into the circulatory system.[9]

GA and 18βGA are known to be the major active components of licorice, and recent studies suggested that they have multiple health benefits.[10] GA can improve ischemia/reperfusion-induced acute renal injury in rats,[11] inhibit CCl_4-induced hepatic fibrosis in rats,[12] inhibit lipopolysaccharide-induced pulmonary injury in mice,[13] and relieve chemodrug-induced genotoxicity,[14] whereas administration of GA and 18βGA can prevent stress ulcers in mice.[15] In a streptozotocin-induced diabetes mellitus rat model, oral administration of 18βGA [100 mg/kg of body weight (BW)] had a hypoglycemic effect similar to that of glibenclamide.[16] Our previous study indicated that GA and 18βGA have excellent antioxidant and anti-inflammatory characteristics that can promote antioxidant enzyme activities in neuronal cells and inhibit reactive oxygen species (ROS)-induced oxidative damage and apoptosis.[17] GA and 18βGA can also inhibit nuclear factor κB (NF-κB) and reduce proinflammatory cytokine secretion through regulating the PI3K pathway, thus achieving an anti-inflammatory function.[18] Other phytochemicals, such as quercetin and hesperidin, can relieve CP toxicity and exhibit renal protective functions by clearing ROS, maintaining

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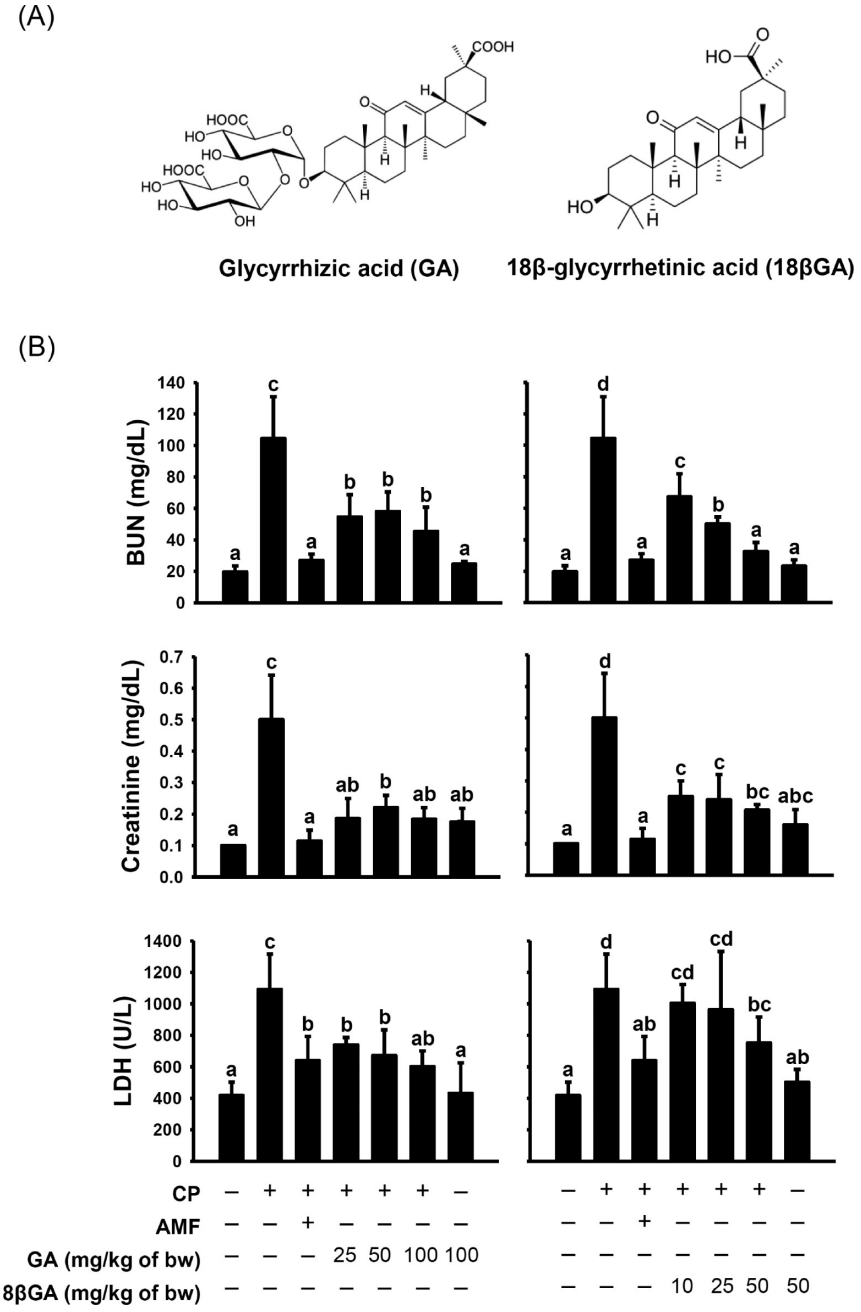

Figure 1. Elevated levels of serum BUN, creatinine, and LDH were reduced by GA and 18βGA in BALB/c mice with CP-induced renal injury. (A) Chemical structures of GA and 18βGA. (B) Mice were gavaged daily with GA (25−100 mg/kg of BW) or 18βGA (10−50 mg/kg of BW) for 8 consecutive days, and a single IP injection of CP (30 mg/kg of BW) was applied on day 6. The positive control group was given a single IP injection of AMF (200 mg/kg of BW) at 0.5 h before the CP challenge. Mice were sacrificed on day 9. Data are presented as the mean ± SD ($n = 8$), and letters (a−c) indicate statistically significant differences in each group ($p < 0.05$). One-way ANOVA tests were used for the statistical analyses.

antioxidant enzyme activities, and reducing inflammatory responses.[1,19] Because oxidative stress and inflammatory responses play key roles in CP-induced nephrotoxicity, this study employed an animal model of CP-induced renal injury to verify the protective effects of GA and 18βGA against chemically induced renal injury and the possible underlying mechanisms of such protective effects.

MATERIALS AND METHODS

Chemicals. CP, GA, 18βGA, Kollisolv PEG E 400, AMF, 1-chloro-2,4-dinitro-benzene (CDNB), glutathione (GSH), glutathione reductase (GSH-Rd), β-nicotinamide adenine dinucleotide phosphate (β-NADPH), sodium azide (NaN_3), and ethanol were obtained from Sigma-Aldrich (St. Louis, MO). The superoxide dismutase (SOD) assay kit was purchased from Faith Technology (Taichung, Taiwan); the GSH assay kit was purchased from Cayman Chemicals (Ann Arbor, MI); the protein assay kit was purchased from Bio-Rad (Hercules, CA); and the anti-nuclear factor E2-related protein (Nrf2), anti-heme oxygenase-1 (HO-1), anti-high-mobility group protein B1 (HMGB1), anti-nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB), anti-tumor necrosis factor (TNF)-α, anti-interleukin (IL)-1β, and anti-IL-6 antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Experimental Animals and Procedures. The 6-week-old male BALB/c mice were purchased from the National Laboratory Animal

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Center (Taipei, Taiwan). The animal experiments in this study were approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University (IACUC approval number 98-76) and followed related regulations of the Animal Care and Use Committee. Animals were housed in animal rooms at the Department of Food Science and Biotechnology of National Chung Hsing University. The temperature and relative humidity (RH) of the animal rooms were maintained at constant levels (22 ± 2 °C and RH of 65 ± 5%). A 12 h light/dark cycle was maintained, and the light cycle was controlled by an automatic timer; the light period was at 06:00−18:00, and the dark period was at 18:00−06:00. After arrival, the experimental mice were immediately weighed, and after a statistical analysis, they were distributed to feeding cages according to a normal distribution. Animals were allowed *ad libitum* access to water and food (laboratory rodent diet 5001, Purina, St. Louis, MO).

The animal groupings and treatments were modified from the methods by Mitazaki et al.[20] and Arjumand et al.[14] After adaptive feeding for 1 week, the 6-week-old male BALB/c mice were continuously fed the sample diets for 8 days (GA at 25, 50, or 100 mg/kg of BW or 18βGA at 10, 25, or 50 mg/kg of BW). After 30 min of sample administration on day 6, an intraperitoneal (IP) injection of CP (30 mg/kg of BW) was given to induce damage, and 30 min prior to the injection of CP on day 6, the positive control group was IP-injected with the chemotherapy protectant, AMF (200 mg/kg of BW). The mice were sacrificed on day 9 to collect samples.

Analysis of Serum Biochemical Values. Serum biochemical values, including BUN, creatinine, and lactate dehydrogenase (LDH), were analyzed using the ADVIA Chemistry Urea Nitrogen Reagent, ADVIA Chemistry Enzymatic Creatinine-2 Reagent, and ADVIA Lactate Dehydrogenase L-P Reagent, respectively (Siemens, NY). The analyses were performed according to protocols in the manuals of the manufacturer.

Histopathological Studies. Renal tissues of BALB/c mice were fixed in 10% formaldehyde immediately following sacrifice, processed for histological examination according to a conventional method, and stained with hematoxylin and eosin (H&E). The morphology of any observed lesions was classified and recorded according to the classification criteria by Shackelford et al.[21]

Analysis of the Activities of Antioxidant Enzymes. Catalase, SOD, glutathione peroxidase (GPx), and glutathione reductase (GRd) activities were measured using an assay kit (Cayman, Ann Arbor, MI) according to the instruction of the manufacturer. The GSH/oxidized glutathione (GSSG) ratio was analyzed using a GSH assay kit purchased from Cayman (Ann Arbor, MI).

Measurement of Malondialdehyde (MDA). The MDA levels in kidney samples of experimental rats were determined using the thiobarbituric acid (TBA) method, with modifications.[22] Briefly, following a preincubation, 0.5 mL of tissue homogenate was mixed with 1 mL of 15% trichloroacetic acid, 0.375% thiobarbituric acid reactive substances (TBARS), and 0.25 mM HCl and then heated in boiling water for 45 min. After centrifugation (2000*g* for 15 min), the absorbance of the butanol phase was read at 535 and 520 nm. The difference between the two values was used to calculate the MDA concentration. An MDA standard was prepared from 1,1,3,3,-tetraethoxypropane. Total protein concentrations were determined by Lowry's method using bovine serum albumin as the standard. The MDA concentration was normalized against the total protein concentration and is expressed in micromoles per milligram of protein.

Immunohistochemical (IHC) Staining. Paraffin blocks of kidney tissues were cut using a microtome into 2 μm thick sections. Sections were floated on water to allow for the tissues to unfold and extend. Tissue sections were placed on 2-aminopropyltriethoxysilane-coated slides, dried at 37 °C, and deparaffinized using xylene. Deparaffinized slides were treated with 3% H_2O_2 and proteinase K (0.5 mg/mL) for 10 min, washed with distilled water 3 times, and incubated in blocking buffer (StartingBlock, Pierce, Rockford, IL) for 5 min. When the slides had slightly dried, they were stained with primary antibodies for 30 min at 40 °C (anti-Nrf2, anti-HO-1, anti-HMGB1, anti-NF-κB, anti-TNF-α, anti-IL-1β, and anti-IL-6 antibodies were used in this study). After the slides were washed with phosphate-buffered saline (PBS) 3 times, they were incubated in EnVision-labeled polymer peroxidase-conjugated anti-immunoglobulin G (IgG, Dako, Glostrup, Denmark) at room temperature for 30 min. Slides were again washed with PBS 3 times and then incubated in diluted 3,3-diaminobenzidine tetrahydrochloride (DAB-4HCl, Dako) for color development. After development, slides were washed with PBS 3 times and counterstained with hematoxylin. Slides were then washed with distilled water 3 times, dehydrated, dried, and mounted. The intensity of antigen expression after staining was quantified using Image-Pro Plus 6.3 image analysis software (Media Cybernetics, Rockville, MD). Results are presented as the positively stained area (%).

Statistical Analysis. All data are expressed as the mean ± standard deviation (SD). An analysis of variance (ANOVA) was used to evaluate differences among multiple groups. Significant differences were subjected to Duncan's test to compare the means of two specific groups. A p value of <0.05 was considered significant.

RESULTS

Evaluation of Renal Function Parameters To Determine the Effects of GA and 18βGA on CP-Induced Renal Injury in BALB/c Mice. The chemical structures of GA and 18βGA are shown in Figure 1A. As shown in Figure 1B, an IP injection of CP (30 mg/kg of BW) induced significant increases in serum BUN, creatinine, and LDH in BALB/c mice. Administration of GA (25, 50, and 100 mg/kg of BW) and 18βGA (10, 25, and 50 mg/kg of BW) significantly reduced CP-induced BUN, creatinine, and LDH levels. The positive control of AMF (200 mg/kg of BW) also showed a protective effect on kidneys exposed to CP-induced acute renal injury. In addition, administration of a high dose of GA or 18βGA alone did not affect levels of BUN, creatinine, or LDH in the kidneys (p < 0.05).

Tissue Pathological Analysis of the Effects of GA and 18βGA on CP-Induced Renal Injury in BALB/c Mice. Acute renal tubular necrosis is an important pathological feature of CP-induced renal injury,[23] and this study used morphological changes in the appearance of the kidneys and renal tubular lesions as indicators to evaluate renal injury. Results showed that CP induced significant lesions that produced whitening of the kidneys in mice. The macroscopic appearances were similar among the GA-, 18βGA-, and AMF-treated sample groups and CP-untreated control group and exhibited a hyperemic dark-maroon color (left panel of Figure 2). The right panel of Figure 2 shows pathological tissue sections with H&E staining (400×) in the GA and 18βGA experimental groups. Changes in tissue morphology indicated that the control group presented a healthy normal morphology of renal glomeruli and tubules, whereas the CP group presented severe renal tubular injury, while the GA and 18βGA groups presented different degrees of protective effects. Further pathological interpretation and classification of the above sections based on the four pathological features of renal tubules, including tubular necrosis, tubular degeneration, hyaline cast, and dilation, were performed according to the classification criteria by Shackelford et al.[21] (Table 1). A statistical analysis of injury scores for the GA and 18βGA experimental groups showed that both compounds significantly protected renal tubules from CP-induced injury (p < 0.05) in dose-dependent manners. In addition, renal tubular injury was found to interfere with the reabsorption of renal filtrate, which might affect BW changes. Administration of a high dose of GA (100 mg/kg of BW) or 18βGA (50 mg/kg of BW) alone did not the affect BW changes in experimental animals (data not shown).

Effects of GA and 18βGA on CP-Induced Lipid Peroxide Accumulation and Alterations of Antioxidant Enzyme

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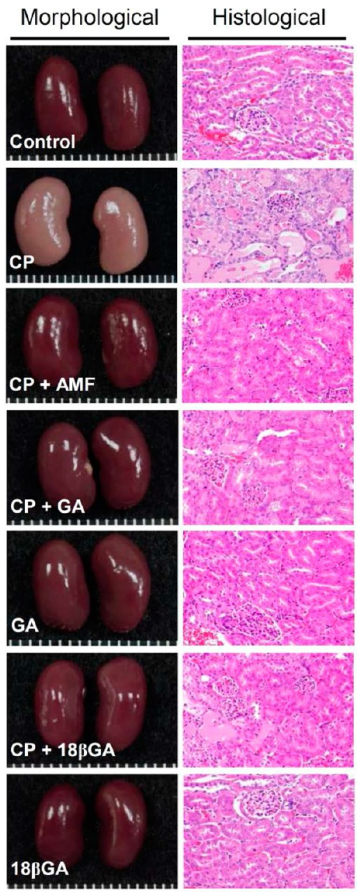

Figure 2. Effects of GA and 18βGA on the kidney morphology and histology in BALB/c mice with CP-induced renal injury. Groups of mice were treated as described in Figure 1, and the data shown are from a representative experiment. Representative macroscopic appearances of the kidneys (left panel) and H&E-stained kidneys (right panel, 400×) were used to assess the renoprotective effects of GA (100 mg/kg of BW) and 18βGA (50 mg/kg of BW) against CP-induced nephrotoxicity in experimental mice.

Activities in the Kidneys of BALB/c Mice. During the pathological process of CP-induced acute renal injury, accumulation of lipid peroxides and reductions in antioxidant enzyme activities in the kidneys play important roles;[24] however, supplementation with antioxidants or activation of the antioxidant defense system can provide a certain degree of protection.[25,26] This study also confirmed that CP promoted formation of MDA and reduced activities of antioxidant enzymes, such as catalase, SOD, and GPx, and the GSH/GSSG ratio in the kidneys. Except for the low dose of GA (25 mg/kg of BW) or 18βGA (10 mg/kg of BW), which showed no effect on GPx activity ($p < 0.05$), AMF and other doses of GA and 18βGA inhibited lipid peroxidation and restored the protective features of the above antioxidant enzymes and GSH/GSSG ratio (Figure 3).

Effects of GA and 18βGA on CP-Induced Antioxidant Protein Expressions in Kidneys of BALB/c Mice. This study used an IHC method to show for the first time that, in a CP-induced renal injury mouse model, the antioxidant transcription factor, Nrf2 (Figure 4A), and its downstream, HO-1 (Figure 4B), were significantly activated in the kidneys. However, GA (50 mg/kg of BW) and 18βGA (25 mg/kg of BW) stimulated antioxidant transcription factor Nrf2 and further stimulated the expression of the above antioxidant proteins in the kidney after CP-induced injury (Figure 4 and Supplementary Figure 1 of the Supporting Information). This result might explain the increased activity of antioxidant enzymes in the kidneys after administration of GA and 18βGA.

Effects of GA and 18βGA on Kidney Inflammation-Related Indicators in BALB/c Mice with CP-Induced Renal Injury. This study also performed IHC analyses and directly used the staining intensity of the detected target molecules in tissues to evaluate alterations in inflammation-related factors in kidney tissues. CP stimulated expression of the NF-κB transcription factor in the kidneys (Figure 5A); however, administration of GA, 18βGA, and AMF all showed a protective effect and significantly inhibited NF-κB (panels A and B of Figure 5). They also had significant inhibitory effects on proinflamma-

Table 1. Histology Injury Scores of CP-Induced Kidney Injury under GA, 18βGA, and AMF Treatment in BALB/c Mice

	injury score[a]			
group[b]	necrosis	hyaline cast	degeneration	dilation
control	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
CP	2.1 ± 0.8 c	2.2 ± 0.8 c	3.0 ± 0.9 d	1.4 ± 0.5 b
AMF + CP	0.8 ± 0.8 ab	0 ± 0 a	0.8 ± 0.8 ab	0 ± 0 a
GA-10 + CP	1.0 ± 0.6 b	1.0 ± 0.6 b	1.8 ± 0.4 c	0 ± 0 a
GA-25 + CP	0.8 ± 0.4 ab	0.6 ± 0.5 ab	1.4 ± 0.5 bc	0 ± 0 a
GA-100 + CP	0.2 ± 0.4 ab	0.4 ± 0.5 ab	0.2 ± 0.4 a	0 ± 0 a
GA-100	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
control	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
CP	2.1 ± 0.8 e	2.2 ± 0.8 b	3.0 ± 0.9 c	1.4 ± 0.5 b
AMF + CP	0.8 ± 0.8 bc	0 ± 0 a	0.8 ± 0.8 ab	0 ± 0 a
18βGA-10 + CP	1.6 ± 0.5 cd	1.8 ± 0.4 b	1.6 ± 0.5 b	0 ± 0 a
18βGA-25 + CP	1.2 ± 0.4 d	1.6 ± 0.5 b	1.2 ± 0.9 b	0.4 ± 0.8 a
18βGA-50 + CP	0.4 ± 0.5 ab	0.6 ± 0.5 a	0 ± 0 a	0 ± 0 a
18βGA-50	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a

[a]Degree of lesions was graded from 0 to 5 depending upon the severity: 0, no significant lesions; 1, minimal (<1%); 2, slight (1−25%); 3, moderate (26−50%); 4, moderate/severe (51−75%); and 5, severe/high (76−100%). Each value is expressed as the mean ± SD ($n = 8$), and letters (a−e) indicate statistically significant differences in each group ($p < 0.05$). One-way ANOVA tests were used for the statistical analysis. [b]CP, 30 mg/kg of BW; AMF, 200 mg/kg of BW; GA-25, 25 mg/kg of BW; GA-50, 50 mg/kg of BW; GA-100, 100 mg/kg of BW; 18βGA-10, 10 mg/kg of BW; 18βGA-25, 25 mg/kg of BW; and 18βGA-50, 50 mg/kg of BW.

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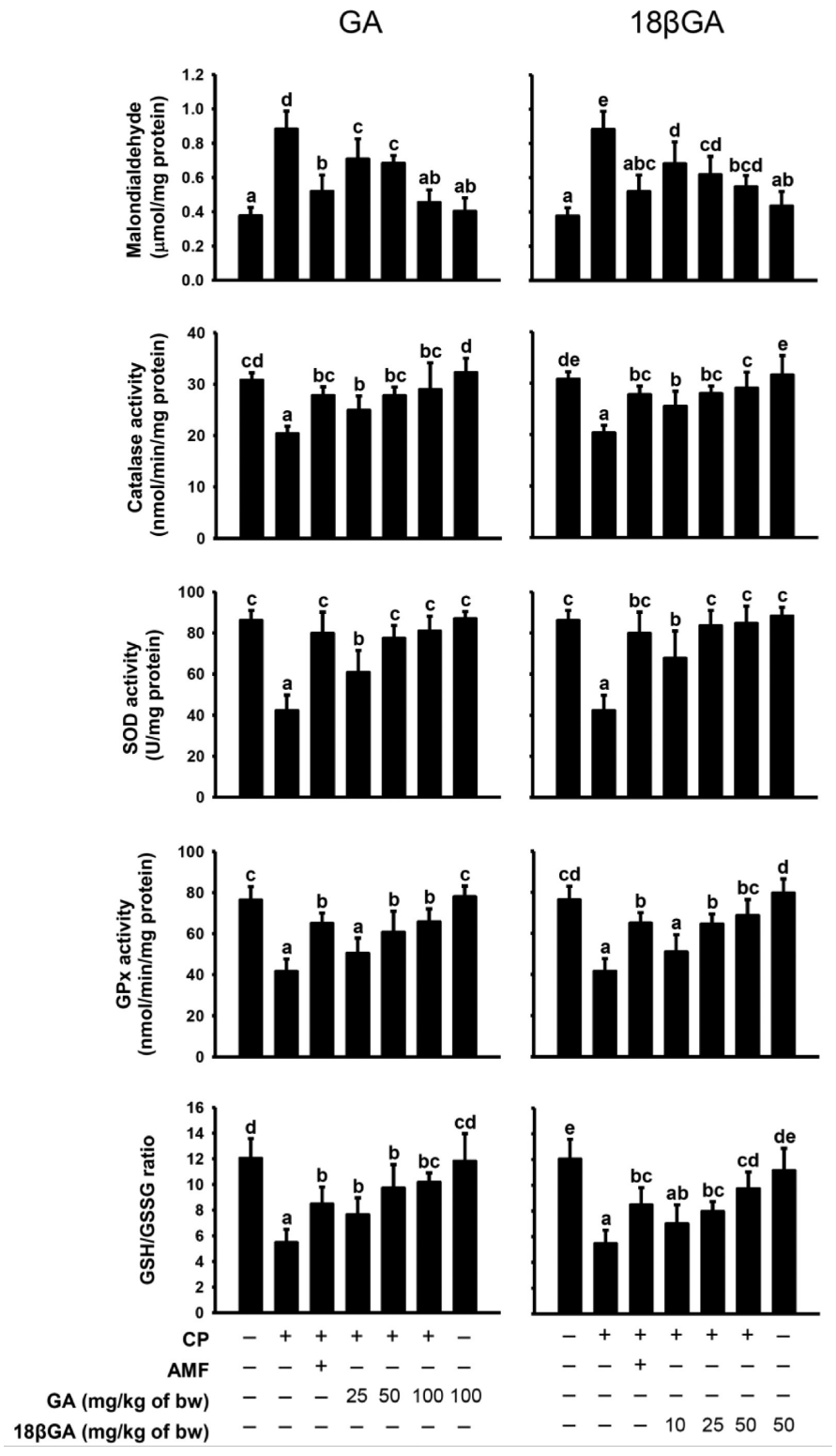

Figure 3. Effects of GA and 18βGA on CP-induced lipid peroxidation and alterations in antioxidant enzyme activities and the GSH/GSSG ratio in renal tissues of BALB/c mice. Groups of mice were treated as described in Figure 1. Data are presented as the mean ± SD (n = 8), and letters (a−e) indicate statistically significant differences in each group (p < 0.05). One-way ANOVA tests were used for the statistical analyses.

tory cytokines, including TNF-α (Figure 5C and Supplementary Figure 2 of the Supporting Information), IL-1β (Figure 5D and Supplementary Figure 2 of the Supporting Information), and IL-6 (Figure 5E and Supplementary Figure 2 of the Supporting Information).

Effects of GA and 18βGA on the Expression of HMGB1 in Kidneys of BALB/c Mice with CP-Induced Renal Injury. HMGB1, a nuclear protein that is usually actively or passively released during stress or necrosis in cells, transmits cell injury signals and activates a series of self-protection mechanisms.[27] Results of the IHC analysis shown in the left panel of Figure 6

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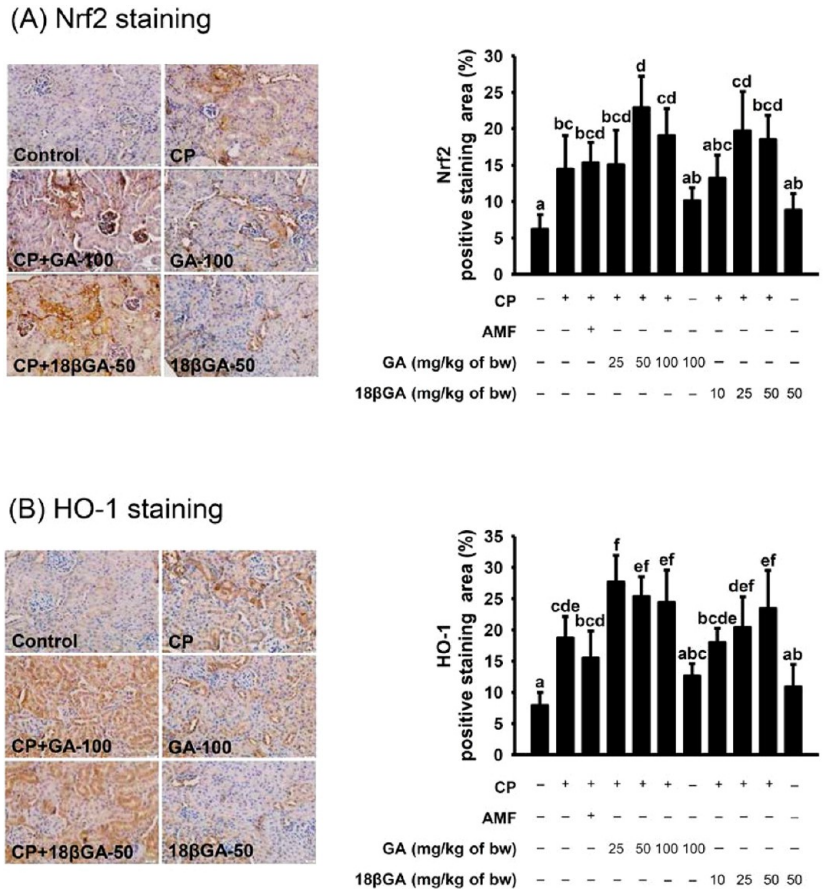

Figure 4. Effects of GA and 18βGA on the IHC expressions of Nrf2 and HO-1 in experimental mice. Groups of mice were treated as described in Figure 1. Renal tissues were analyzed by IHC staining for Nrf2 (A, left panel) and HO-1 (B, left panel). Nrf2- (A, right panel) and HO-1- (B, right panel) stained areas were quantified, and numbers of positively stained areas (%) per 400× field were averaged across eight fields for each section. Each value is expressed as the mean ± SD ($n = 8$), and letters (a−d) indicate statistically significant differences in each group ($p < 0.05$). One-way ANOVA tests were used for the statistical analyses.

indicated that, after CP-induced tissue injury to kidneys of BALB/c mice, HMBG1 showed a typical staining result, indicating diffuse release from nuclei. GA and 18βGA each significantly inhibited the accumulation of HMGB1 in the cytoplasm in a dose-dependent manner, and their inhibitory effects were greater than that of AMF, a known protectant (right panel of Figure 6). In summary, GA and 18βGA were able to decrease expressions of NF-κB, TNF-α, IL-1β, and IL-6 and release of HMGB1; in addition, they provided a protective function by increasing Nrf2 and HO-1 levels.

DISCUSSION

Since approval of CP by the U.S. Food and Drug Administration (FDA) in 1978 as a chemodrug, it has provided great therapeutic contributions to treating solid tumors, such as testicular cancer. Chemotherapy usually causes strong side effects in patients, including serious damage to normal organs, often leading to cessation of treatment; therefore, chemotherapy causes a bottleneck in current cancer treatment. The major clinical side effect of CP is nephrotoxicity, and patients usually show increased serum BUN and creatinine levels in 10 days after an injection.[4] Oxidative stress is the major cause of nephrotoxicity, and CP induces the production of a large amount of ROS in renal tubular cells. Although mitochondria in cells continue to clear ROS by activating antioxidant enzymes (such as catalase, SOD, GPx, and GST), eventually, antioxidant enzyme activities decrease, intracellular GSH is depleted, and mitochondrial dysfunction is induced, which causes cell necrosis and impaired renal function.[28] CP also directly stimulates renal tubular cells to secret TNF-α, which causes severe inflammatory responses, aggravates renal tubular cell damage, directly causes blood vessel injury, reduces blood flow in the kidneys, induces ischemic necrosis of cells, and eventually results in renal failure.[2]

Studies on applying phytochemicals to ameliorate drug side effects are still emerging. The literature shows that quercetin may decrease CP-induced NF-κB and TNF-α expressions and reduce kidney inflammation.[19,29] Lycopene may restore CP-depleted antioxidant enzyme activities through activating the Nrf2/HO-1 signaling pathway.[24] Hesperidin has antioxidant and anti-inflammatory effects and might also decrease CP-induced renal tubular necrosis and high levels of serum BUN and creatinine.[30] Those results show that, during the process of CP-induced renal injury, effective control of excess inflammatory responses and oxidative damage had definite positive effects on renal function maintenance and disease prevention. Previous studies showed that GA and 18βGA have multiple biological activities, such as antioxidant and anti-inflammatory effects,[17,18,31] and are closely associated with the mechanism underlying the aforementioned CP-induced renal injury. Therefore, we speculated that GA and 18βGA might have protective potentials to relieve the nephrotoxicity caused by CP. AMF is a drug currently approved to relieve nephrotoxicity caused by CP treatment. Clinical studies

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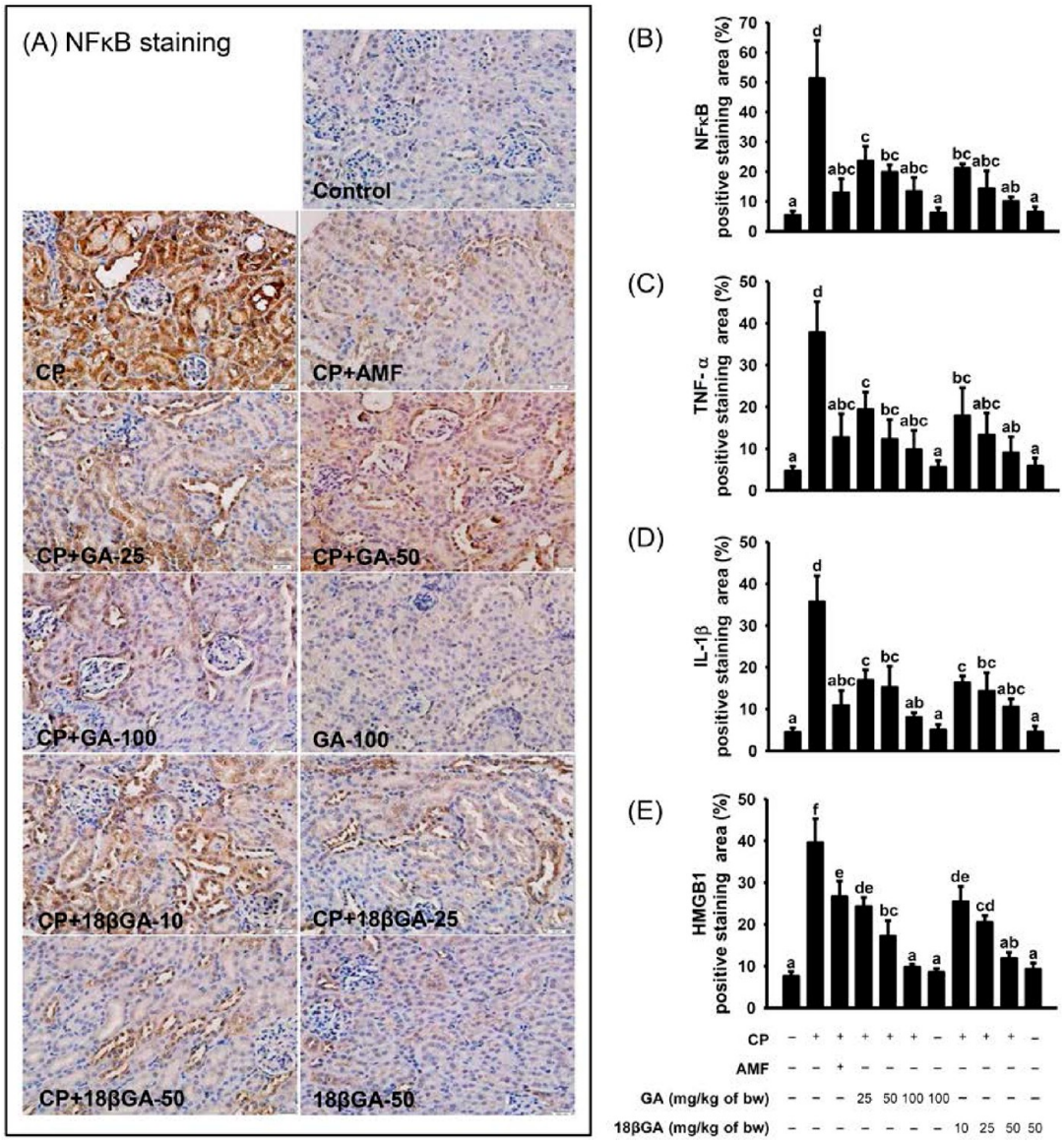

Figure 5. Effects of GA and 18βGA on CP-induced proinflammatory mediator expressions in renal tissues. Groups of mice were treated as described in Figure 1. (A) Renal tissues were analyzed by IHC staining for NF-κB. (B) NF-κB-, (C) TNF-α-, (D) IL-1β-, and (E) IL-6-stained areas were quantified, and numbers of positively stained areas (%) per 400× field were averaged across eight fields for each section. Each value is expressed as the mean ± SD (n = 8), and letters (a–f) indicate statistically significant differences in each group ($p < 0.05$). One-way ANOVA tests were used for the statistical analysis. CP, 30 mg/kg of BW; AMF, 200 mg/kg of BW; GA-25, 25 mg/kg of BW; GA-50, 50 mg/kg of BW; GA-100, 100 mg/kg of BW; 18βGA-10, 10 mg/kg of BW; 18βGA-25, 25 mg/kg of BW; and 18βGA-50, 50 mg/kg of BW.

have confirmed that AMF has inhibitory effects on the accumulation of nephrotoxicity in lung adenocarcinoma patients.[5,6] Therefore, this study selected AMF as the drug in the control group for use as a baseline control of all injury indicators in mice with CP-induced renal injury.

Activation of the Nrf2 is a defense mechanism after toxic insult. In response to CP, the current work also confirmed that expressions of Nrf2 protein were increased (Figure 4A) and the activities of antioxidant enzymes as well as the GSH/GSSG ratio were declined (Figure 3), reflective of more oxidative stress. Instead, not only GA but also 18βGA supplementation was found to significantly improve the changes associated with CP nephrotoxicity, as also evident by the increased level of antioxidant enzymes. A previous study showed that HO-1 expression was upregulated by these two phytochemicals to eliminate ROS formation.[31] Therefore, the expected induction of antioxidant enzymes by GA or 18βGA in CP-treated mice may be at least partly attributed to the functional activation of Nrf2/HO-1 signaling. However, the free radical scavenging ability of GA and 18βGA cannot be ruled out as a mechanism underlying the antioxidant enzyme-inducing effect in response to CP. The protective effects of GA and 18βGA may also be attributable to their ability to scavenge ROS and subsequently ameliorate CP-mediated oxidative stress and appear to be linked to the differential levels of antioxidant enzymes in CP-treated mice.

Previous studies showed that licorice plant extracts can inhibit ochratoxin-A-induced renal injury.[32] GA also showed protective effects against gentamicin-induced acute renal injury in rats.[33] In an ischemic model, GA also decreased neuronal cell apoptosis[17] and acute renal failure in animals.[11] Results of this study also confirmed that GA and 18βGA effectively inhibited CP-induced expressions of proinflammatory agents (NF-κB, TNF-α, IL-1β,

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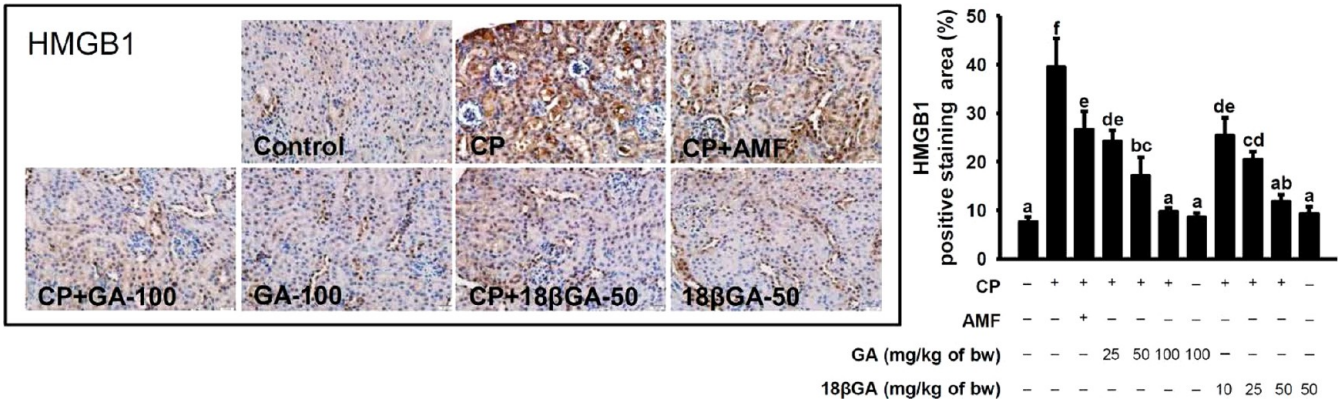

Figure 6. Effects of GA and 18βGA on CP-induced HMGB1 release from nuclei to the cytoplasm in renal tissues. Groups of mice were treated as described in Figure 1. Renal tissues were analyzed by IHC staining for HMGB1 (left panel); HMGB1-stained areas were quantified; and numbers of positively stained areas (%) per 400× field were averaged across eight fields for each section (right panel). Each value is expressed as the mean ± SD (n = 8), and letters (a–f) indicate statistically significant differences in each group ($p < 0.05$). One-way ANOVA tests were used for the statistical analyses. CP, 30 mg/kg of BW; AMF, 200 mg/kg of BW; GA-100, 100 mg/kg of BW; and 18βGA-50, 50 mg/kg of BW.

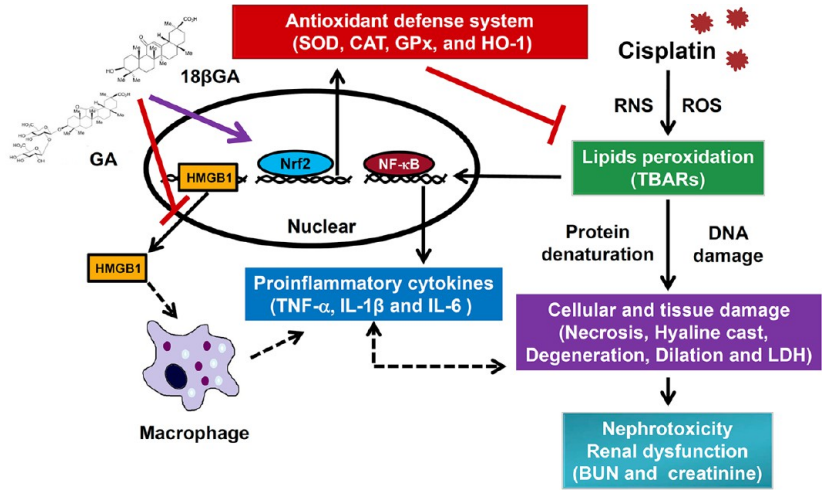

Figure 7. Proposed mechanisms of GA and 18βGA on CP-induced nephrotoxicity in BALB/c mice.

IL-6, and HMGB1) in kidney tissues of BALB/c mice, restored the activities of antioxidant enzymes and the GSH/GSSG ratio, and suppressed CP-induced oxidative stress in the kidneys. Therefore, the conclusion of this study is that GA and 18βGA can be used to relieve CP-induced chemical renal injury because of their antioxidant and anti-inflammatory features.

It is known that HMBG1 is a chaperone protein of nuclear DNA and a secreted protein. HMBG1 can be passively released from necrotic cells or actively released from inflammatory cells, act as an extracellular damage-associated molecular pattern (DAMP) molecule, and is an important mediator of inflammatory responses.[27] Although the association between CP and HMGB1 in cancer treatment is not clear, studies indicated that CP promoted HMGB1 expression in human myeloma cells[34] to produce drug resistance. Sitia et al.[35] suggested that GA interfered with the aggregation of chemotactic neutrophils and other inflammatory cells caused by HMGB1 in hepatitis B virus transgenic mice and relieved hepatic lesions. In addition, GA directly interacts with HMGB1 to inhibit the chemoattractant activity of HMGB1 on inflammatory cells and reduce HMGB1-induced inflammatory responses.[36] The results of this study also found that GA and 18βGA reduced the release of HMGB1 from cell nuclei during CP-induced injury. This result suggests a possible cause of the inhibitory effects of GA and 18βGA on many proinflammatory cytokines during renal injury.

The doses employed in the current work (25–100 mg/kg of BW for GA and 10–50 mg/kg of BW for 18βGA) were based on previous animal studies showing the effectiveness of GA or 18βGA to treat hyperglycemia[16] and chemical-induced hepatotoxicity.[37,38] 18βGA is a metabolic product of GA by way of intestinal bacterial enzymes. Our pilot experiment showed that 100 mg of 18βGA/kg of BW had an unexpected effect of causing a decrease in mice activities and yellowish skin (data not shown). Therefore, this study selected 50 mg of 18βGA/kg of BW, because that dose caused no abnormal conditions in mice. A comparison of the protective effects against renal injury between GA and 18βGA at 50 mg/kg of BW each showed that the biological activity of 18βGA was better than that of GA. This result indicates that 18βGA can possibly be used as an adjuvant factor that has the potential to delay side effects during chemotherapy. Kao et al.[31] indicated that 18βGA had a better anti-inflammatory effect in a cell culture model than GA. GA and 18βGA have different anti-inflammatory mechanisms, with GA mainly activating the PI3K/Akt/GSK3β pathway and 18βGA mainly activating the glucocorticoid receptor. This difference might be explained by the structure of 18βGA, which is much closer to that of steroids; therefore, 18βGA may be able to

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activate glucocorticoid receptors to exhibit an improved anti-inflammatory effect.[39]

In addition to GA and 18βGA, there are some constitutes in licorice, such as chalconoids (liquiritin, isoliquiritin, liquiritigenin, and isoliquiritigenin), isoflavonoids (dehydroglyasperin C, dehydroglyasperin D, and glabridin), 18βGA derivative (carbenoxolone), and some unique compounds (licoricidin, licorisoflavan A, and licochalcone A) that have been well-studied and biologically characterized.[10] These effects have been associated with the anti-inflammatory, antioxidative, antimicrobial, antivirus, cell protective, and chemopreventive properties of the compounds, which may also have potential to be developed into a natural chemoprotectant, and they should be analyzed through further investigations in the future.

In summary, CP-induced nephrotoxicity is produced by a combination of interactions among many pathways. GA and 18βGA are phytochemicals in licorice with multiple physiological activities, and a possible mechanism underlying their ability to delay CP-induced physiological renal injury is shown in Figure 7. Our results indicate that GA and 18βGA have the potential to be developed into natural adjuvants to relieve CP side effects in patients undergoing chemotherapy, and they should be analyzed through further clinical validations and investigations in the future.

■ ASSOCIATED CONTENT

Supporting Information

Effects of GA and 18βGA on the IHC expressions of Nrf2 and HO-1 in experimental mice (Supplemental Figure 1) and effects of GA and 18βGA on CP-induced proinflammatory mediator expressions in renal tissues (Supplemental Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

AMF, amifostine; BUN, blood urea nitrogen; CP, cisplatin; GA, glycyrrhizic acid; 18βGA, 18β-glycyrrhetinic acid; HMGB1, high-mobility group protein B1; HO-1, heme oxygenase-1; IHC, immunohistochemical; LDH, lactate dehydrogenase; MDA, malondialdehyde; Nrf2, nuclear factor E2-related protein; ROS, reactive oxygen species

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CLINICAL AND LABORATORY OBSERVATION ON THE EFFECT OF GLYCYRRHIZIN IN ACUTE AND CHRONIC VIRAL HEPATITIS

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Twenty cases each of acute and chronic viral hepatitis were treated with potassium glycyrrhizinate, and another 20 such cases were treated with inosine and Poly I:C as control group. Results showed that improvement of liver function, decrease of SGPT levels and negative conversion of HBsAg and HBeAg were more marked in the glycyrrhizin group than in the control. Short-term follow-up of the 20 acute cases in the glycyrrhizin group showed clinical cure in 17 and marked improvement in 3, while the 20 cases in the control group showed clinical cure in 7, marked improvement in one, improvement in 7 and no effect in 5. Of the chronic cases, short-term follow-up of the 20 cases in the glycyrrhizin group showed clinical cure in 15, marked improvement in 2, improvement in one and no effect in 2, while the 20 cases in the control group showed clinical cure in 2, improvement in 5 and no effect in 13. Animal experiments revealed that glycyrrhizinic acid lessened the liver damage induced by carbon tetrachloride in rats, reduced the serum levels of SGPT and triglyceride content in liver homogenate, and promoted the detection of serum αFP. In experimental fatty liver and liver cirrhosis, glycyrrhizinic acid markedly lessened the fatty changes and fibrohyperplasia. However, resorption of fibrous tissue was not promoted in the rats with liver cirrhosis.

Based on the successful results of Sancao Decoction (Glycyrrhiza, Oldenlandia and Prunella Spike) in treating acute and chronic viral hepatitis,[1] we have recently screened for the effective ingredient of the decoction. The results of clinical and animal observation on the effect of glycyrrhizin are reported as follows.

CLINICAL OBSERVATION

Case Selection and Grouping

Eighty patients with acute and chronic hepatitis diagnosed according to the criteria set by the national conference on viral hepatitis held in 1978 were admitted and randomly divided into 2 groups of equal number: the glycyrrhizin and the control groups. There were 20 cases of acute hepatitis and 20 of chronic hepatitis in each group. Twelve males and 8 females comprised the acute cases in the glycyrrhizin group. These ranged in age between 14 and 61 (average 31.2). Average duration of illness before admission was 7 days. Sixteen males and 4 females made up the chronic cases in the glycyrrhizin group, who ranged in age between 17 and 54 (average 27.9). Duration of illness was 6 months to 3 years, with an average of 15 months.

Drug Preparation

Glycyrrhizin was prepared by the pharmacy laboratory of our hospital. Glycyrrhiza slices were boiled in water and the de-

coction was concentrated. Crude glycyrrhizinic acid was precipitated by addition of the concentrated sulfuric acid. The precipitate was dissolved in acetone, with 20% potassium hydroxide added later, producing tripotassium glycyrrhizinate. The latter was refluxed with glacial acetic acid. The solution so formed was made to stand at 0-10°C to allow for crystallization of monopotassium glycyrrhizinate. The crystals were washed with acetic acid and ethanol, and then dried in vacuum. The glycyrrhizin obtained was a yellow powder which was packed as capsules each containing the equivalent of 7.5 gm of the crude drug.

Treatment and Observation

Each patient in the glycyrrhizin group received one capsule of glycyrrhizin twice a day, plus vitamins C (0.2 gm), K (8 mg) and E (20 mg) t.i.d. per os. Each patient in the control group received intramascular injection of inosine 100 mg once daily, and Poly I:C 2 mg twice a week, with the same oral dosage of vitamins C, K and E as in the glycyrrhizin group. The course of treatment for the 2 groups was similarly scheduled, i.e. 30 days for acute hepatitis and 90 days for chronic hepatitis. Liver function and immunoglobulins were determined before and after the treatment in acute cases, while the tests were performed monthly in chronic cases. HBsAg and HBeAg were determined before and after the treatment in all cases.

Results

The effect was evaluated according to the degree of improvement in liver function. The criteria for short-term clinical cure: all items of liver function became normal; marked improvement: SGPT<200u (King's), TTT<10u, ZnTT<14u; improvement: SGPT>200u TTT>10u, ZnTT>14u; no effect: liver function not improved or even worse. Results showed that better effect was obtained in the glycyrrhizin group than in the control in both acute and chronic cases (Table 1). The improvement of liver function and normalization of immunoglobulins were also more marked in the glycyrrhizin group than in the control (Table 2).

Table 3 shows that in the glycyrrhizin group 3 and 1 of the 20 acute cases were positive for HBsAg and HBeAg respectively before treatment, of the 20 chronic cases 17 and 10 were positive for HBsAg and HBeAg respectively before treatment. Of the acute patients after treatment, HBsAg and HBeAg returned to negative in 2 and 1 case respectively; of the chronic patients after treatment, HBsAg and HBeAg became negative in 8 and 5 cases respectively. Of the 20 acute patients in the control group 5 and 2 were positive for HBsAg and HBeAg respectively before treatment; of the 20 chronic cases before treatment, 17 and 14 were positive for HBsAg and HBeAg respectively. However, none of the acute or chronic cases became negative after treatment. This indicated that about 50% of acute and chronic cases with positive HBsAg and HBeAg returned to negative after glycyrrhizin treatment.

LABORATORY STUDY

Effect of Glycyrrhizin on Experimental Hepatitis in Rats

Twenty-eight healthy Sprague-Dawley rats weighing 150-200 gm were randomly

Table 1. Comparison of glycyrrhizin and control groups

Effect	Acute hepatitis		Chronic hepatitis	
	Glycyrrhizin group (20 cases)	Control group (20 cases)	Glycyrrhizin group (20 cases)	Control group (20 cases)
Clinical cure	17	7	15	2
Marked improvement	3	1	2	0
Improvement	0	7	1	5
No effect	0	5	2	13
P values	$P < 0.001$ ($X^2 = 17.2$)		$P < 0.001$ ($X^2 = 22.7$)	

Remarks: Numbers in the Table indicate No. of cases

Table 2. Comparison of laboratory findings in glycyrrhizin and control groups before and after treatment

	Acute hepatitis				Chronic hepatitis			
	Glycyrrhizin group (20 cases)		Control group (20 cases)		Glycyrrhizin group (20 cases)		Control group (20 cases)	
	Before	After	Before	After	Before	After	Before	After
Icteric index (u)	37.7±3.3	***6.5±3.4	30.4±4.2	**7.7±5.0	27.4±5.0	**4.0±5.5	29.4±2.9	25.2±3.0
TTT (u)	10.9±2.1	*3.8±2.3	13.7±6.0	17.5±6.7	17.5±4.4	*2.9±4.2	13.4±4.0	15.3±3.8
ZnTT (u)	15.1±3.0	**2.8±2.0	16.0±2.8	10.8±3.0	17.5±4.0	*2.5±5.0	17.8±5.2	16.6±4.9
SGPT (u)	569.3±4.8	***80.2±3.7	511.4±1.2	***105.3±1.8	216.5±4.7	***20.3±4.0	344.4±3.9	***285.3±4.0
IgG (mg%)	2262.1±7.8	***1637.3±5.0	2164.9±2.8	***2001.0±4.0	2132.6±4.2	***1307.1±5.1	1932.0±1.0	***2010.3±1.5
IgA (mg%)	281.0±5.1	***190.6±5.2	320.1±3.0	***298.1±1.0	219.3±5.0	***108.5±4.0	229.2±5.4	218.5±4.0
IgM (mg%)	361.1±3.6	***77.3±4.0	219.2±2.9	*199.0±7.0	223.3±1.0	***145.4±7.9	197.8±1.0	200.0±2.0

Remarks: Numbers in the Table are M±SE
* Compared with before treatment $P < 0.05$;
** Compared with before treatment $P < 0.01$;
*** Compared with before treatment $P < 0.001$.

Table 3. Comparison of negative conversion of HBsAg, HBeAg in the glycyrrhizin and control groups

Grouping		Acute hepatitis				Chronic hepatitis			
		HBsAg		HBeAg		HBsAg		HBeAg	
		+	−	+	−	+	−	+	−
Glycyrrhizin group	Before treating	3	17	1	19	17	3	10	10
	After treating	1	19	0	20	9	11	5	15
Control group	Before treating	5	15	2	18	17	3	14	6
	After treating	5	15	2	18	17	3	14	6

placed in 3 groups, 8 in the normal group and 10 each in the pathological and glycyrrhizin groups. On the 1st and 5th day of the experiment, rats in the pathological and glycyrrhizin groups received hypodermic injection of carbon tetrachloride 0.5 ml/100 gm body weight. From the 2nd experimental day rats in the glycyrrhizin group received 30 mg of glycyrrhizin injection once daily, while an equivalent amount of normal saline was injected to the rats in the pathological group. The rats were sacrificed on the 8th day and a portion of the liver of each rat was prepared as homogenate; triglyceride was determined. The remaining liver was fixed in neutral-buffered formalin. Sections were stained with hemotoxylin-eosin and Sudan III for pathological study. The items for observation were the same as described in a previous report.[2] SGPT and αFP (RPHA) levels were determined in the rat sera.

The laboratory findings are shown in Tables 4 and 5. Pathological examination showed hepatic cellular necrosis, vacuolar and fatty changes, the changes being less marked in the glycyrrhizin group. SGPT activity and glyceride content in homogenate from the glycyrrhizin group were markedly less in the glycyrrhizin group than in the pathological group ($P<0.01$). All 10 rats in the glycyrrhizin group were positive for αFP

Table 4. Effect of glycyrrhizin on pathological changes in acute hepatitis in rats

Groups	No. of rats	Cellular necroses				Loose vacuolar changes				Fatty degeneration			
		−	+	++	+++	−	+	++	+++	−	+	++	+++
Pathological group	10	1	4	4	1	1	5	3	1	0	1	6	3
Glycyrrhizin group	10	5	5	0	0	3	6	1	0	0	4	6	0

Table 5. Effect of glycyrrhizin on the SGPT activity and triglyceride content in liver homogenate of rats with experimental acute hepatitis

Groups	SGPT (unit)	Triglyceride (mg/gm wet weight)
Pathological group	355.7±76.2(10)	81.80±6.84(10)
Glycyrrhizin group	*113.6±20.8(10)	*54.23±7.23(10)
Normal group	143.8±13.3(8)	37.18±3.54(8)

Remarks: Numbers in the Table are M±SE

* Compared with the pathological group, $P < 0.01$

1:10. Two were 1:100 positive, but only 2 rats in the pathological group were positive for αFP 1:10. Significant difference was found between the 2 groups ($P<0.001$). A previous report[3] demonstrated that hepatic regeneration was related to the αFP levels in serum, and the present results suggested that glycyrrhizin could promote hepatic cellular regeneration.

Effect of Glycyrrhizin on Rat Fatty Liver

Selection and grouping of the rats were similarly arranged as in the above study. Rats in both glycyrrhizin and pathological groups were fed a diet containing high lipid and low protein (80% maize, 20% fat, 0.5% cholesterol and 33% ethanol). Hypodermic injection of carbon tetrachloride 0.5ml/100 gm body weight was given on the 1st day of experiment and followed by injections of 40% carbon tetrachloride solution in cottonseed oil 0.3 ml/100 gm body weight every 3 days for two weeks. In the glycyrrhizin group, the same dosage was started on the 2nd day of experiment. The rats were sacrificed at the end of 2nd week, and homogenate was prepared with a portion of the liver for determination of triglyceride. The remainder of the liver was fixed and stained with hemotoxylin-eosin and Sudan III for light microscopy. β-lipoprotein was determined in the rats' sera.

Light microscopy revealed fatty degeneration which was particularly severe in the pathological group, i.e. fatty liver (+++) in all 7 rats, while among the 7 rats in the glycyrrhizin group fatty degeneration (+) was found in 2, (++) in 4, and fatty liver (+++) in only one — a significant difference between the 2 groups ($\chi^2=10.5$, $P<0.05$). As shown in Table 6, triglyceride content in homogenate from the glycyrrhizin group showed marked decrease over the pathological group ($P<0.01$), but serum β-lipoprotein was markedly increased in the glycyrrhizin group ($P<0.01$). This suggested that glycyrrhizin had lipotropic action.[4]

Effect of Glycyrrhizin on the Prevention of Liver Cirrhosis in Animals

Healthy rats weighing 230-280 gm were similarly divided into 3 groups. The animal-

Table 6. Effect of glycyrrhizin on the triglyceride content in liver homogenate and serum β-lipoprotein in animals with fatty liver

Groups	Triglyceride (mg/gm wet weight)	β-lipoprotein (mg%)
Pathological group	149.43±18.07(7)	470.6± 54.9(7)
Glycyrrhizin group	*83.33±16.37(7)	*943.6±126.8(7)
Normal group	20.75± 3.93(8)	280.6± 15.9(3)

Remarks: Numbers in the Table are M±SE

* Compared with the pathological group. $P < 0.01$

model of liver cirrhosis was induced by multiple pathogenic factors. Animals in both glycyrrhizin and pathological groups were fed a diet of maize (20% fat was added in the previous 2 weeks) containing 0.5% cholesterol. 30% ethanol was given for drinks. Hypodermic injection of carbon tetrachloride 0.5 mg/100 gm body weight was given daily from the 1st day of experiment and continued for 6 weeks. In the glycyrrhizin group, daily hypodermic injection of 30 mg glycyrrhizin was started on the 2nd day of experiment. Animals were sacrificed at the end of the 6th week and liver samples were taken from the left lobe. The liver was fixed, sectioned and stained with hemotoxylineosin and Van Gieson staining. Degree of fibro-hyperplasia was determined. The items of observation were similar as in a previous report.[5] The remaining part of liver was kept for determination of hydroxyproline and collagen content was thus assessed. Sera of the rats were prepared for protein electrophoresis.

Laboratory findings showed that of the 10 rats in the pathological group, 3 had hepatic fibro-hyperplasia (++) and 7 had liver cirrhosis; of 11 rats in the glycyrrhizin group, hepatic fibro-hyperplasia was (−) in 5, (+) in 2, (++) in 3 and liver cirrhosis in one only. There was significant difference between the 2 groups ($\chi^2=11.48$, $P<0.01$). As shown in Table 7, the liver collagen content in the glycyrrhizin group was lower than in the pathological group ($P<0.05$), suggesting that glycyrrhizin inhibited hepatic fibro-hyperplasia and so reduced the incidence of liver cirrhosis. Although serum gamma globulin was lower in the glycyrrhizin group, there was no significant difference, suggesting that liver interstitial inflammatory response was not markedly reduced by glycyrrhizin.

Table 7. Effect of glycyrrhizin on the hepatic collagen and serum gamma globulin in animals with liver cirrhosis

globulin (%)	Collagen (mg/gm wet weight)	Gamma globulin (%)
Pathological group	33.5±5.6(10)	22.2±1.1(8)
Glycyrrhizin group	*21.5±2.2(11)	14.4±0.7(9)
Normal group	15.1±2.1(8)	15.5±1.1(8)

Remarks: Numbers in the Table are M±SE

* Compared with the pathological group, $P < 0.05$

Effect of Glycyrrhizin on the Resorption of Fibrous Tissue after Formation of Liver Cirrhosis

Animal-model of liver cirrhosis was similarly induced. When liver cirrhosis was formed at the 42nd day of experiment, daily hypodermic injection of 30 mg glycyrrhizin was given for 3 weeks. These animals were sacrificed at the end of the 9th week, liver samples were taken for pathologic examination and hydroxyproline was determined, thus assessing collagen content. In the glycyrrhizin group a 16-hour (4 p.m. to 8 a.m.) urine specimen was collected before and 2 weeks after administration of the drug for hydroxyproline determination. The results obtained were compared with those of the other 2 groups.

Laboratory findings showed fibro-hyperplasia (+) in one, (++) in one and (+++) in 3 of the total of 5 rats in the glycyrrhizin group, while fibro-hyperplasia (++) was found in 2 and (+++) in 5 of the total of 7 rats in the pathological group. There was no significant difference between the 2 groups. The values of liver collagen and urinary hydroxyproline appear in Table 8.

Table 8. Effect of glycyrrhizin on the hepatic collagen and urinary hydroxyproline contents after the formation of liver cirrhosis

Groups	Collagen (mg/gm wet weight)	Urinary hydroxyproline (ng/ml)	
		Before treating	After treating
Pathological group	19.2±1.4(6)	7.1±0.9(6)	2.7±0.7(6)
Glycyrrhizin group	18.2±2.3(7)	4.7±0.3(6)	3.0±0.4(6)
Normal group	9.8±1.1(5)	6.3±1.3(6)	6.0±1.0(6)

The findings showed no significant difference of collagen and hydroxyproline between the 2 groups, indicating that glycyrrhizin effected neither degradation nor resorption of collagen.

DISCUSSION

1. This series of acute and chronic viral hepatitis treated with glycyrrhizin showed markedly shorter period of treatment, better recovery of liver function and reduction of SGPT activity, plus a negative conversion of HBsAg and HBeAg as compared with the group treated with other drugs. Animals with experimental acute and chronic hepatitis treated with glycyrrhizin showed less pathological changes and rapid repair of necrotic tissue, marked decrease of SGPT activity, increase of β-lipoprotein and decrease of triglyceride accumulation in liver, all compatible with clinical observation.

2. It is currently believed that disseminated fibro-hyperplasia is the primary cause of liver cirrhosis,[6,7] and the fibro-hyperplasia is related to cellular changes and necrosis. Hepatic cellular necrosis is virtually an essential factor in chronic hepatitis and liver cirrhosis. Fibro-hyperplasia would be active and fibrous tissues aggregated to form collagenous fibers if the necrosis was not rapidly repaired.[8] In hepatitis the non-dialysable substance released from lymphocytes and phagocytes may stimulate fibroblasts to synthesize collagen. Also, the inflammatory changes further reduce the hepatic cellular regeneration,[8] and the patient is likely to develop chronic hepatitis or even liver cirrhosis. Laboratory findings demonstrated that glycyrrhizin may not only markedly lessen fatty degeneration and necrosis of hepatic cells but also reduce inflammatory infiltration. Clinically, liver function is improved, SGPT activity reduced, and IgG, IgA, and IgM recovered, while detection rate of αFP increases. These changes tally with the previous report[9] and provide the rationale for the use of glycyrrhizin in treating acute and chronic hepatitis.

Glycyrrhizin is demonstrated to have an effect similar to that of corticosteroid. Corticosteroid directly decreases prolyl hydroxylase activity and inhibits the synthesis of collagen,[10] while glycyrrhizin inhibits lysyl oxidase (monoamine oxidase) in liver, which affects the synthesis of collagen.[11] Glycyrrhizin inhibits the synthesis of collagen, and this may relate to the aforementioned actions.

3. In other countries at present, corticosteroid and D-penicillinase are used to treat chronic active hepatitis and liver cirrhosis. These two agents have non-specific anti-inflammatory and fibro-hyperplasia inhibiting action. Corticosteroid and D-penicillinase inhibit the synthesis of collagen through their inhibitory action on prolyl hydroxydase and lysyl oxidase respectively, but severe adverse effect is common when the period of administration is prolonged.[7] Glycyrrhizin is similar in effect to corticosteroid and D-penicillinase, but no adverse effect has so far been encountered, and it therefore appears to be the better drug for treating acute and chronic hepatitis and preventing liver cirrhosis. The mechanism of negative conversion of HBsAg needs further study.

4. Promotion of resorption of fibrous tissue in liver is the critical measure for treating cirrhosis. It has been demonstrated that collagenous fibers can be reabsorbed due to the effect of collagenase in cirrhosis.[12] This series of animal experiments showed neither marked changes of fibrous tissue nor significant difference of urinary hydroxyproline between the glycyrrhizin and control groups, indicating that resorption of fibrous tissue was not promoted by glycyrrhizin.

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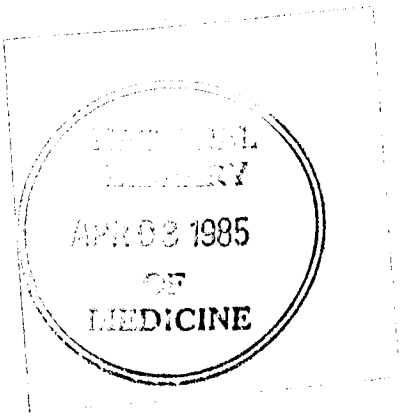

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Deaths: Final Data for 2013

by Jiaquan Xu, M.D.; Sherry L. Murphy, B.S.; Kenneth D. Kochanek, M.A.; and Brigham A. Bastian, B.S., Division of Vital Statistics

Abstract

Objectives—This report presents final 2013 data on U.S. deaths, death rates, life expectancy, infant mortality, and trends, by selected characteristics such as age, sex, Hispanic origin, race, state of residence, and cause of death.

Methods—Information reported on death certificates, which are completed by funeral directors, attending physicians, medical examiners, and coroners, is presented in descriptive tabulations. The original records are filed in state registration offices. Statistical information is compiled in a national database through the Vital Statistics Cooperative Program of the Centers for Disease Control and Prevention's National Center for Health Statistics. Causes of death are processed in accordance with the *International Classification of Diseases, Tenth Revision.*

Results—In 2013, a total of 2,596,993 deaths were reported in the United States. The age-adjusted death rate was 731.9 deaths per 100,000 U.S. standard population, a record low figure, but the decrease in 2013 from 2012 was not statistically significant. Life expectancy at birth was 78.8 years, the same as in 2012. Age-specific death rates decreased in 2013 from 2012 for age groups 15–24 and 75–84. Age-specific death rates increased only for age group 55–64. The 15 leading causes of death in 2013 remained the same as in 2012, although Accidents (unintentional injuries), the 5th leading cause of death in 2012, became the 4th leading cause in 2013, while Cerebrovascular diseases (stroke), the 4th leading cause in 2012, became the 5th leading cause of death in 2013. The infant mortality rate of 5.96 deaths per 1,000 live births in 2013 was a historically low value, but it was not significantly different from the 2012 rate.

Conclusions—Although statistically unchanged from 2012, the decline in the age-adjusted death rate is consistent with long-term trends in mortality. Life expectancy in 2013 remained the same as in 2012.

Keywords: mortality • cause of death • life expectancy • vital statistics

Highlights

Mortality experience in 2013

- In 2013, a total of 2,596,993 resident deaths were registered in the United States.
- The age-adjusted death rate, which accounts for the aging of the population, was 731.9 deaths per 100,000 U.S. standard population.
- Life expectancy at birth was 78.8 years.
- The 15 leading causes of death in 2013 were:

    1. Diseases of heart (heart disease)
    2. Malignant neoplasms (cancer)
    3. Chronic lower respiratory diseases
    4. Accidents (unintentional injuries)
    5. Cerebrovascular diseases (stroke)
    6. Alzheimer's disease
    7. Diabetes mellitus (diabetes)
    8. Influenza and pneumonia
    9. Nephritis, nephrotic syndrome and nephrosis (kidney disease)
    10. Intentional self-harm (suicide)
    11. Septicemia
    12. Chronic liver disease and cirrhosis
    13. Essential hypertension and hypertensive renal disease (hypertension)
    14. Parkinson's disease
    15. Pneumonitis due to solids and liquids

- In 2013, the infant mortality rate was 5.96 infant deaths per 1,000 live births.
- The 10 leading causes of infant death were:

    1. Congenital malformations, deformations and chromosomal abnormalities (congenital malformations)
    2. Disorders related to short gestation and low birth weight, not elsewhere classified (low birth weight)

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3. Newborn affected by maternal complications of pregnancy (maternal complications)
4. Sudden infant death syndrome (SIDS)
5. Accidents (unintentional injuries)
6. Newborn affected by complications of placenta, cord and membranes (cord and placental complications)
7. Bacterial sepsis of newborn
8. Respiratory distress of newborn
9. Diseases of the circulatory system
10. Neonatal hemorrhage

Trends

- The age-adjusted death rate declined to a record low in 2013, although the decrease from 2012 to 2013 was not significant.
- Life expectancy for the total population was 78.8 years in 2013, the same as in 2012.
- Life expectancy did not change for any of the major race and ethnicity populations from 2012 to 2013.
- Life expectancy for females was 4.8 years higher than for males. The difference in life expectancy between the sexes has narrowed since 1979, when it was 7.8 years, but it has remained at 4.8 years since 2010.
- The 15 leading causes of death were the same in 2013 as they were in 2012, although unintentional injuries and stroke exchanged positions in the ranking.
- Age-adjusted death rates decreased significantly in 2013 from 2012 for 4 of the 15 leading causes of death and increased for 6 of the 15 leading causes.
- Rates for the two leading causes—heart disease and cancer—continued their long-term decreasing trends. Significant decreases also occurred for stroke and Alzheimer's disease. Significant increases occurred in 2013 from 2012 for Chronic lower respiratory diseases, Influenza and pneumonia, Septicemia, Chronic liver disease and cirrhosis, hypertension, and Parkinson's disease.
- Within external causes of injury death, unintentional poisoning was the leading mechanism of injury mortality in 2013, followed by unintentional motor vehicle traffic-related injuries. During 2002–2010, unintentional motor vehicle traffic-related injuries was the leading mechanism of injury mortality, followed by unintentional poisoning, but beginning in 2011, the number of deaths from unintentional poisoning was higher than the number from unintentional motor vehicle traffic-related injuries; see CDC's Web-based Injury Statistics Query and Reporting System (WISQARS) at http://www.cdc.gov/injury/wisqars/index.html.
- Differences in mortality between the non-Hispanic black and non-Hispanic white populations persisted. The age-adjusted death rate was 1.2 times greater for the non-Hispanic black population than for the non-Hispanic white population.
- The differences in life expectancy among the Hispanic, non-Hispanic white, and non-Hispanic black populations in 2013 were the same as in 2012. The difference in life expectancy between the non-Hispanic black and non-Hispanic white populations was 3.8 years, between the non-Hispanic black and Hispanic populations was 6.5 years, and between the non-Hispanic white and Hispanic populations was 2.7 years.
- The infant mortality rate declined 0.3% in 2013 from 2012, to a record low of 5.96 infant deaths per 1,000 live births, but the decline was not statistically significant.

Introduction

This report presents detailed 2013 data on deaths and death rates according to a number of demographic and medical characteristics. These data provide information on mortality patterns among residents of the United States by such variables as age, sex, Hispanic origin, race, state of residence, and cause of death. Information on these mortality patterns is key to understanding changes in the health and wellbeing of the U.S. population (1). Separate companion reports present additional details on leading causes of death and life expectancy in the United States (2,3).

Mortality data in this report can be used to monitor and evaluate the health status of the United States in terms of current mortality levels and long-term mortality trends, as well as to identify segments of the U.S. population at greater risk of death from specific diseases and injuries. Differences in death rates among various demographic subpopulations, including race and ethnicity groups, may reflect subpopulation differences in factors such as socioeconomic status, access to medical care, and the prevalence of specific risk factors in a particular subpopulation.

Methods

Data in this report are based on information from all resident death certificates filed in the 50 states and the District of Columbia. More than 99% of deaths occurring in this country are believed to be registered (4). Tables showing data by state also provide information for Puerto Rico, Guam, American Samoa, and the Commonwealth of the Northern Mariana Islands (Northern Marianas). Cause-of-death statistics presented in this report are classified in accordance with the *International Classification of Diseases, Tenth Revision* (ICD–10) (5). A discussion of the cause-of-death classification is provided in Technical Notes at the end of the report.

Mortality data on specific demographic and medical characteristics cover all 50 states and the District of Columbia. Measures of mortality in this report include the number of deaths; crude, age-specific, and age-adjusted death rates; infant, neonatal, and postneonatal mortality rates; life expectancy; and rate ratios. Changes in death rates in 2013 compared with 2012, and differences in death rates across demographic groups in 2013, are tested for statistical significance. Unless otherwise specified, reported differences are statistically significant. Additional information on these statistical methods, random variation and relative standard error, the computation of derived statistics and rates, population denominators, and the definition of terms is presented in Technical Notes.

The populations used to calculate death rates shown in this report for 1991–2013 were produced under a collaborative arrangement with the U.S. Census Bureau. Populations for 2010–2013 and the intercensal period 2001–2009 are consistent with the 2010 census (6–10). Reflecting the latest guidelines issued in 1997 by the Office of Management and Budget (OMB), the 2000 and 2010 censuses included an option for persons to report more than one race as appropriate for themselves and household members (11); see Technical Notes for

detailed information on the 2013 multiple-race reporting area and methods used to bridge responses for those who report more than one race. Beginning with deaths occurring in 2003, some states allowed for multiple-race reporting on the death certificate. Multiple-race data for these states are bridged to single-race categories; see Technical Notes. Once all states are collecting data on race according to the 1997 OMB guidelines, use of the bridged-race algorithm is expected to be discontinued.

The population data used to compile death rates by race in this report are based on special estimation procedures and are not true counts (see Technical Notes, "Race and Hispanic origin"). This is the case even for the 2000 and 2010 populations. The estimation procedures used to develop these populations contain some error. Smaller population groups are affected much more than larger population groups (12). Data presented in this report and other mortality tabulations are available from the National Center for Health Statistics (NCHS) website, http://www.cdc.gov/nchs/deaths.htm. Availability of mortality microdata is described in Technical Notes.

Results and Discussion

Deaths and death rates

In 2013, a total of 2,596,993 resident deaths were registered in the United States—53,714 more deaths than in 2012. The crude death rate for 2013 (821.5 deaths per 100,000 population) was 1.4% higher than the 2012 rate (810.2) (Tables A, 1, 3, 4, 14, and 15).

The age-adjusted death rate in 2013 was 731.9 deaths per 100,000 U.S. standard population—a record low value, although it was not significantly different from 2012 (Table 1). Age-adjusted death rates are constructs that show what the level of mortality would be if no changes occurred in the age composition of the population from year to year. (For a discussion of age-adjusted death rates, see Technical Notes.) Thus, age-adjusted death rates are better indicators than unadjusted (crude) death rates for examining changes in the risk of death over a period of time when the age distribution of the population is changing. Age-adjusted death rates also are better indicators of relative risk when comparing mortality across geographic areas or between sex or race subgroups of the population that have different age distributions; see Technical Notes. Since 1980, the age-adjusted death rate has decreased significantly every year except 1983, 1985, 1988, 1993, 1999, 2005, 2008, and 2013 (Figure 1 and Table 1).

Race—In 2013, age-adjusted death rates for the major race groups (Table 1) were:

- White population: 731.0 deaths per 100,000 U.S. standard population
- Black population: 860.8

In 2013, the age-adjusted death rate for the black population was 1.2 times that for the white population (Table B). The average risk of death for the black population was 17.8% higher than for the white population (Table 1). From 1960 through 1982, rates for the black and white populations declined by similar percentages (22.6% and 26.5%, respectively). From 1983 through 1988, rates diverged, increasing 3.5% for the black population and decreasing 2.0% for the white population. The disparity in age-adjusted death rates between the black and white populations was greatest from 1988 through 1996 (1.4 times greater for the black population). Since 1996, the disparity between the two populations has narrowed, as the age-adjusted rate for the black population declined 27.0% while the rate for the white population declined 15.9% (Table 1 and Figure 2).

In 2013, age-adjusted death rates did not change significantly for major race and sex groups compared with 2012 (Tables A and 1).

In general, age-adjusted death rates declined from 1980 through 2013 for white males and females and for black males and females. The rate decreased an average of 1.3% per year for white males, 0.7% for white females, 1.4% for black males, and 1.1% for black females during 1980–2013 (Table 1).

Rates for the American Indian or Alaska Native (AIAN) and Asian or Pacific Islander (API) populations should be interpreted with caution because of reporting problems regarding correct identification of race on both the death certificate and in population censuses and surveys (13).

Counts of deaths for the AIAN population are substantially underreported (by about 30%) on the death certificate relative to self-reporting while alive (13). Thus, the age-adjusted death rates that are shown for the AIAN population (e.g., Tables 1 and 16) do not lend themselves to valid comparisons against other races.

Year-to-year trends for the AIAN population present valid insight into changes in mortality affecting this group, if it is reasonable to assume that the level of underreporting of AIAN deaths has remained more or less constant over past years (13). The age-adjusted death rate for the AIAN population fluctuated from 1980 through 1999, peaking in 1993 at 796.4 deaths per 100,000 U.S. standard population (Table 1). Since 1999, the rate has trended downward, declining 24.2% from 1999 to 2013. The rate for the AIAN population decreased 0.6% from 2012 (595.3) to 2013 (591.7), although the change was not significant (Table A).

In 2013, the age-adjusted death rate for the API population was 405.4 deaths per 100,000 U.S. standard population. The level of underreporting of deaths for the API population (about 7%) is not as high as for the AIAN population (13), but this underreporting still creates enough of a challenge that any comparisons of this population with other races must be interpreted with caution. The age-adjusted death rate for the API population peaked at 586.5 in 1985. The rate fluctuated from 1985 through 1993 before starting a persistent downward trend, decreasing 28.3% from 1993 to 2013 (Table 1).

Hispanic origin—Problems of race and Hispanic-origin reporting affect Hispanic death rates and the comparison of rates for the Hispanic and non-Hispanic populations; see Technical Notes. Mortality for Hispanics is somewhat understated because of net underreporting of Hispanic origin on the death certificate (by an estimated 5%), while the non-Hispanic white and non-Hispanic black populations are not affected by problems of underreporting (13,14); see Technical Notes. Underreporting of Hispanic origin on the death certificate is relatively stable across age groups (13).

The age-adjusted death rate in 2013 was 535.4 for the Hispanic population, 747.1 for the non-Hispanic white population, and 885.2 for

Table A. Percentage change in death rates and age-adjusted death rates in 2013 from 2012, by age, race, and sex: United States

[Based on death rates on an annual basis per 100,000 population, and age-adjusted rates per 100,000 U.S. standard population; see Technical Notes. Rates are based on populations estimated as of July 1 using postcensal estimates; see Technical Notes. Data for specified races other than white and black should be interpreted with caution because of inconsistencies between reporting race on death certificates and on censuses and surveys; see Technical Notes. Race categories are consistent with the 1977 Office of Management and Budget (OMB) standards]

Age (years)	All races			White[1]			Black[1]			American Indian or Alaska Native[1,2]			Asian or Pacific Islander[1,3]		
	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female
All ages	Percent change														
Crude	1.4	1.8	1.0	1.5	1.8	1.1	1.5	1.6	1.4	1.8	1.5	2.1	3.2	4.4	1.8
Age-adjusted	–0.1	–0.2	–0.2	0.0	–0.1	0.0	–0.5	–0.5	–0.5	–0.6	–0.2	–0.8	–0.4	0.8	–1.7
Under 1 year[4]	–0.8	–0.2	–1.6	–0.6	1.4	–3.1	0.4	–2.4	3.8	–16.0	–6.6	–28.2	–5.4	–5.1	–5.6
1–4	–3.0	–2.1	–3.4	–5.3	–4.0	–7.4	–0.5	–2.4	1.2	17.9	21.3	12.3	21.3	18.4	24.3
5–14	3.2	1.4	3.7	3.4	4.5	1.9	–2.8	–8.0	5.7	–13.5	–28.0	13.5	23.5	23.3	22.2
15–24	–2.4	–2.8	–1.1	–2.2	–2.9	0.0	–2.9	–2.3	–5.5	–9.6	–9.2	–10.3	0.0	1.7	–3.8
25–34	0.7	0.8	0.3	0.3	0.4	0.0	2.6	2.7	1.7	–2.7	–1.5	–5.4	0.8	–1.3	5.3
35–44	0.8	0.5	1.2	1.1	0.4	2.2	0.2	2.1	–2.6	1.4	–1.7	6.3	–1.6	–3.8	2.2
45–54	0.2	0.0	0.4	0.8	0.6	1.1	–2.1	–2.5	–1.5	1.4	1.1	1.5	0.4	1.8	–1.8
55–64	0.7	0.8	0.6	0.6	0.7	0.5	1.0	0.8	1.2	1.9	–0.1	5.0	–0.6	2.3	–4.7
65–74	0.0	0.0	–0.1	0.1	0.1	0.2	–0.4	–0.4	–0.5	–5.3	–5.3	–5.4	–0.9	1.6	–3.8
75–84	–0.6	–0.5	–0.8	–0.6	–0.4	–0.9	0.0	–0.8	0.5	–0.1	0.8	–1.1	0.8	0.7	0.7
85 and over	–0.1	–0.4	–0.1	0.1	–0.3	0.3	–1.5	–0.9	–1.8	2.0	4.8	0.3	–1.5	0.3	–2.7

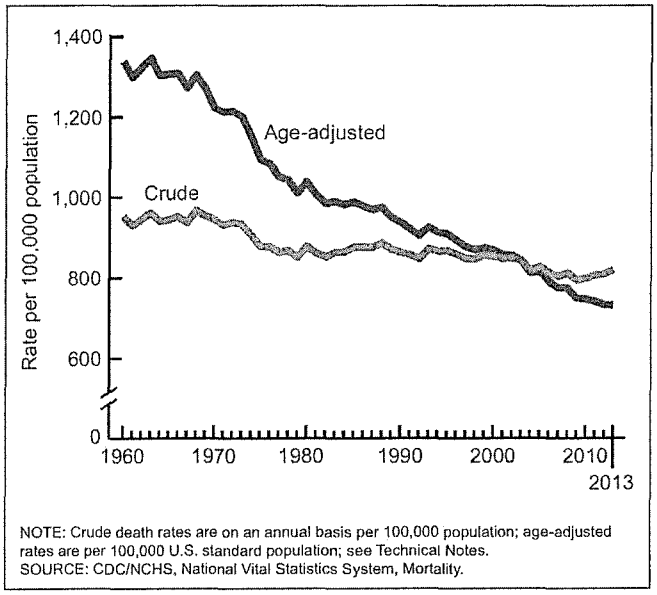
[1]Multiple-race data were reported by 42 states and the District of Columbia in 2012 and 2013. The multiple-race data for these reporting areas were bridged to the single-race categories of the 1977 OMB standards, for comparability with other reporting areas; see Technical Notes.
[2]Includes Aleut and Eskimo persons.
[3]Includes Chinese, Filipino, Hawaiian, Japanese, and other Asian or Pacific Islander persons.
[4]Death rates for "Under 1 year" (based on population estimates) differ from infant mortality rates (based on live births).

the non-Hispanic black population. Changes in death rates from 2012 to 2013 for race and ethnicity groups were not significant (Tables C, 2, and 17).

The age-adjusted death rate in 2013 was 639.8 for Hispanic males, 448.6 for Hispanic females, 876.8 for non-Hispanic white males, 638.4 for non-Hispanic white females, 1,083.3 for non-Hispanic black males, and 740.6 for non-Hispanic black females. Changes in rates from 2012 to 2013 for race and ethnicity populations by sex were not significant. (Tables C and 2).

Within the Hispanic population, the age-adjusted death rate for males was 1.4 times the rate for females in 2013 (Table 2). The male-to-female death rate ratio for the Hispanic population was unchanged from the ratio in 2012. The corresponding male-to-female ratio was 1.4 for the non-Hispanic white population and 1.5 for the non-Hispanic black population in 2013. The male-to-female ratios for non-Hispanic white and non-Hispanic black populations were also unchanged from 2012. Age-adjusted death rates in 2013 for selected Hispanic subgroups (Table 5), in order of relative magnitude, were:

- Puerto Rican population: 615.8 deaths per 100,000 U.S. standard population
- Mexican population: 561.7
- Cuban population: 529.4
- Central and South American population: 366.7



NOTE: Crude death rates are on an annual basis per 100,000 population; age-adjusted rates are per 100,000 U.S. standard population; see Technical Notes.
SOURCE: CDC/NCHS, National Vital Statistics System, Mortality.

Figure 1. Crude and age-adjusted death rates: United States, 1960–2013

Death rates by age and sex

Age-specific death rates decreased significantly from 2012 to 2013 for age groups 15–24 and 75–84. The only significant increase in age-specific death rates was for age group 55–64. Changes in rates for the other age groups were not significant (Tables A, 9, and 11; Figure 3).

Table B. Number of deaths, percentage of total deaths, death rates, and age-adjusted death rates for 2013, percentage change in age-adjusted death rates in 2013 from 2012, and ratio of age-adjusted death rates by sex and by race for the 15 leading causes of death for the total population in 2013: United States

[Crude death rates on an annual basis per 100,000 population; age-adjusted rates per 100,000 U.S. standard population; see Technical Notes. Rates are based on populations estimated as of July 1 using postcensal estimates; see Technical Notes. The asterisks (*) preceding the cause-of-death codes indicate that they are not part of the *International Classification of Diseases, Tenth Revision* (ICD–10); see Technical Notes. Race categories are consistent with the 1977 Office of Management and Budget (OMB) standards]

Rank[1]	Cause of death (based on ICD–10)	Number	Percent of total deaths	2013 crude death rate	Age-adjusted death rate			
					2013	Percent change 2012 to 2013	Ratio: Male to female	Ratio: Black[2] to white
...	All causes	2,596,993	100.0	821.5	731.9	–0.1	1.4	1.2
1	Diseases of heart (I00–I09,I11,I13,I20–I51)	611,105	23.5	193.3	169.8	–0.4	1.6	1.3
2	Malignant neoplasms (C00–C97)	584,881	22.5	185.0	163.2	–2.0	1.4	1.2
3	Chronic lower respiratory diseases (J40–J47)	149,205	5.7	47.2	42.1	1.4	1.2	0.7
4	Accidents (unintentional injuries) (V01–X59,Y85–Y86)	130,557	5.0	41.3	39.4	0.8	2.0	0.8
5	Cerebrovascular diseases (I60–I69)	128,978	5.0	40.8	36.2	–1.9	1.0	1.4
6	Alzheimer's disease (G30)	84,767	3.3	26.8	23.5	–1.3	0.7	0.8
7	Diabetes mellitus (E10–E14)	75,578	2.9	23.9	21.2	0.0	1.5	2.0
8	Influenza and pneumonia (J09–J18)	56,979	2.2	18.0	15.9	10.4	1.3	1.1
9	Nephritis, nephrotic syndrome and nephrosis (N00–N07, N17–N19,N25–N27)	47,112	1.8	14.9	13.2	0.8	1.4	2.1
10	Intentional self-harm (suicide) (*U03,X60–X84,Y87.0)	41,149	1.6	13.0	12.6	0.0	3.7	0.4
11	Septicemia (A40–A41)	38,156	1.5	12.1	10.7	3.9	1.2	1.8
12	Chronic liver disease and cirrhosis (K70,K73–K74)	36,427	1.4	11.5	10.2	3.0	2.0	0.7
13	Essential hypertension and hypertensive renal disease (I10,I12,I15)	30,770	1.2	9.7	8.5	3.7	1.1	2.1
14	Parkinson's disease (G20–G21)	25,196	1.0	8.0	7.3	4.3	2.3	0.5
15	Pneumonitis due to solids and liquids (J69)	18,579	0.7	5.9	5.2	2.0	1.8	0.9
...	All other causes (residual)	537,554	20.7	170.0	...	...	...	...

... Category not applicable.

[1]Based on number of deaths; see Technical Notes.

[2]Multiple-race data were reported by 42 states and the District of Columbia in 2013. The multiple-race data for these reporting areas were bridged to the single-race categories of the 1977 OMB standards for comparability with other reporting areas; see Technical Notes.

The death rate for males declined significantly only for age group 15–24. The only significant increase in rates for males was for age group 55–64. Changes in the rates for males in other age groups were not significant. The only significant change in rates for females was a decrease for age group 75–84.

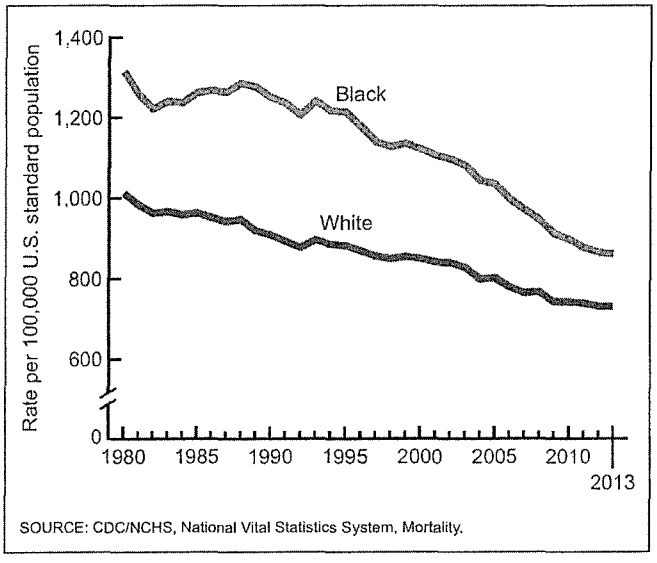


Figure 2. Age-adjusted death rates, by race: United States, 1980–2013

Race—In 2013, age-specific death rates declined significantly for white males in age group 15–24, and increased for age group 55–64 (Table A). For the black male population in 2013, the only statistically significant change was a 2.5% decrease for age group 45–54. For AIAN and API males, rates did not change significantly for any age group. Other observed changes for males by race were not statistically significant.

For white females, age-specific death rates increased significantly in 2013 for those aged 35–44 and decreased for those aged 75–84. For black females in 2013, the only statistically significant change was a decrease for age group 85 and over. For API females, rates did not change significantly for any age group. The only significant change in rates for AIAN females was a decrease for those under age 1 year. Other observed changes for females by race were not statistically significant.

Hispanic origin—For the total Hispanic population in 2013 compared with 2012, age-specific death rates decreased significantly for age groups 55–64 and 85 and over (Table C). Rates for Hispanic males decreased for age groups 15–24 and 55–64. For Hispanic

Table C. Percentage change in death rates and age-adjusted death rates in 2013 from 2012, by age, Hispanic origin, race for non-Hispanic population, and sex: United States

[Based on death rates on an annual basis per 100,000 population, and age-adjusted rates per 100,000 U.S. standard population; see Technical Notes. Rates are based on populations estimated as of July 1 using postcensal estimates; see Technical Notes. Race and Hispanic origin are reported separately on the death certificate. Persons of Hispanic origin may be of any race. Data for Hispanic persons are not tabulated separately by race. Data for non-Hispanic persons are tabulated by race. Data for Hispanic origin should be interpreted with caution because of inconsistencies between reporting Hispanic origin on death certificates and on censuses and surveys; see Technical Notes]

Age (years)	All origins[1]			Hispanic			Non-Hispanic[2]			Non-Hispanic white[3]			Non-Hispanic black[3]		
	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female
All ages	Percent change														
Crude	1.4	1.8	1.0	2.3	2.3	2.5	1.6	1.9	1.1	1.7	2.1	1.3	1.7	1.8	1.6
Age-adjusted	–0.1	–0.2	–0.2	–0.7	–0.6	–0.9	0.0	0.0	0.0	0.2	0.1	0.1	–0.2	–0.3	–0.2
Under 1 year[4]	–0.8	–0.2	–1.6	–0.4	–1.6	1.1	–0.7	0.2	–1.9	–0.6	2.4	–4.4	0.5	–2.1	3.9
1–4	–3.0	–2.1	–3.4	–4.6	–1.7	–8.0	–2.2	–2.3	–2.1	–4.8	–3.6	–6.0	–1.5	–3.8	0.6
5–14	3.2	1.4	3.7	–2.7	–4.9	–1.0	4.6	2.6	6.4	6.8	8.3	5.0	–2.1	–7.6	6.1
15–24	–2.4	–2.8	–1.1	–3.6	–4.6	0.4	–2.1	–2.3	–1.3	–1.7	–2.6	0.5	–2.8	–1.9	–5.6
25–34	0.7	0.8	0.3	1.0	–0.1	3.5	0.6	1.0	–0.1	0.3	0.6	–0.7	2.7	2.8	1.9
35–44	0.8	0.5	1.2	2.5	0.4	6.4	0.8	0.7	0.9	1.1	0.6	1.9	0.5	2.4	–2.3
45–54	0.2	0.0	0.4	0.6	1.5	–1.3	0.4	0.2	0.9	1.1	0.7	1.7	–1.7	–2.0	–1.3
55–64	0.7	0.8	0.6	–1.9	–2.3	–1.3	1.0	1.1	0.9	0.9	1.0	0.7	1.2	0.9	1.6
65–74	0.0	0.0	–0.1	0.9	1.9	–0.6	0.0	–0.1	0.0	0.1	–0.1	0.2	–0.2	–0.3	–0.2
75–84	–0.6	–0.5	–0.8	–0.8	–0.8	–0.8	–0.5	–0.4	–0.7	–0.5	–0.3	–0.9	0.2	–0.7	0.8
85 and over	–0.1	–0.4	–0.1	–1.7	–1.9	–1.6	0.1	–0.2	0.1	0.3	–0.1	0.5	–1.2	–0.3	–1.6

[1]Figures for origin not stated are included in "All origins" but not distributed among specified origins.

[2]Includes races other than white and black.

[3]Race categories are consistent with the 1977 Office of Management and Budget (OMB) standards. Multiple-race data were reported by 42 states and the District of Columbia in 2012 and 2013; see Technical Notes. The multiple-race data for these reporting areas were bridged to the single-race categories of the 1977 OMB standards for comparability with other reporting areas; see Technical Notes.

[4]Death rates for "Under 1 year" (based on population estimates) differ from infant mortality rates (based on live births).

females, the only significant change was a 6.4% increase for age group 35–44. Other observed changes were not statistically significant.

Expectation of life at birth and at specified ages

Life expectancy at birth represents the average number of years that a group of infants would live if the group was to experience throughout life the age-specific death rates present in the year of birth.

Life table data shown in this report for data years 2001–2013 are based on a revised methodology first presented with final data reported for 2008. The life table methodology was revised by changing the smoothing technique used to estimate the life table functions at the oldest ages. This revision improves on the methodologies used previously; see Technical Notes.

The methods used to produce life expectancies by Hispanic origin are based on death rates adjusted for misclassification (see Technical Notes). In contrast, the age-specific and age-adjusted death rates shown in this report for the Hispanic population are not adjusted for misclassification of Hispanic origin. Thus, the report shows Hispanic deaths and death rates as collected by the registration areas; these match those produced using the mortality data file.

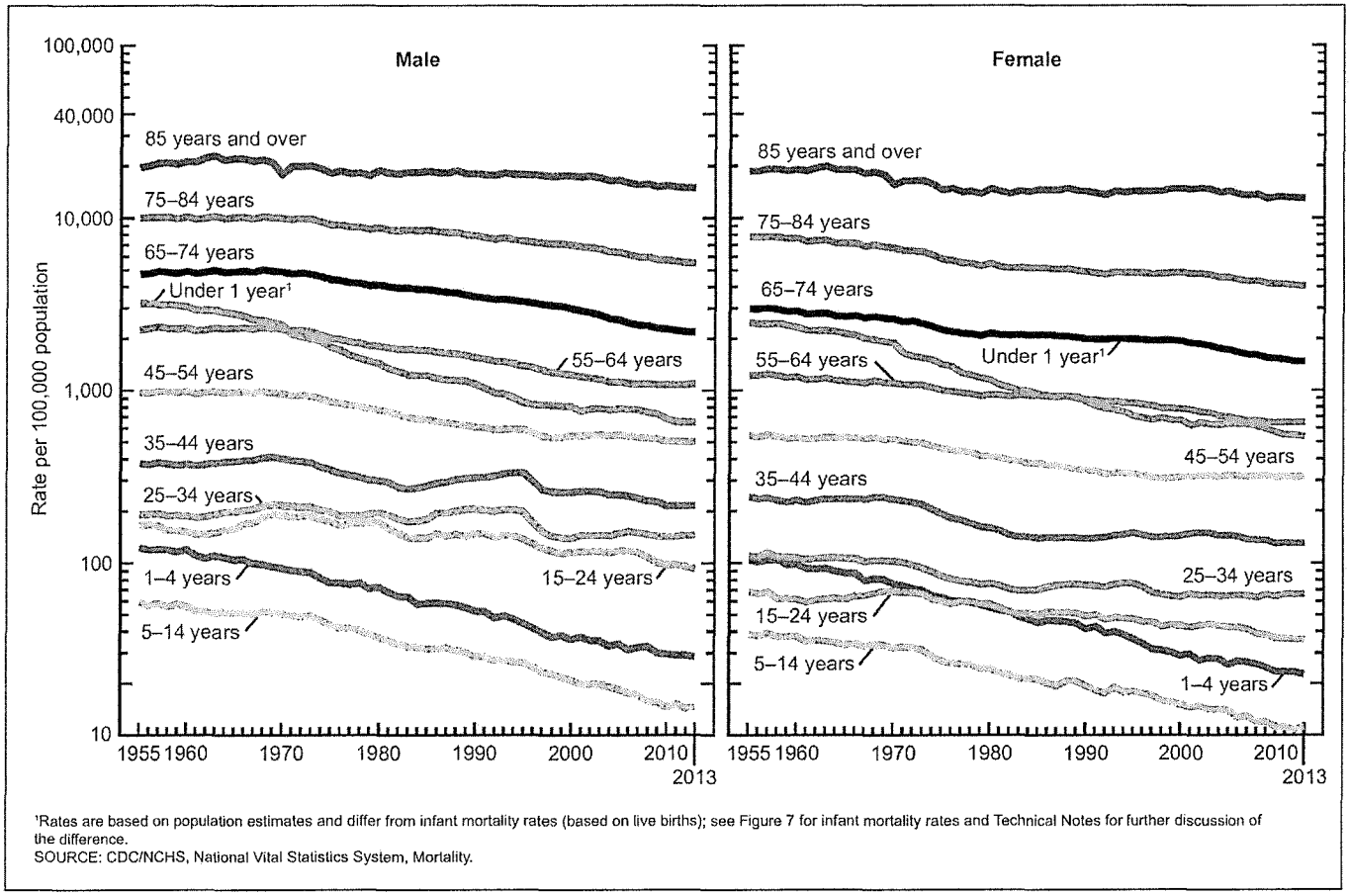
Life tables were generated for both sexes and by each sex for the following populations:

- Total U.S. population
- Black population
- White population
- Hispanic population
- Non-Hispanic white population
- Non-Hispanic black population

In 2013, life expectancy at birth for the U.S. population was 78.8 years, the same as in 2012 (Tables 6–8). The trend in U.S. life expectancy since 1900 has been one of gradual improvement, with occasional single-year decreases. In 2013, life expectancy was the same as in 2012 for females (81.2 years) and males (76.4 years). From 1900 through the late 1970s, the gap in life expectancy between the sexes widened (Figure 4) (3), from 2.0 to 7.8 years (data prior to 1975 are not shown). Since its peak in the 1970s, the gap between sexes has been narrowing. In 2013, the difference in life expectancy between the sexes was 4.8 years, unchanged since 2010.

Life expectancy was unchanged in 2013 from 2012 for the black population (75.5 years) and the white population (79.1 years). The difference in life expectancy between the white and black populations in 2013 was 3.6 years (Table 8). The white-black gap has been narrowing gradually, from a peak of 7.1 years in 1993 to the current record low (Figure 4). This continues a long-term decline in the white-black difference in life expectancy that was interrupted from 1983 through 1993 when the gap widened.

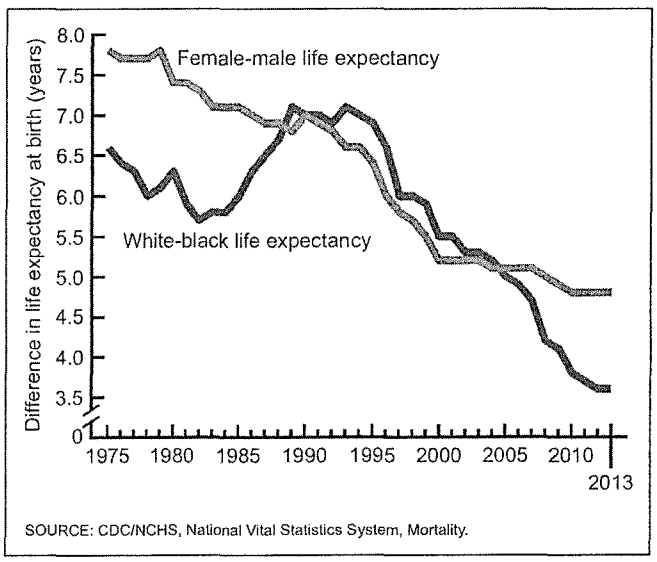
Life expectancy for white males has increased or remained the same nearly every year since 1975 (Figure 5). In contrast, life expectancy for black males declined every year from 1985 through 1989, then resumed the long-term trend of increase for most years from 1990 through 2013 (Table 8). For white females, life expectancy increased in most years from 1975 through 1998. In 1999, life expectancy for white females briefly fell slightly below 1998's then-record high, but



¹Rates are based on population estimates and differ from infant mortality rates (based on live births); see Figure 7 for infant mortality rates and Technical Notes for further discussion of the difference.
SOURCE: CDC/NCHS, National Vital Statistics System, Mortality.

Figure 3. Death rates, by age and sex: United States, 1955–2013

began to increase again in 2001. From 1989 through 1992, during 1994, and from 1996 through 1998, life expectancy for black females increased. In 1999, life expectancy for black females declined, as it did for white females, only to begin climbing again in 2000.



SOURCE: CDC/NCHS, National Vital Statistics System, Mortality.

Figure 4. Differences in female-male and white-black life expectancy: United States, 1975–2013

Life expectancy for the Hispanic population was 81.6 years in 2013, the same as in 2012 (Tables 7 and 8). Life expectancy figures for the Hispanic population have been available starting with data for 2006 (15). Since that year, life expectancy for the Hispanic population has increased by 1.3 years. In 2013, life expectancy for the Hispanic female population was 83.8 years, a 0.1-year decrease from 2012. Life expectancy for the Hispanic male population in 2013 was unchanged from 2012 at 79.1 years. The difference in life expectancy between the sexes for the Hispanic population was 4.7 years, a 0.1-year decrease from the 2012 gap.

Among the six Hispanic origin-race-sex groups (Tables 7 and 8) in 2013, Hispanic females had the highest life expectancy at birth (83.8 years), followed by non-Hispanic white females (81.2), Hispanic males (79.1), non-Hispanic black females (78.1), non-Hispanic white males (76.5), and non-Hispanic black males (71.8).

Life expectancy data by race include persons of Hispanic and non-Hispanic origin; life expectancy data by Hispanic origin include persons of any race. Life expectancy is higher when the Hispanic population is included in the race group. For example, life expectancy was 75.5 years for the black population, but was 75.1 for the non-Hispanic black population. Similarly, life expectancy for the white population was 79.1, but was 78.9 for the non-Hispanic white population.

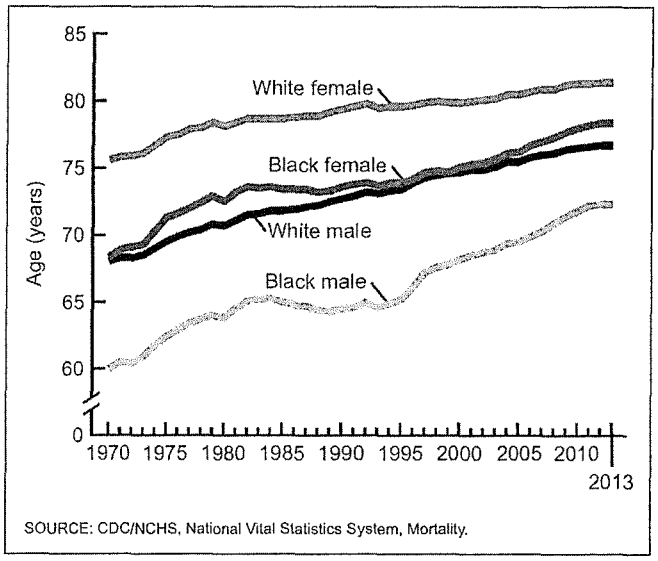


Figure 5. Life expectancy, by race and sex: United States, 1970–2013

Life expectancy for both males and females was more than 2 years higher for the Hispanic population than for the non-Hispanic white and non-Hispanic black populations. Various hypotheses have been proposed to explain favorable mortality outcomes among Hispanic persons. The most prevalent hypotheses are the healthy migrant effect, which argues that Hispanic immigrants are selected for their good health and robustness, the "salmon bias" effect, which posits that U.S. residents of Hispanic origin may return to their country of origin to die or when ill, and the "cultural effects," which argues that culturally influenced family structure, lifestyle behaviors, and social networks may confer a protective barrier against the negative effects of low socioeconomic and minority status (16,17).

Life tables shown in this report may be used to compare life expectancies at selected ages from birth to 100 years. For example, on the basis of mortality experienced in 2013, a person aged 50 could expect to live an average of 31.6 more years, for a total of 81.6 years. A person aged 65 could expect to live an average of 19.3 more years, for a total of 84.3, and a person aged 85 could expect to live an average of 6.6 more years, for a total of 91.6 (Tables 6 and 7).

Leading causes of death

The 15 leading causes of death in 2013 accounted for 79.3% of all deaths in the United States (Tables B and 9). The leading causes of death in 2013 remained the same as in 2012, although unintentional injuries, the 5th leading cause of death in 2012, became the 4th leading cause in 2013; and stroke, the 4th leading cause of death in 2012, became the 5th leading cause in 2013. Causes of death are ranked according to the number of deaths; for ranking procedures, see Technical Notes. By rank, the 15 leading causes of death in 2013 were:

1. Diseases of heart (heart disease)
2. Malignant neoplasms (cancer)
3. Chronic lower respiratory diseases
4. Accidents (unintentional injuries)
5. Cerebrovascular diseases (stroke)
6. Alzheimer's disease
7. Diabetes mellitus (diabetes)
8. Influenza and pneumonia
9. Nephritis, nephrotic syndrome and nephrosis (kidney disease)
10. Intentional self-harm (suicide)
11. Septicemia
12. Chronic liver disease and cirrhosis
13. Essential hypertension and hypertensive renal disease (hypertension)
14. Parkinson's disease
15. Pneumonitis due to solids and liquids

The pattern of mortality varies greatly with age. As a result, the shifting age distribution of a population can significantly influence changes in crude death rates over time. Age-adjusted death rates, in contrast, eliminate the influence of such differences in the population age structure. Therefore, whereas causes of death are ranked according to the number of deaths, age-adjusted death rates are used to depict trends for leading causes of death in this report because they are better than crude rates for showing changes in mortality over time and among causes of death (Figure 6).

From 2012 to 2013, the age-adjusted death rate declined significantly for 4 of the 15 leading causes of death and increased for 6 leading causes. The age-adjusted death rate for the leading cause of death, heart disease, decreased 0.4%. The age-adjusted death rate for cancer decreased 2.0% (Tables B and 9). Deaths from these two diseases combined accounted for 46.1% of deaths in the United States in 2013. Except for a relatively small increase in 1993, mortality from heart disease has declined steadily since 1980 (Figure 6). The age-adjusted death rate for cancer, the second leading cause of death, has shown a gradual but consistent downward trend since 1993 (Figure 6).

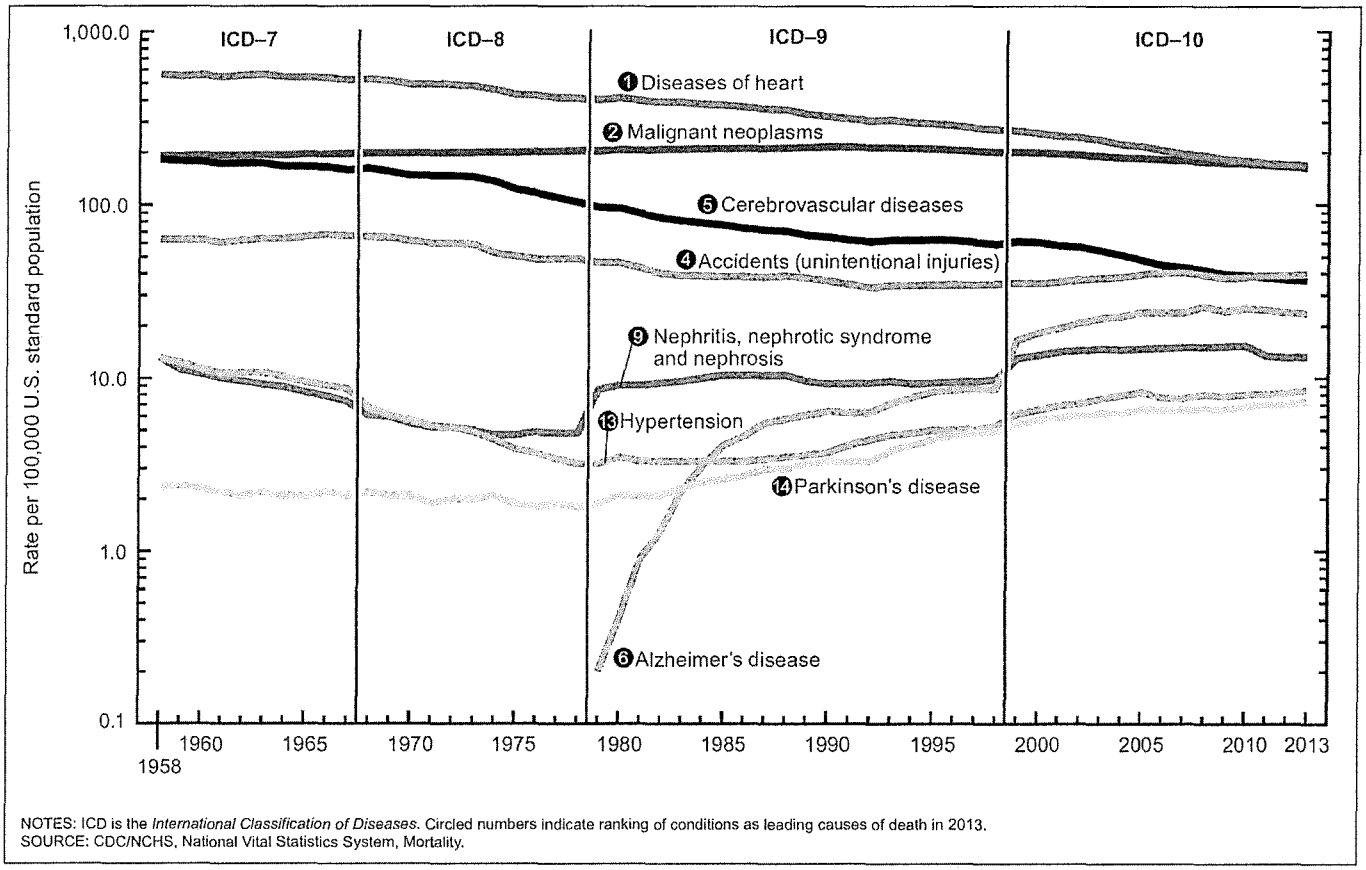
Other leading causes of death that showed significant decreases in 2013 relative to 2012 were stroke (1.9%) and Alzheimer's disease (1.3%).

The age-adjusted death rate increased significantly between 2012 and 2013 for six leading causes: Chronic lower respiratory diseases (1.4%), Influenza and pneumonia (10.4%), Septicemia (3.9%), Chronic liver disease and cirrhosis (3.0%), hypertension (3.7%), and Parkinson's disease (4.3%).

Observed changes from 2012 to 2013 in the age-adjusted death rate for unintentional injuries, diabetes, kidney disease, suicide, and Pneumonitis due to solids and liquids were not significant.

Assault (homicide), the 16th leading cause of death since 2010, dropped from among the 15 leading causes of death in 2010 but is still a major issue for some age groups. In 2013, homicide remained among the 15 leading causes of death for age groups 1–4 (3rd), 5–14 (5th), 15–24 (3rd), 25–34 (3rd), 35–44 (5th), and 45–54 (13th). From 2012 to 2013, the ranking of homicide for these age groups changed only for those aged 1–4, rising from the 4th leading cause in 2012 to the 3rd leading cause in 2013, and for those aged 5–14, dropping from the 4th leading cause in 2012 to the 5th leading cause in 2013.

Although Human immunodeficiency virus (HIV) disease has not been among the 15 leading causes of death since 1997 (18), it is still considered a major public health problem for some age groups. Historically, for all ages combined, HIV disease mortality reached its highest level in 1995 after a period of increase from 1987 through 1994. Subsequently, the rate for this disease decreased an average of 33.0% per year from 1995 through 1998, and 6.4% per year from



NOTES: ICD is the *International Classification of Diseases*. Circled numbers indicate ranking of conditions as leading causes of death in 2013.
SOURCE: CDC/NCHS, National Vital Statistics System, Mortality.

Figure 6. Age-adjusted death rates for selected leading causes of death: United States, 1958–2013

1999 through 2013 (19). In 2013, HIV disease remained among the 15 leading causes of death for age groups 15–24 (13th), 25–34 (8th), 35–44 (9th), 45–54 (10th), and 55–64 (14th). Among these age groups, the ranking of HIV disease changed between 2012 and 2013 for those aged 15–24, rising from 14th leading cause in 2012 to 13th leading cause in 2013; those aged 25–34, dropping from 6th leading cause in 2012 to 8th leading cause in 2013; and those aged 45–54, dropping from 9th leading cause in 2012 to 10th leading cause in 2013 (20).

Enterocolitis due to *Clostridium difficile* (*C. difficile*)—a predominantly antibiotic-associated inflammation of the intestines caused by *C. difficile*, a gram-positive, anaerobic, spore-forming bacillus—is of growing concern. The disease is often acquired in hospitals or other health care facilities with long-term patients or residents (21,22). The number of deaths from *C. difficile* climbed from 793 deaths in 1999 to a high of 8,085 deaths in 2011 (19,20). In 2013, the number of deaths from *C. difficile* was 7,665, continuing to decline after a slight decrease in 2012. In 2013, the age-adjusted death rate for this cause was 2.1 deaths per 100,000 standard population, a decrease of 4.5% from the rate in 2012 (2.2). In 2013, *C. difficile* ranked as the 18th leading cause of death for the population aged 65 and over. Approximately 90% of deaths from *C. difficile* occurred among people aged 65 and over (Table 10).

Changes in mortality levels by age and cause of death can have a major effect on changes in life expectancy. While changes in causes of death occurred in 2013 from 2012, life expectancy at birth for the total population did not change. Decreases in mortality from cancer, homicide, stroke, and heart disease were offset by increases in mortality from Influenza and pneumonia, chronic lower respiratory diseases, Septicemia, and unintentional injuries. (In other words, if mortality for these causes of death had not increased as much as they did in 2013, the life expectancy for the total population might have increased.) Life expectancy at birth for both males and females did not change between 2012 and 2013. For males, decreases in mortality from cancer, homicide, Alzheimer's disease, and stroke were offset by increases in mortality from Influenza and pneumonia, hypertension, Chronic liver disease and cirrhosis, and Septicemia. Similarly for the female population, decreases in mortality for cancer, heart disease, stroke, and Congenital malformations were offset by increases in mortality from Influenza and pneumonia, Chronic lower respiratory diseases, Septicemia, and perinatal conditions. (For a discussion of the major causes contributing to the change in life expectancy, see Technical Notes.)

The relative risk of death in one population group compared with another can be expressed as a ratio. Ratios based on age-adjusted death rates show that males have higher rates than females for 13 of the 15 leading causes of death (Table B), with rates for males being at least twice as great as those for females for 4 of these leading causes. The largest ratio was for suicide (3.7). Other large ratios were evident for Parkinson's disease (2.3); unintentional injuries and Chronic liver disease and cirrhosis (2.0 each); Pneumonitis due to solids and liquids (1.8); heart disease (1.6); diabetes (1.5); cancer and kidney disease (1.4 each); Influenza and pneumonia (1.3); chronic lower respiratory diseases and Septicemia (1.2 each); and hypertension (1.1). Age-adjusted rates were lower for males than for females for one leading cause, Alzheimer's disease (0.7).

Age-adjusted death rates for the black population were higher than for the white population for 8 of the 15 leading causes of death (Table B). The largest ratios were for kidney disease and hypertension (2.1 each). Other causes for which the ratio was high include diabetes (2.0); Septicemia (1.8); stroke (1.4); heart disease (1.3); cancer (1.2); and Influenza and pneumonia (1.1). For 7 of the leading causes, age-adjusted rates were lower for the black population than for the white population. The smallest black-to-white ratio was for suicide (0.4); that is, the risk of dying from suicide was more than double for the white population than for the black population. Other conditions with a low black-to-white ratio were Parkinson's disease (0.5); Chronic lower respiratory diseases and Chronic liver disease and cirrhosis (0.7 each); unintentional injuries and Alzheimer's disease (0.8 each); and Pneumonitis due to solids and liquids (0.9).

The difference in life expectancy between the white and black populations was 3.6 years in 2013, the same as in 2012 (Table B). The difference between white and black life expectancy remained constant due to offsetting improvements in mortality from specific causes for the white and black populations. For example, the white population experienced greater improvements in mortality from cancer, homicide, HIV disease, and kidney disease; while the black population experienced greater improvements in mortality from unintentional injuries, perinatal conditions, Alzheimer's disease, and aortic aneurysm (data not shown).

Death rates for the AIAN population are not adjusted for misclassification. Given that the rates for the AIAN population are underestimated by about 30% (13), disparities in the age-adjusted death rates should be interpreted with caution when making comparisons across races.

For the API population, death rates are not adjusted for misclassification and are underestimated by about 7% due to underreporting on death certificates (13). Therefore, even though the level of underestimation for this population is not as great as for the AIAN population, similar caution should be exercised when interpreting rate disparities involving the API population and other races.

Death rates for the population of Hispanic origin are not adjusted for misclassification (see Technical Notes). Because these rates are both unadjusted for misclassification and underestimated by about 5.0% (13), caution should be exercised when interpreting rate disparities in the Hispanic and non-Hispanic populations.

Life table partitioning analysis indicates that the difference of 2.7 years in life expectancy between the Hispanic and non-Hispanic white populations is mostly explained by lower death rates from cancer, heart disease, Chronic lower respiratory diseases, unintentional injuries, and suicide experienced by the Hispanic population. (For a discussion of the major causes contributing to the difference in life expectancy, see Technical Notes.)

Leading causes of death in 2013 for the total population and for specific subpopulations are examined in more detail in a companion *National Vital Statistics Report* on leading causes by age, race, Hispanic origin, and sex (2).

Injury mortality by mechanism and intent

In 2013, a total of 192,945 deaths were classified as injury-related (Table 18). Injury data are presented using the external cause-of-injury mortality matrix for ICD–10, as jointly conceived by the International Collaborative Effort (ICE) on Injury Statistics and the Injury Control and Emergency Health Services section, known as ICEHS, of the American Public Health Association (23,24). The ICD codes for injuries have two essential dimensions: the mechanism of the injury and its manner or intent. The mechanism involves the circumstances of the injury (e.g., fall, motor vehicle traffic, or poisoning). The manner or intent involves whether the injury was purposefully inflicted (where it can be determined) and, when intentional, whether the injury was self-inflicted (suicide) or inflicted upon another person (assault). In the List of 113 Selected Causes of Death, the focus is on manner or intent, with subcategories showing selected mechanisms. The matrix has two distinct advantages for the analysis of injury mortality data: It contains a comprehensive list of mechanisms, and data can be displayed by mechanism with subcategories of intent, or vice versa. Four major mechanisms of injury in 2013—poisoning, motor-vehicle traffic, firearm, and fall—accounted for 76.3% of all injury deaths.

Poisoning—In 2013, 48,545 deaths occurred as the result of poisonings, 25.2% of all injury deaths (Table 18). The age-adjusted death rate for poisoning in 2013 (15.2 deaths per 100,000 U.S. standard population) increased significantly, 4.1% from the rate in 2012 (14.6). The majority of poisoning deaths were either unintentional (80.0%) or suicides (13.7%). However, 6.1% of poisoning deaths were of undetermined intent. The age-adjusted death rate for unintentional poisoning increased 6.1%, from 11.5 in 2012 to 12.2 in 2013, and has nearly tripled since 1999 (data prior to 2013 are not shown but are available through CDC WONDER at http://wonder.cdc.gov/).

Motor-vehicle traffic—In 2013, motor-vehicle traffic-related injuries resulted in 33,804 deaths, accounting for 17.5% of all injury deaths (Table 18). The age-adjusted death rate for these injuries decreased significantly, 3.7%, from 10.9 per 100,000 standard population in 2012 to 10.5 in 2013.

Firearm—In 2013, 33,636 persons died from firearm injuries in the United States (Tables 18 and 19), accounting for 17.4% of all injury deaths in that year. The age-adjusted death rate from firearm injuries (all intents) did not change significantly in 2013 from 2012. The two major component causes of firearm injury deaths in 2013 were suicide (63.0%) and homicide (33.3%). The age-adjusted death rate for firearm homicide decreased 5.3%, from 3.8 in 2012 to 3.6 in 2013. The rate for firearm suicide did not change significantly.

Fall—In 2013, 31,240 persons died as the result of falls, 16.2% of all injury deaths (Table 18). The age-adjusted death rate for falls increased 2.3%, from 8.6 in 2012 to 8.8 in 2013. The overwhelming majority of fall-related deaths (96.7%) were unintentional.

Drug-induced mortality

In 2013, a total of 46,471 persons died of drug-induced causes in the United States (Tables 10, 12, and 13). This category includes deaths from poisoning and medical conditions caused by use of legal or illegal drugs, as well as deaths from poisoning due to medically prescribed and other drugs. It excludes unintentional injuries, homicides, and other causes indirectly related to drug use, as well as newborn deaths due to the mother's drug use. (For a list of drug-induced causes, see Technical Notes; also see the discussion of poisoning mortality that uses the more narrow definition of poisoning as an injury in the preceding "Injury mortality by mechanism and intent" section.)

In 2013, the age-adjusted death rate for drug-induced causes for the total population increased significantly, 5.8%, from 13.8 in 2012

to 14.6 in 2013 (Internet Tables I–3 and I–4). For males in 2013, the age-adjusted death rate for drug-induced causes was 1.6 times the rate for females. The age-adjusted death rate for black females was 46.5% lower than for white females, and the rate for black males was 30.0% lower than for white males. The rate for drug-induced causes increased 5.9% for males and 3.7% for females in 2013 from 2012.

Among the major race-sex and race-ethnicity-sex groups, the age adjusted death rates for drug-induced causes increased significantly in 2013 from 2012 for white males (5.3%), white females (5.0%), black males (12.0%), Hispanic males (7.5%), non-Hispanic white males (5.1%), non-Hispanic white females (5.1%), and non-Hispanic black males (13.3%).

Alcohol-induced mortality

In 2013, a total of 29,001 persons died of alcohol-induced causes in the United States (Tables 10, 12, and 13). This category includes deaths from dependent and nondependent use of alcohol, as well as deaths from accidental poisoning by alcohol. It excludes unintentional injuries, homicides, and other causes indirectly related to alcohol use, as well as deaths due to fetal alcohol syndrome (for a list of alcohol-induced causes, see Technical Notes).

The age-adjusted death rate for alcohol-induced causes for the total population increased significantly, 2.5%, from 8.0 in 2012 to 8.2 in 2013 (Tables I–5 and I–6). For males, the age-adjusted death rate for alcohol-induced causes in 2013 was 2.9 times the rate for females. Compared with the rate for the white population, the rate for the black population was 31.0% lower.

Among the major race-sex and race-ethnicity-sex groups, the age-adjusted rate for alcohol-induced death increased significantly in 2013 from 2012 for white males (3.1%), non-Hispanic white males (3.3%), and non-Hispanic white females (4.3%). No other major race-sex and race-ethnicity-sex groups experienced significant changes.

State of residence

Mortality patterns vary considerably by state (Tables 19 and 22). The state with the highest age-adjusted death rate in 2013 was Mississippi (959.6 per 100,000 U.S. standard population), with a rate 31.1% above the national average (731.9). The state with the lowest age-adjusted death rate was Hawaii (590.8 per 100,000 standard population), with a rate 19.3% below the national average. The age-adjusted death rate for Mississippi was 62.4% higher than the rate for Hawaii.

Variations in mortality by state are associated with differences in socioeconomic status, race, and ethnicity composition, as well as with differences in risk for specific causes of death (25).

Infant mortality

In 2013, a total of 23,440 deaths occurred in children under age 1 year (Tables D and 21). This number represents 189 fewer infant deaths in 2013 than in 2012. The infant mortality rate was 5.96 per 1,000 live births, the neonatal mortality rate (deaths of infants aged 0–27 days per 1,000 live births) was 4.04, and the postneonatal mortality rate (deaths of infants aged 28 days through 11 months per 1,000 live births) was 1.93 in 2013 (Figure 7; see Technical Notes for information on alternative data sources). Changes in the infant, neonatal, and postneonatal rates from 2012 to 2013 were not statistically significant.

The 10 leading causes of infant death in 2013 accounted for 69.0% of all infant deaths in the United States (Table E). By rank, the 10 leading causes were:

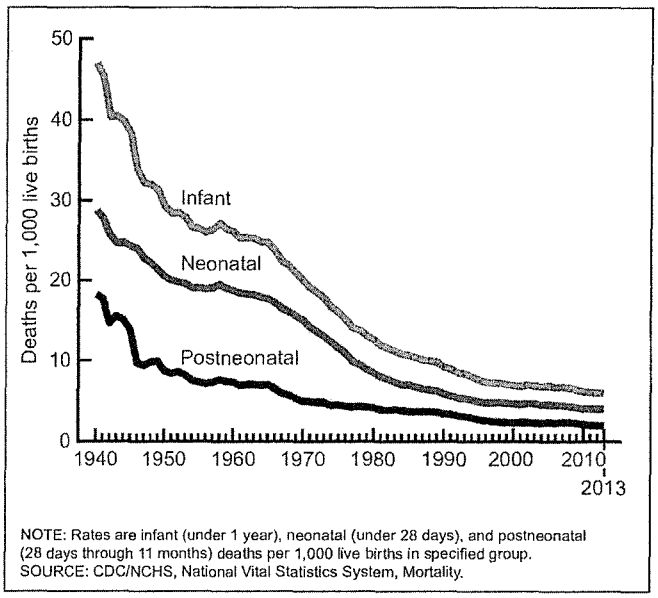
1. Congenital malformations, deformations and chromosomal abnormalities
2. Disorders related to short gestation and low birth weight, not elsewhere classified
3. Newborn affected by maternal complications of pregnancy
4. Sudden infant death syndrome (SIDS)
5. Accidents (unintentional injuries)
6. Newborn affected by complications of placenta, cord and membranes
7. Bacterial sepsis of newborn
8. Respiratory distress of newborn
9. Diseases of the circulatory system
10. Neonatal hemorrhage

Table D. Number of infant, neonatal, and postneonatal deaths and mortality rates, by sex: United States, 2012–2013

[Rates are infant (under 1 year), neonatal (under 28 days), and postneonatal (28 days–11 months) deaths per 1,000 live births in specified group]

Infant age and sex	2013		2012		Percent change[1] from 2012 to 2013
	Number	Rate	Number	Rate	
Infant					
Total	23,440	5.96	23,629	5.98	–0.3
Male	13,119	6.52	13,139	6.50	0.3
Female	10,321	5.38	10,490	5.43	–0.9
Neonatal					
Total	15,867	4.04	15,850	4.01	0.7
Male	8,800	4.37	8,764	4.34	0.7
Female	7,067	3.68	7,086	3.67	0.3
Postneonatal					
Total	7,573	1.93	7,779	1.97	–2.0
Male	4,319	2.15	4,375	2.16	–0.5
Female	3,254	1.70	3,404	1.76	–3.4

[1]Based on a comparison of the 2013 and 2012 mortality rates.



NOTE: Rates are infant (under 1 year), neonatal (under 28 days), and postneonatal (28 days through 11 months) deaths per 1,000 live births in specified group.
SOURCE: CDC/NCHS, National Vital Statistics System, Mortality.

Figure 7. Infant, neonatal, and postneonatal mortality rates: United States, 1940–2013

In 2013, the 10 leading causes of infant death remained the same as in 2012 (20), although SIDS dropped from 3rd leading cause to 4th leading cause and Newborn affected by complications of pregnancy rose from 4th leading cause to 3rd leading cause. Changes in rates by cause of death among the 10 leading causes were not statistically significant (Table E).

Race cited on the death certificate is considered to be relatively accurate for white and black infants (13). For other race groups, however, race may be misreported on the death certificate (26). Generally, infant mortality rates calculated from the linked file of live births and infant deaths provide better measures of infant mortality by race (26); see Technical Notes. In addition, infant mortality rates by specified Hispanic origin and race for non-Hispanic origin that are based on the mortality file may be somewhat understated and are better measured using data from the linked file of live births and infant deaths (26); see Technical Notes. Infant mortality data presented in this report use the general mortality file, not the linked file of live births and infant deaths.

The ratio of male to female infant mortality rates was 1.2, the same as in 2012. The ratio of black to white infant mortality rates was 2.2 in 2013, also the same as in 2012. The infant mortality rate did not change significantly in 2013 from 2012 for major race groups (Table 20).

Hispanic infant mortality—Infant mortality rates for the population of Hispanic origin are not adjusted for misclassification; see Technical Notes. Because these rates are not adjusted for misclassification, caution should be exercised when interpreting rate disparities between the Hispanic and non-Hispanic populations (13). In 2013, the infant mortality rate for Hispanic infants was 5.27 deaths per 1,000 live births. By comparison, for non-Hispanic white infants, the infant mortality rate was 4.96; and for non-Hispanic black infants, the infant mortality rate was 11.61 (data not shown). The infant mortality rate did not change significantly in 2013 from 2012 for the Hispanic, non-Hispanic white, and non-Hispanic black populations. Among Hispanic subgroups, the infant mortality rate was 6.71 per 1,000 live births for Puerto Rican, 6.00 for Mexican, 2.92 for Cuban, and 3.30 for Central and South American populations.

Additional mortality tables based on 2013 final data

For data year 2013, trend data on drug-induced causes, alcohol-induced causes, and firearm-related injuries are available as supplemental tables (Tables I–1–I–6) from the NCHS website at

Table E. Number of infant deaths, percentage of total infant deaths, and infant mortality rates for 2013, and percentage change in infant mortality rates from 2012 to 2013 for the 10 leading causes of infant death in 2013: United States

[Rates are infant deaths per 100,000 live births]

Rank[1]	Cause of death (based on ICD–10)	Number	Percent of total deaths	Rate	Percent change[2] from 2012 to 2013
...	All causes	23,440	100.0	596.1	–0.3
1	Congenital malformations, deformations and chromosomal abnormalities (Q00–Q99)	4,758	20.3	121.0	–3.1
2	Disorders related to short gestation and low birth weight, not elsewhere classified (P07)	4,202	17.9	106.9	0.6
3	Newborn affected by maternal complications of pregnancy (P01)	1,595	6.8	40.6	6.6
4	Sudden infant death syndrome (R95)	1,563	6.7	39.7	–6.6
5	Accidents (unintentional injuries) (V01–X59)	1,156	4.9	29.4	–0.7
6	Newborn affected by complications of placenta, cord and membranes (P02)	953	4.1	24.2	–6.2
7	Bacterial sepsis of newborn (P36)	578	2.5	14.7	2.8
8	Respiratory distress of newborn (P22)	522	2.2	13.3	3.9
9	Diseases of the circulatory system (I00–I99)	458	2.0	11.6	–6.5
10	Neonatal hemorrhage (P50–P52,P54)	389	1.7	9.9	–7.5
...	All other causes (residual)	7,266	31.0	184.8	...

... Category not applicable.
[1]Based on number of deaths; see Technical Notes.
[2]Based on a comparison of the 2013 infant mortality rate with the 2012 infant mortality rate.

http://www.cdc.gov/nchs/data/nvsr/nvsr64/nvsr64_02_tables.pdf. Similarly, mortality data by educational attainment, marital status, and injury at work are also available as supplemental tables (Tables I–7–I–10).

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List of Detailed Tables

List of Internet Tables

(Available from http://www.cdc.gov/nchs/data/nvsr/nvsr64/nvsr64_02_tables.pdf)

Table 11. Death rates for 113 selected causes, Enterocolitis due to *Clostridium difficile*, drug-induced causes, alcohol-induced causes, and injury by firearms, by age: United States, 2013—Con.

[Rates per 100,000 population in specified group. Populations used for computing death rates are postcensal estimates based on the 2010 census estimated as of July 1, 2013; see Technical Notes. The asterisks (*) preceding the cause-of-death codes indicate that they are not part of the *International Classification of Diseases, Tenth Revision* (ICD–10); see Technical Notes]

Cause of death (based on ICD–10)	All ages[1]	Age group (years)										
		Under 1 year[2]	1–4	5–14	15–24	25–34	35–44	45–54	55–64	65–74	75–84	85 and over
Diseases of pericardium and acute myocarditis . . . (I30–I31,I40)	0.3	*	*	*	0.1	0.1	0.1	0.3	0.3	0.5	1.2	2.4
Heart failure . . . . . (I50)	20.6	*	*	*	0.1	0.3	0.8	2.9	8.9	28.4	119.3	607.1
All other forms of heart disease . . (I26–I28,I34–I38,I42–I49,I51)	40.9	6.7	0.8	0.3	1.5	3.6	8.1	17.6	34.9	75.2	227.8	863.1
Essential hypertension and hypertensive renal disease . (I10,I12,I15)	9.7	*	*	*	0.1	0.3	1.0	3.5	8.0	17.3	53.7	231.6
Cerebrovascular diseases . . . . . (I60–I69)	40.8	2.7	0.2	0.2	0.3	1.2	4.2	12.4	28.9	74.2	268.9	906.0
Atherosclerosis . . . . . (I70)	2.1	*	*	*	*	*	*	0.4	0.9	2.9	11.8	63.0
Other diseases of circulatory system . . . . . (I71–I78)	6.0	*	*	*	0.1	0.4	1.0	2.2	5.6	14.4	39.2	103.3
Aortic aneurysm and dissection . . . . . (I71)	3.1	*	*	*	0.1	0.3	0.8	1.4	3.2	7.8	20.8	45.3
Other diseases of arteries, arterioles and capillaries . . . (I72–I78)	2.9	*	*	*	0.1	0.1	0.2	0.8	2.4	6.7	18.4	58.0
Other disorders of circulatory system . . . . . (I80–I99)	1.4	0.6	*	*	0.1	0.4	0.9	1.4	1.9	2.8	5.8	16.0
Influenza and pneumonia . . . . . (J09–J18)	18.0	4.5	0.6	0.3	0.4	1.0	2.2	5.1	12.2	29.5	103.7	441.0
Influenza . . . . . (J09–J11)	1.2	0.5	0.2	0.1	0.1	0.2	0.4	0.6	1.0	1.6	5.2	26.4
Pneumonia . . . . . (J12–J18)	16.9	4.0	0.4	0.2	0.3	0.9	1.8	4.5	11.2	27.9	98.6	414.7
Other acute lower respiratory infections . . . . . (J20–J22,U04)	0.1	0.7	*	*	*	*	*	*	0.1	0.1	0.3	2.1
Acute bronchitis and bronchiolitis . . . . . (J20–J21)	0.1	0.7	*	*	*	*	*	*	0.1	0.1	0.2	1.6
Other and unspecified acute lower respiratory infections . . (J22,U04)	0.0	*	*	*	*	*	*	*	*	*	*	0.4
Chronic lower respiratory diseases . . . . . (J40–J47)	47.2	0.6	0.4	0.4	0.4	0.7	1.9	10.6	40.5	141.2	367.0	699.3
Bronchitis, chronic and unspecified . . . . . (J40–J42)	0.2	0.5	0.1	*	*	*	*	0.1	0.2	0.3	1.0	4.8
Emphysema . . . . . (J43)	2.6	*	*	*	*	*	0.1	0.7	2.7	8.8	20.4	31.1
Asthma . . . . . (J45–J46)	1.1	*	0.2	0.4	0.3	0.5	0.9	1.2	1.5	1.7	3.4	11.6
Other chronic lower respiratory diseases . . . . . (J44,J47)	43.2	*	*	*	*	0.1	0.9	8.5	36.1	130.3	342.2	651.9
Pneumoconioses and chemical effects. . . . . (J60–J66,J68)	0.3	*	*	*	*	*	*	*	0.1	0.6	2.3	4.4
Pneumonitis due to solids and liquids . . . . . (J69)	5.9	*	*	*	0.1	0.2	0.4	1.3	3.2	9.0	36.7	152.6
Other diseases of respiratory system . (J00–J06,J30–J39,J67,J70–J98)	11.1	7.0	0.7	0.2	0.3	0.5	1.2	3.3	10.2	29.3	79.8	171.3
Peptic ulcer . . . . . (K25–K28)	0.9	*	*	*	*	*	0.2	0.6	1.2	2.2	5.3	14.8
Diseases of appendix . . . . . (K35–K38)	0.1	*	*	*	*	*	*	0.1	0.1	0.3	0.6	1.5
Hernia . . . . . (K40–K46)	0.6	0.5	*	*	*	*	0.1	0.2	0.5	1.2	3.5	13.3
Chronic liver disease and cirrhosis . . . . . (K70,K73–K74)	11.5	*	*	*	0.1	1.6	6.2	20.1	30.4	28.1	29.9	23.0
Alcoholic liver disease . . . . . (K70)	5.7	*	*	*	0.1	1.3	4.5	12.9	16.4	10.5	6.3	2.7
Other chronic liver disease and cirrhosis . . . . . (K73–K74)	5.8	*	*	*	*	0.3	1.7	7.2	14.0	17.6	23.6	20.3
Cholelithiasis and other disorders of gallbladder . . . . . (K80–K82)	1.1	*	*	*	*	0.1	0.1	0.3	0.7	2.3	7.1	23.3
Nephritis, nephrotic syndrome and nephrosis . . . . . (N00–N07,N17–N19,N25–N27)	14.9	2.2	*	*	0.1	0.6	1.5	4.6	12.6	33.8	99.0	285.4
Acute and rapidly progressive nephritic and nephrotic syndrome . . . . . (N00–N01,N04)	0.1	*	*	*	*	*	*	*	0.1	0.3	0.8	2.7
Chronic glomerulonephritis, nephritis and nephropathy not specified as acute or chronic, and renal sclerosis unspecified . . . . . (N02–N03,N05–N07,N26)	0.1	*	*	*	*	*	*	0.1	0.1	0.2	0.4	0.9
Renal failure . . . . . (N17–N19)	14.7	2.1	*	*	0.1	0.6	1.5	4.5	12.4	33.3	97.8	281.6

See footnotes at end of table.

Table 12. Number of deaths from 113 selected causes, Enterocolitis due to *Clostridium difficile*, drug-induced causes, alcohol-induced causes, and injury by firearms, by race and sex: United States, 2013—Con.

[Data for specified races other than white and black should be interpreted with caution because of inconsistencies between reporting race on death certificates and on censuses and surveys; see Technical Notes. The asterisks (*) preceding cause-of-death codes indicate that they are not part of the *International Classification of Diseases, Tenth Revision* (ICD–10); see Technical Notes]

Cause of death (based on ICD–10)	All races			White[1]			Black[1]			American Indian or Alaska Native[1,2]			Asian or Pacific Islander[1,3]		
	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female
Other forms of chronic ischemic heart disease (I20,I25)	249,484	140,400	109,084	215,572	121,784	93,788	26,626	14,291	12,335	1,397	864	533	5,889	3,461	2,428
Atherosclerotic cardiovascular disease, so described (I25.0)	59,475	35,906	23,569	48,859	29,458	19,401	8,726	5,275	3,451	502	317	185	1,388	856	532
All other forms of chronic ischemic heart disease (I20,I25.1–I25.9)	190,009	104,494	85,515	166,713	92,326	74,387	17,900	9,016	8,884	895	547	348	4,501	2,605	1,896
Other heart diseases (I26–I51)	196,460	91,091	105,369	169,324	77,950	91,374	22,651	10,916	11,735	828	430	398	3,657	1,795	1,862
Acute and subacute endocarditis (I33)	1,255	766	489	1,038	629	409	174	113	61	18	9	9	25	15	10
Diseases of pericardium and acute myocarditis (I30–I31,I40)	872	453	419	688	363	325	150	72	78	10	7	3	24	11	13
Heart failure (I50)	65,120	28,513	36,607	57,353	25,005	32,348	6,548	2,946	3,602	241	106	135	978	456	522
All other forms of heart disease (I26–I28,I34–I38,I42–I49,I51)	129,213	61,359	67,854	110,245	51,953	58,292	15,779	7,785	7,994	559	308	251	2,630	1,313	1,317
Essential hypertension and hypertensive renal disease (I10,I12,I15)	30,770	12,963	17,807	24,149	9,940	14,209	5,455	2,496	2,959	183	94	89	983	433	550
Cerebrovascular diseases (I60–I69)	128,978	53,691	75,287	107,909	44,203	63,706	16,269	7,338	8,931	595	253	342	4,205	1,897	2,308
Atherosclerosis (I70)	6,685	2,714	3,971	5,995	2,419	3,576	529	225	304	35	16	19	126	54	72
Other diseases of circulatory system (I71–I78)	18,956	9,971	8,985	16,124	8,509	7,615	2,282	1,167	1,115	91	41	50	459	254	205
Aortic aneurysm and dissection (I71)	9,846	5,753	4,093	8,485	4,980	3,505	1,027	590	437	39	18	21	295	165	130
Other diseases of arteries, arterioles and capillaries (I72–I78)	9,110	4,218	4,892	7,639	3,529	4,110	1,255	577	678	52	23	29	164	89	75
Other disorders of circulatory system (I80–I99)	4,443	2,165	2,278	3,492	1,683	1,809	859	437	422	25	15	10	67	30	37
Influenza and pneumonia (J09–J18)	56,979	26,804	30,175	49,013	22,907	26,106	5,567	2,696	2,871	375	190	185	2,024	1,011	1,013
Influenza (J09–J11)	3,697	1,697	2,000	3,288	1,506	1,782	278	127	151	30	9	21	101	55	46
Pneumonia (J12–J18)	53,282	25,107	28,175	45,725	21,401	24,324	5,289	2,569	2,720	345	181	164	1,923	956	967
Other acute lower respiratory infections (J20–J22,U04)	285	110	175	252	94	158	30	14	16	1	–	1	2	2	–
Acute bronchitis and bronchiolitis (J20–J21)	226	90	136	199	76	123	24	12	12	1	–	1	2	2	–
Other and unspecified acute lower respiratory infections (J22,U04)	59	20	39	53	18	35	6	2	4	–	–	–	–	–	–
Chronic lower respiratory diseases (J40–J47)	149,205	70,317	78,888	136,682	63,757	72,925	9,918	5,073	4,845	757	370	387	1,848	1,117	731
Bronchitis, chronic and unspecified (J40–J42)	664	272	392	580	237	343	66	29	37	2	1	1	16	5	11
Emphysema (J43)	8,284	4,321	3,963	7,589	3,905	3,684	545	317	228	41	21	20	109	78	31
Asthma (J45–J46)	3,630	1,410	2,220	2,415	842	1,573	1,006	470	536	34	16	18	175	82	93
Other chronic lower respiratory diseases (J44,J47)	136,627	64,314	72,313	126,098	58,773	67,325	8,301	4,257	4,044	680	332	348	1,548	952	596
Pneumoconioses and chemical effects (J60–J66,J68)	806	767	39	764	728	36	32	29	3	9	9	–	1	1	–
Pneumonitis due to solids and liquids (J69)	18,579	10,140	8,439	16,458	8,989	7,469	1,609	857	752	76	44	32	436	250	186
Other diseases of respiratory system (J00–J06, J30–J39,J67,J70–J98)	35,217	17,825	17,392	30,954	15,822	15,132	3,188	1,451	1,737	248	124	124	827	428	399
Peptic ulcer (K25–K28)	2,988	1,535	1,453	2,551	1,286	1,265	295	164	131	17	11	6	125	74	51
Diseases of appendix (K35–K38)	371	216	155	306	176	130	51	34	17	5	1	4	9	5	4

See footnotes at end of table.

Table 12. Number of deaths from 113 selected causes, Enterocolitis due to *Clostridium difficile*, drug-induced causes, alcohol-induced causes, and injury by firearms, by race and sex: United States, 2013—Con.

[Data for specified races other than white and black should be interpreted with caution because of inconsistencies between reporting race on death certificates and on censuses and surveys; see Technical Notes. The asterisks (*) preceding cause-of-death codes indicate that they are not part of the *International Classification of Diseases, Tenth Revision* (ICD–10); see Technical Notes]

Cause of death (based on ICD–10)	All races			White[1]			Black[1]			American Indian or Alaska Native[1,2]			Asian or Pacific Islander[1,3]		
	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female	Both sexes	Male	Female
Hernia (K40–K46)	1,932	772	1,160	1,718	687	1,031	173	68	105	12	5	7	29	12	17
Chronic liver disease and cirrhosis (K70,K73–K74)	36,427	23,709	12,718	31,871	20,884	10,987	3,053	1,955	1,098	944	511	433	559	359	200
Alcoholic liver disease (K70)	18,146	12,991	5,155	15,861	11,478	4,383	1,372	930	442	703	419	284	210	164	46
Other chronic liver disease and cirrhosis (K73–K74)	18,281	10,718	7,563	16,010	9,406	6,604	1,681	1,025	656	241	92	149	349	195	154
Cholelithiasis and other disorders of gallbladder (K80–K82)	3,377	1,605	1,772	2,903	1,382	1,521	318	144	174	39	18	21	117	61	56
Nephritis, nephrotic syndrome and nephrosis (N00–N07,N17–N19,N25–N27)	47,112	23,493	23,619	37,270	18,800	18,470	8,393	3,976	4,417	302	143	159	1,147	574	573
Acute and rapidly progressive nephritic and nephrotic syndrome (N00–N01,N04)	399	205	194	329	164	165	60	34	26	1	–	1	9	7	2
Chronic glomerulonephritis, nephritis and nephropathy not specified as acute or chronic, and renal sclerosis unspecified (N02–N03,N05–N07,N26)	254	132	122	215	118	97	24	9	15	3	1	2	12	4	8
Renal failure (N17–N19)	46,425	23,136	23,289	36,694	18,500	18,194	8,307	3,931	4,376	298	142	156	1,126	563	563
Other disorders of kidney (N25,N27)	34	20	14	32	18	14	2	2	–	–	–	–	–	–	–
Infections of kidney (N10–N12,N13.6,N15.1)	641	191	450	534	157	377	73	23	50	12	3	9	22	8	14
Hyperplasia of prostate (N40)	558	558	...	507	507	...	37	37	...	3	3	...	11	11	...
Inflammatory diseases of female pelvic organs (N70–N76)	129	...	129	99	...	99	20	...	20	3	...	3	7	...	7
Pregnancy, childbirth and the puerperium (O00–O99)	1,138	...	1,138	713	...	713	364	...	364	16	...	16	45	...	45
Pregnancy with abortive outcome (O00–O07)	27	...	27	15	...	15	10	...	10	1	...	1	1	...	1
Other complications of pregnancy, childbirth and the puerperium (O10–O99)	1,111	...	1,111	698	...	698	354	...	354	15	...	15	44	...	44
Certain conditions originating in the perinatal period (P00–P96)	12,084	6,723	5,361	7,437	4,162	3,275	4,055	2,215	1,840	105	69	36	487	277	210
Congenital malformations, deformations and chromosomal abnormalities (Q00–Q99)	9,583	5,052	4,531	7,559	3,994	3,565	1,590	815	775	119	77	42	315	166	149
Symptoms, signs and abnormal clinical and laboratory findings, not elsewhere classified (R00–R99)	37,752	16,275	21,477	32,310	13,636	18,674	4,522	2,199	2,323	253	126	127	667	314	353
All other diseases (residual)	320,065	129,448	190,617	278,876	112,075	166,801	33,391	13,965	19,426	1,915	903	1,012	5,883	2,505	3,378
Accidents (unintentional injuries) (V01–X59,Y85–Y86)	130,557	81,916	48,641	112,803	70,161	42,642	13,413	9,017	4,396	1,833	1,177	656	2,508	1,561	947
Transport accidents (V01–V99,Y85)	37,938	27,102	10,836	31,292	22,410	8,882	5,007	3,647	1,360	715	467	248	924	578	346
Motor vehicle accidents (V02–V04,V09.0,V09.2, V12–V14,V19.0–V19.2,V19.4–V19.6,V20–V79, V80.3–V80.5,V81.0–V81.1,V82.0–V82.1,V83–V86, V87.0–V87.8,V88.0–V88.8,V89.0,V89.2)	35,369	25,048	10,321	29,118	20,662	8,456	4,731	3,431	1,300	664	426	238	856	529	327
Other land transport accidents (V01,V05–V06,V09.1, V09.3–V09.9,V10–V11,V15–V18,V19.3,V19.8–V19.9, V80.0–V80.2,V80.6–V80.9,V81.2–V81.9,V82.2–V82.9, V87.9,V88.9,V89.1,V89.3,V89.9)	1,000	804	196	814	650	164	137	117	20	20	15	5	29	22	7

See footnotes at end of table.

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The effects of glycyrrhizin on experimental acute pancreatitis in rats

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Abstract. – INTRODUCTION: Although physiopathology of acute pancreatitis (AP) is not fully understood, the roles of reactive oxygen species (ROS) and changes of cytokines have been determined.

AIM: To investigate anti-inflammatory and anti-oxidant effects of glycyrrhizin (GL) on taurocholate-induced AP in rats.

MATERIALS AND METHODS: Thirty six rats were randomly divided into three groups as sham, AP and AP+GL (n=12 per group). AP was induced by 1 ml/kg body weight using 5% taurocholate injection into the biliopancreatic duct in groups II and III after clamping the hepatic duct. In groups III, GL (20 mg/kg) was given by oral gavage twice daily for 4 days. Group I and II did not receive any treatment. After the rats were killed; blood samples were taken to measure amylase, lipase, calcium, albumin, urea, glucose, AST and LDH assays before killing. Pancreatic tissue samples were also taken for biochemical analyses and histopathology.

RESULTS: Amylase, lipase, AST and urea levels were significantly lower in the AP+GL group than in the AP group. Cytokines including IL-6, TNF-α and MPO levels were significantly lower in the AP+GL group than in the AP group. Even so there is no statistically difference between in the AP+GL group and the AP group in terms of pancreatic tissue IL-1β, IL-6 and TNF-α levels.

DISCUSSION: GL treatment significantly decreased pancreatic tissue MPO activities and MDA levels in the AP+GL group compared with the other groups ($p = 0.001$ and $p = 0.05$, respectively). Acinar cell necrosis, hemorrhage, and edema determined that were significantly lower in the AP+GL group than in the AP group ($p < 0.001$).

CONCLUSIONS: GL treatment for acute necrotizing pancreatitis in rats suppressed the levels of pro-inflammatory cytokines, and caused a clear recovery of histological changes.

Key Words:
Glycyrrhizin, Acute Pancreatitis, Pro-inflammatory cytokines.

Introduction

Acute pancreatitis (AP) is a disease with high morbidity and mortality. It has a relative frequency ranging 10 to 80 cases per 100 000 population in the world. AP is caused by gallstones or alcohol abuse in 80 percent of patients. AP resulting systemic effects is local inflammation in pancreatic tissue. Although physiopathology of AP is not fully understood, the roles of reactive oxygen species (ROS) and changes of cytokines have been determined. Increased levels of pro-inflammatory cytokines such as tumor necrosis factor-a (TNF-a), interleukin (IL)-1 and IL-6 are responsible to local tissue damage in pancreas and multiple organs' failure. Productions of ROS cause the changes in the fundamental components of the cytoplasm, membrane lipid peroxidation, and protein damage in pancreas activating digestive enzymes[1-6].

Glycyrrhizinic acid or glycyrrhizin (GL) is a natural sweetener derived from the roots a Licorice (Glycyrrhyza glabra L. Leguminosae) plant which is dried in the sun. It has been using as herbal medicine since ancient times throughout the world[7]. A

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number of components including triterpenes, saponins, flavonoids, isoflavonoids and chalcones have been isolated from licorice with glycyrrhizic acid being considered the main biologically active component. Licorice root, a traditional drug, has been used for centuries in treatment of diseases of lung, peptic ulcer, hepatitis C and skin[8].

Background

Nowadays, clinical and experimental studies have revealed that GL has antiviral, antimicrobial, anti-inflammatory, anti-oxidant as well as many pharmacological properties. Some studies have showed that it has anticancer activities, immunomodulatory, hepatoprotective effects and the protective effects on the heart[9-11].

The anti-inflammatory properties of β-Glycyhrritinic acid and GL were shown in several studies[12-14]. Recently, some glycyrrihetinic acid derivatives have shown their inhibitory activity against IL-1b-induced prostaglandin E2 production in normal human dermal fibroblasts[10].

There are many studies to create an experimental model of acute pancreatitis using invasive and non-invasive models such as sodium taurocholate, ethanol, caerulein, L-Arginine induction of choline-deficient, diet containing DL-ethionine and ischaemia/reperfusion. Taurocholate-induced pancreatitis model was chosen for the study. This model is appropriate for studies of systemic issues. The retrograde injection of salts into the pancreatic duct of animals is not an easy, effective and reproducible model for creating a severe, rapidly evolving variety of acute haemorrhagic pancreatitis and lethal[15-17].

Aim

The aim of this study is to investigate anti-inflammatory and anti-oxidant effects of GL on taurocholate-induced AP in rats.

Materials and Methods

This experimental study protocol was approved by the Institutional Animal Use and Care Committee of the Gulhane Medical Academy, Turkey and was performed in accordance with the standard guidelines for the care and handling of animals.

Animals

Thirty-six male Sprague-Dawley rats weighing from 200 to 250 g were obtained from Gulhane School of Medicine Research Center, Ankara, Turkey. Animals were kept at constant room temperature in a 12-h light-dark cycle with free access to water and standard rat chow at least 1 week before the experiments. Animals were randomly divided into three groups such as Group I (sham), Groups II (AP) and III (AP+GL).

Induction of Pancreatitis

Animals were anaesthetized by intraperitoneal administration of 50 mg/kg Ketamine (Ketalar® Parke Davis, Eczacıba ı, Istanbul, Turkey) and 5 mg/kg Xylazine (Rompun®; Bayer AG, Leverkusen, Germany), and then laparotomy was performed to all groups through a midline incision. The common biliopancreatic ducts of animals were cannulated with a 28 gauge ½-inch, microfine catheter, except group I. Then, 1 ml/kg of 5 percent sodium taurocholate (Sigma, St. Louis, MO, USA)[18] was slowly infused into the common biliopancreatic duct, and the infusion pressure was kept below 30 mmHg, as measured with a mercury manometer calibrated system[16], and monitored with a monitoring kit (Transpac IV Safeset, Abbott, Dublin, Ireland) attached to the infusion line with a three-way stopcock. When the infusion was finished for group and II and III, abdomen was closed in two layers for all groups.

Study Protocol

Group I (sham) underwent laparotomy with manipulation of the pancreas without induction of pancreatitis and received 10 ml/kg saline intravenously (single dose). Groups II (AP) and III (AP+GL) were underwent laparotomy with induction of pancreatitis. Group II did not receive any treatment. In Group III, after 6 hour the induction of AP, GL (20 mg/kg) was given by oral gavage twice daily for 4 days. On the fifth day of induction, all animals were killed with intracardiac pentobarbital (200 mg/kg) injection. Blood samples were taken from the heart before killing. Pancreatic tissue samples were also taken for biochemical analyses and histopathology.

Tissue Preparation

The frozen pancreatic tissues were homogenized in 50 mM phosphate buffer (pH 7.4) by means of a homogenizator (Heidolph Diax 900; Heidolph Elektro GmbH, Kelhaim, Germany) on an ice cube. Homogenates were centrifuged at 7530 × g in 4°C for 10 min. The protein content of pancreatic homogenates was measured according to the method described by Lowry et al[19].

Biochemical Analyses

A Hitachi 917 autoanalyzer (Boehringer Mannheim, Mannheim, Germany) was used for amylase, lipase, calcium, albumin, urea, glucose, aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) assays.

TNF-α, IL-1b and IL-6 Assays

Blood samples were collected and centrifuged (at 3000 rpm for 5 min). The sera were stored at -40°C. TNF-α, IL-1beta and IL-6 levels were measured in serum samples using quantitative sandwich enzyme linked immunosorbent assay kits (R&D Systems Inc., Minneapolis, MN, USA).

Histopathologic Analysis

A part of the pancreatic tissue from each rat was fixed in 10% neutral buffered formalin and embedded in paraffin. Two pathologists who were blinded to the treatment protocol scored the tissue sections stained with haematoxylin and eosin (H&E) for acinar necrosis, inflammation and perivascular inflammation, hemorrhage and edema in 20 fields. The scores of each histological examination were summed up, with a maximum score of 24 as defined by Schmidt et al[20].

Evaluation of Oxidative Stress

Pancreatic tissue samples were homogenized in cold KCl solution (1.5%) in a glass homogenizer on ice, centrifuged and supernatant was used for analyses. Tissue malondialdehyde (MDA) concentration was measured by method as described by Ohkawa et al[21]. MDA level was expressed as nmol/mg protein. Glutathione peroxidase (GPx) activity was measured by the method of Paglia and Valentine[22] and was expressed as U/mg protein. Myeloperoxidase (MPO) levels were measured by ELISA kit (Cusabio Biotech Co., Ltd. Wuhan, Hubei Province, China) and were expressed as ng/ml and ng/mg protein.

Statistical Analysis

All statistical measurements were made by using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). All results were expressed as median (25%-75%). The Kruskal-Wallis test is used in all groups' comparisons and then Mann-Whitney U test was used to compare the groups in pairs, which had significant results. $p < 0.05$ values were considered significant.

Results

Serum biochemical parameters are compared in Table I. The serum amylase, lipase, AST and urea levels were significantly lower in the AP+GL group than in the AP group. However, in the AP+GL group serum albumin and calcium levels were higher than in the AP group ($p = 0.001$ and $p < 0.001$).

The serum cytokines including IL-6, TNF-α and MPO levels were compared among the groups (Table II). All of them were significantly lower in the AP+GL group than in the AP group. Even so there was no significant difference between the AP+GL and sham groups for all of these parameters. There was no statistically difference between in the AP+GL group and the AP group in terms of pancreatic tissue IL-1β, IL-6 and TNF-α level (Figure 1). GL treatment significantly decreased pancreatic tissue MPO activities and MDA levels in the AP+GL group compared with the AP group ($p = 0.001$ and $p = 0.05$, respectively) (Table III).

Table I. Biochemical parameters in the all experimental groups.

Parameters	Group 1 (Sham)	Group 2 (AP)	Group 3 (AP+GL)	p*
Amylase (U/L)	1271.5 (1256-1876)	2047 (1763-2372)	1711 (1410-2144)	0.015
Lipase (U/L)	8.5 (7-13)	13.55 (9-18)	11.55 (7-13)	0.015
Albumin (g/dL)	2.89 (2.73-3.91)	2.01 (1.78-3.27)	3.19 (2.16-3.24)	0.001
AST (U/L)	154.5 (118-444)	268.5 (108-680)	167 (89-884)	0.043
Calcium (mg/dL)	10.45 (8.1-10.65)	7.89 (6.78-9.87)	9.92 (8.1-10.65)	< 0.001
LDH (U/L)	1005 (217-3929)	1551 (474-6008)	1480 (661-4078)	0.686
Urea (mg/dL)	43 (38-50)	49 (39-58)	45 (41-54)	0.043
Glucose (mg/dL)	193 (145-273)	225.5 (157-261)	196.5 (136-282)	0.386

AP: acute pancreatitis; GL: glycyrrhizin; AST: aspartate aminotransferase; LDH: lactate dehydrogenase. All data were expressed as median (25%-75%). *Kruskal-Wallis test.

Table II. Serum cytokines' levels in all groups.

Parameters	Group 1 (Sham)	Group 2 (AP)	Group 3 (AP+GL)	p*
IL-1β (pg/mL)	62.4 (31.8-143)	125.5 (49-431)	77.1 (15.15-388)	0.083
IL-6 (pg/mL)	45.4 (14.3-71.6)	70.73 (3.5-112.8)	51.9 (30.4-116.4)	0.018
TNF-α (pg/mL)	10.35 (8.13-12.55)	21.3 (10.51-26.7)	10.9 (9.8-14.35)	0.001
MPO (ng/mL)	0.19 (0.56-1.80)	1.30 (0-1.35)	0.42 (0.02-2.01)	0.001

AP: acute pancreatitis; GL: glycyrrhizin; TNF: tumor necrosis factor; IL: interleukin; MPO: myeloperoxidase. All data were expressed as median (25%-75%). *Kruskal-Wallis test.

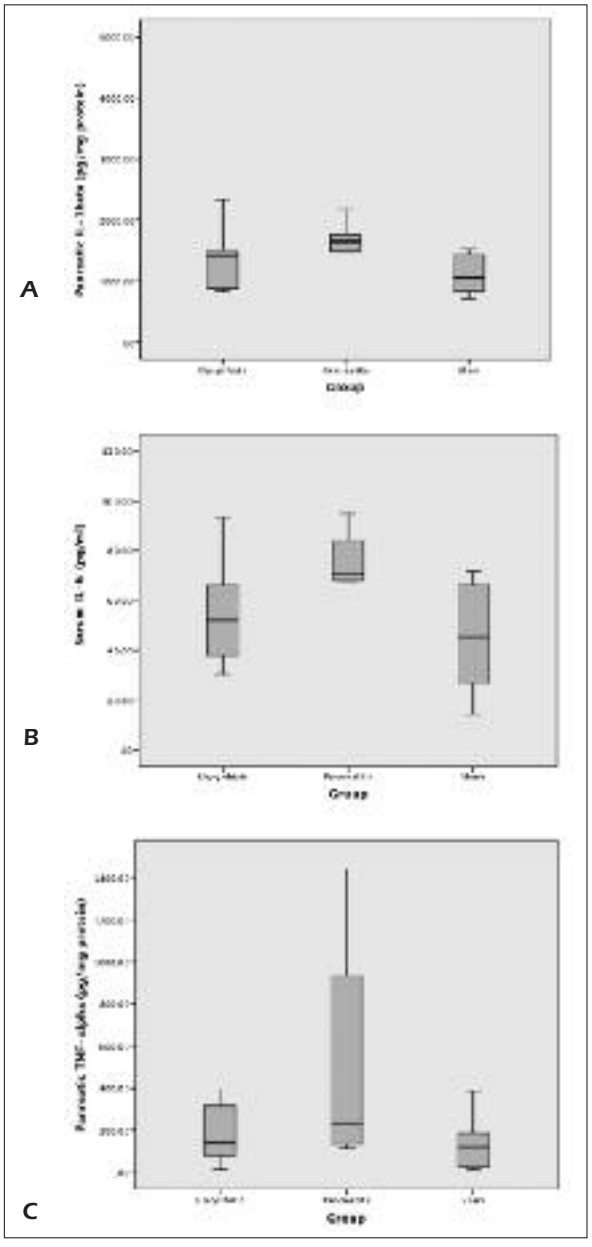

Figure 1. Pancreatic tissue cytokines' levels. ***A,*** IL-1β levels, $p = 0.100$ (AP vs AP+GL). ***B,*** IL-6 levels, $p = 0.800$ (AP vs AP+GL). ***C,*** TNF-α levels, $p = 0.13$ (AP vs AP+GL).

Histopathological scores are also shown in Table IV. The histopathology evaluations of the groups determined that acinar cell necrosis, hemorrhage, and edema were significantly lower in the AP+GL group than in the AP group ($p < 0.001$). The level of inflammation-perivascular inflammatory cell infiltration was significantly higher in the AP+GL group than in the sham group ($p < 0.001$). Figure 2 shows histologic images of all groups. Histological examination of pancreatic tissues confirmed amelioration by treatment GL on AP.

Discussion

AP is a disease with high morbidity and mortality. However, the standard treatment for AP is still mainly based on supportive treatments including vital signs are followed, intravascular volume is maintained, electrolyte balance is achieved, analgesics are provided and treatment for possible complications. No clear benefit from the administration of medications, such as aprotinin, gabexate mesylate, glucagon and calcitonin, to treat AP has been demonstrated[20,23].

Following the extravasations of pancreatic secretions through the tissue spaces, proteolytic enzymes such as activated trypsinogen start the process of auto digestion of pancreatic tissue[23,24]. Tissue edema develops and it is along with microcirculation failure and ischemia at the cellular level. Circulatory failure leads to increased severity of the inflammation and accumulation of toxic mediators within pancreas and monocytes-macrophages releases some cytokines[25-28].

Substances such as activated protein C[3], cyclooxygenase inhibitors[29], melatonin[30,31], allopurinol[32] and octreotide[33] were used to prevent the pancreatic damage caused by released ROS and cytokines in experimental studies.

Table III. Pancreatic tissue oxidative stress parameters in all groups.

Parameters	Group 1 (Sham)	Group 2 (AP)	Group 3 (AP+GL)	p*
MPO (ng/mg protein)	5.48 (1.03-13.94)	23.11 (1.4-74)	9.12 (1.7-16.6)	0.001
MDA (nmol/ mg protein)	2.46 (0.13-9.97)	8.86 (2.5-43.6)	4.78 (2.43-12.9)	0.050
GPx (U/mg protein)	1.28 (0.8-2.84)	1.47 (1.07-3.52)	1.21 (0.8-2.84)	0.225

AP: acute pancreatitis; GL: glycyrrhizin; MPO: myeloperoxidase; MDA: malondialdehyde; GPx: glutathione peroxidase. All data were expressed as median (25%-75%). *Kruskal-Wallis test.

Table IV. Histological injury scores in pancreatic tissues in all experimental groups.

Parameters	Group 1 (Sham)	Group 2 (AP)	Group 3 (AP+GL)	p*
Acinar cell necrosis	0 (0-1)	3.5 (1-4)	2 (1-3)	< 0.001
Hemorrhage	0 (0-1)	2 (1-4)	1 (0-4)	< 0.001
Inflammation and perivascular infiltration	0 (0-1)	3 (1-4)	1 (0-2)	0.060
Edema	0 (0-1)	3 (1-4)	2 (1-3)	< 0.001

AP: acute pancreatitis; GL: glycyrrhizin. All data were expressed as median (25%-75%). *Kruskal-Wallis test.

In this study, acute necrotizing pancreatitis was induced by retrograde infusion of sodium taurocholic acid (5%) into the biliopancreatic duct, as Aho et al[18,34] described. The application route and dose for glycyrrhizin was selected according to the data presented in the literature[35,36]. GL is believed to be partly responsible for its anti-inflammatory effect[37]. Akamatsu et al[38] showed that GL is not a scavenger of ROS but it exerts an anti-inflammatory action by inhibiting the generation of ROS by neutrophils. In experimental studies, it was determined that glycyrrhizin inhibe phospholipase A2 of the arachidonic acid cascade and reduce cytokines in rats, secondary to AP[3,39].

The sensitivity and specificity of the diagnosis of AP are higher than 90% in patients with appropriate clinical signs. Serum amylase and lipase levels increase within 24 h following the acute attack and decrease gradually to the normal values[40]. In our work, serum amylase, lipase, AST, LDH and urea levels were significantly lower in the AP+GL group than in the AP group. However, in the AP+GL group serum albumin and calcium levels were higher than in the AP group. After pancreatitis induction, GL treatment significantly increased serum albumin and calcium levels. Nagai et al[41] showed that GL suppressed the elevation lipid peroxides, AST, LDH and decreased morphological damage in the liver. In another study, Shiki et al[42] also showed that GL deceased release of AST and inhibit phospholipase A in the liver.

Failure in the microcirculation led to a histopathologically significant increase in acinar cell necrosis, hemorrhage, inflammation and perivascular inflammatory cell infiltration and edema level in AP. Glycyrrhizin might protect the organ function by regulating the excessive inflammatory reactions and attenuated organ injury

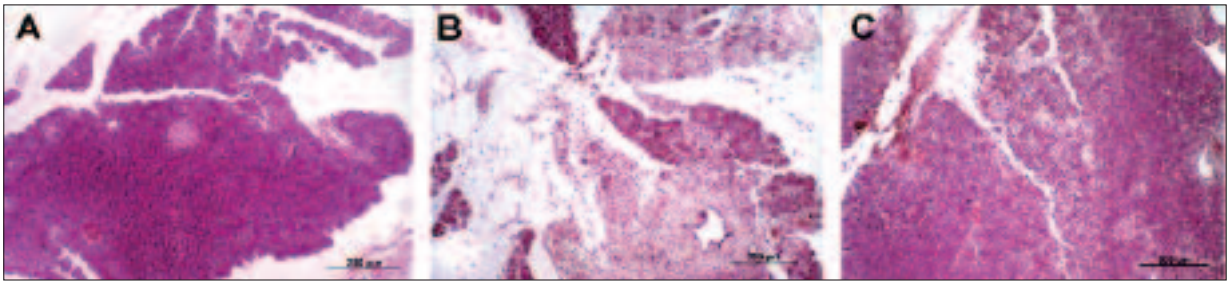

Figure 2. Histological images of pancreatic tissues in the *(A)* Sham, *(B)* Acute pancreatitis and *(C)* Glycyrrhizin groups. (H&E, Scale bars 200 μm).

induced by lipopolysaccharide[43]. After administration of GL in the treatment group showed that a significant decrease in these parameters except level of inflammation and perivascular inflammatory cell infiltration compared with the AP group.

Stimulation of production of either acute phase proteins and adhesion molecules or several inflammatory cytokines including TNF-α, IL-1β and IL-6 occurs after taurocholic acid activation in acute pancreatitis[34]. In this study, the concentrations of cytokines including TNF-α, IL-1β and IL-6 were increased in the AP group rats. Our results have shown that GL inhibited the release of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in serum and pancreatic tissue. In addition to these parameters, we have found high MPO levels in the AP group. MPO activity has been used to determine quantitatively the extent of neutrophils infiltration. Our findings show that GL also reduces MPO levels after AP such as caffeic acid phenethyl ester[44]. The anti-inflammatory and anti-oxidant effects of GL may explain our results. Akamatsu et al[38] and Wang and Nixon[45] found that glycyrrhizin inhibited the generation of ROS by neutrophils which were the potent mediator of tissue inflammation in the in vitro study. Wang et al[43] showed that GL decreased the inflammatory cytokines through inhibiting their gene and protein expression.

As a result, retrograde infusion of 5% taurocholate into the biliopancreatic duct in rats induces AP biochemically and histologically. GL treatment following this procedure suppressed the levels of pro-inflammatory cytokines, and caused a clear recovery of histological changes.

Conclusions

GL might be beneficial in decreasing the severity of AP. However, further investigations are needed to demonstrate the potential roles of glycyrrhizin in taurocholate-induced acute necrotizing pancreatitis.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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BRAIN RESEARCH

Research Report

Activity of mannitol and hypertonic saline therapy on the oxidant and antioxidant system during the acute term after traumatic brain injury in the rats

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ABSTRACT

In this study, our objective is to investigate the effects of mannitol and 7.5% hypertonic saline (HS) therapy on the levels of malondialdehyde (MDA), catalase and glutathione peroxidase (GSH-Px) in the early stages of experimental head traumas in rats. Rats included in the study were divided into four groups: Group I Control, Group II Trauma, Group III Mannitol, and Group IV 7.5% Hypertonic Saline. Rats in Group II were subject to head trauma only. Mannitol was injected intraperitoneally to rats in Group III after head trauma and 7.5% HS was injected intraperitoneally to rats in Group IV after head trauma. Rats were sacrificed 4 h after administration of mannitol or 7.5% HS, and the levels of MDA catalase and GSH-Px in brain tissues extracted from rats were determined. MDA levels in the trauma group were significantly increased compared with the control group ($p<0.01$), whereas there was a reduction in catalase and GSH-Px levels, although these differences were not significant. By contrast, in the mannitol group, MDA, catalase and GSH-Px levels were lower than the levels in the trauma group, and these reductions were statistically significant ($p<0.05$). The MDA, catalase and GSH-Px levels of the 7.5% HS group were lower than those of the trauma group; however, this reduction was not statistically significant. It was concluded that mannitol and 7.5% HS therapies that are used to reduce intracranial pressure and to increase the use of catalase, an antioxidant enzyme, and GSH-Px, are likely to reduce cellular damage by reducing the formation of MDA, the levels of which are known to be indicative of cellular level oxidant damage.

1. Introduction

Trauma to the brain causes tissue damage by primary and secondary injury to the neural tissue. Primary injury due to initial mechanical trauma results in physical disruption of vessels, neurons, and their axons. Secondary injury involves loss of autoregulation, increased intracranial pressure, systemic hypotension, hemorrhage, thrombosis, edema,

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Abbreviations: HS, hypertonic saline; MDA, malondialdehyde; GHS-Px, glutathione peroxidase; TBI, Traumatic brain injury

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inflammation, and vasospasm, leading to hypoperfusion and ischemia. Traumatic brain injury (TBI) results in cellular metabolic alterations and inflammatory responses, which lead to marked increases in the production of reactive oxygen species (Farooqui and Horrocks, 1998; Lewen et al., 2000; Povlishock and Kontos, 1992; Tyurin et al., 2000). The detrimental effects of free oxygen radicals are countered by the antioxidant enzymes catalase and GSH-Px (Choi et al., 2006). In cases of TBI, osmotherapy remains the mainstay of pharmacological intervention in neurological and neurosurgical intensive care for the treatment of cerebral edema and intracranial hypertension. Several osmotic agents have been used in clinical practice, including mannitol, saline, glycerol, urea, and sorbitol (Cao et al., 2000; Lewen et al., 2000; Mirski et al., 2000; Pleban et al., 1982). Mannitol has been the osmotic agent of choice since the 1960s because it does not cross the blood–brain barrier. Osmotic agents have potent anti-edema action, and presumably act by drawing water from interstitial and intracellular spaces into the intravascular compartment. More recently, HS solutions have received renewed attention and use in clinical practice (Vassar et al., 1993; Wade et al., 1997).

This study was planned to investigate the prevention of cell damage in rats with experimental head trauma, to determine if mannitol and HS reduce oxidative damage and to characterize their effects on antioxidant enzymes.

2. Results

During the experiment five rats died and three responded to resuscitation. The remaining rats survived all stages of the experiment. Values for entire groups are shown in Table 1. MDA values of the trauma group (mean 46.52±13.68 μmol/l) were significantly greater than levels of the control group (mean 17.07±1.08 μmol/l; $p<0.01$). Compared with levels of in the trauma group, MDA values in the mannitol group (mean 23.15±1.56 μmol/l) and 7.5% HS group (mean 30.51±4.45 μmol/l) reduced. However, this reduction was only statistically significant in the mannitol group ($p<0.05$).

Comparison of catalase activities between the control group (92.82±32.38) and the trauma groups (46.52±13.68) revealed that catalase values were lower in the trauma group; however, the difference was not statistically significant. Catalase values in the mannitol (14.11±3.33 μmol/l) and 7.5% HS (39.90±13.22 μmol/l) groups were reduced compared with the trauma group (92.82±32.38±μmol/l); however, the reduction was not statistically significant. The catalase values of the mannitol group were significantly reduced compared with the control group ($p<0.05$).

Comparison of GSH-Px values of the control (mean 4555.57±1736.02 μmol/l), trauma (mean 63.06±24.53 μmol/l), mannitol (mean 14.11±3.33 μmol/l) and 7.5% HS (mean: 39.90±13.22 μmol/l) groups revealed the values of the mannitol and 7.5% HS groups to be less than the values of the control and trauma groups; however these differences were not statistically significant.

3. Discussion

Two types of brain injury are thought to occur after an impact. The first is the primary injury, which occurs at the time of impact; the second is known as secondary injury and is a result of the pathophysiological alterations secondary to trauma, such as hypotension and ischemia, and some reactants produced by the primary injury (Adamides et al., 2006; Ikeda et al., 1989). Some studies report that free radicals have a role in cerebral ischemia and trauma. Free oxygen radicals either disrupt the blood–brain barrier or cause brain edema by affecting the neurons (Hall and Braughler, 1993; Santos et al., 2005). Antioxidant enzymes such as superoxide dismutase, catalase, and GSH-Px are present in mammalian cells, and they protect the cells from the toxic effects of free radicals. When free radicals are produced in the cell membrane, the cell membrane lipids undergo peroxidation, which can ultimately cause cell death. Increased antioxidant enzyme activity is a protective response to free radical formation. There is extensive experimental evidence to support the early occurrence and pathophysiological importance of oxygen radical formation and cell membrane lipid peroxidation in the injured nervous system. Free-radical-induced damage has been proposed to be involved in traumatic cell injury and cell death, whereas free radical scavengers such as catalase and GSH-Px are associated with partial amelioration of traumatic injury (Ikeda et al., 1989; Marmarou et al., 1994; Soustiel et al., 2006).

Hyperosmolar therapy has been used to reduce increased intracranial pressure from several causes, including TBI. Mannitol is often used as it has been shown to be safe, well tolerated, and easily prepared. More recently, there has been increased interest in the use of HS for both resuscitation and maintenance in head-injury patients. Multiple animal models and human trials have evaluated its safety and efficacy, highlighting its potential beneficial effect on the control of increased intracranial pressure. Data from studies involving resuscitation after hemorrhagic shock show improved survival of patients with TBI who received HS compared with those who received other resuscitation fluids (Horn et al., 1999; Toung et al., 2005; Vassar et al., 1993; Wade et al., 1997).

Table 1 – Statistical analysis

	Control ($n=10$)	Trauma ($n=8$)	Mannitol ($n=9$)	7.5% SF ($n=10$)
MDA (μmol/l)	17.07±1.08	46.52±13.68[a**]	23.15±1.56[b*]	30.51±4.45
Catalase (KU/l)	92.82±32.38	63.06±24.53	14.11±3.33[c*]	39.90±13.22
GSH-Px (IU/l)	4555.57±1736.02	4075.74±1551.56	1381.12±115.41	2498.40±322.55

Data presented as mean±SE.
[a]$p<0.01$, [b,c]$p<0.05$; a, c refer to comparison with control group, and b refers to the significance of comparison between the trauma and mannitol groups.

There are many reports highlighting that the administration of mannitol and HS solution after a brain injury is likely to reduce intracranial pressure and reduce cerebral edema (Horn et al., 1999; Vassar et al., 1993; Wade et al., 1997). However, there are not enough data from clinical studies of oxidative damage at a cellular level to demonstrate the effects of antioxidant enzymes. In the present study, we planned to investigate how to avoid cell damage and the effects of mannitol and HS on cranial edemas, oxidative damage, and antioxidant enzymes.

Hypertonic stress suppresses neutrophil functions. In trauma patients, hemorrhagic shock results in excessive neutrophil activation. The ensuing ischemia–reperfusion injury leads to tissue destruction and serious posttraumatic complications, such as acute respiratory distress syndrome and multiple organ failure syndrome. In light of the critical role of neutrophils in the development of such posttraumatic complications, therapeutic approaches to control neutrophil activation have become a central focus in trauma research (Choi et al., 2006; Orlic et al., 2002).

Cellular MDA content may increase owing to lipid peroxidation in the membranes of cells producing free oxygen radicals after a brain injury. Catalase and GSH-Px are antioxidant enzymes with key roles in reducing production of MDA, which has an oxidative effect on cells (Goth, 1991; Lewen et al., 2000). In the present study, it was observed that mannitol and 7.5% HS therapy, which was applied to rats with a head trauma, reduced the catalase and GSH-Px values soon after the injury. The reductions in catalase and GSH-Px values could be correlated with the administration of mannitol and 7.5% HS. These substances were administered to protect the cells from the oxidative effects of MDA, whereas they are likely to accelerate the catalase and GSH-Px metabolism. When the use of these substances increased, there was a reduction in catalase and GSH-Px values. We consider that HS, but not as much as mannitol, is moderately effective in cellular level in TBI rats by reducing MDA, CAT and GSH-Px.

In the trauma group, it was shown that tissue MDA levels reduced with increases in catalase and GSH-Px metabolic pathways in rats who received mannitol and 7.5% HS therapy. It was also found that, compared with the administration of 7.5% HS solution, early administration of mannitol after a head trauma was more effective in reducing oxidative damage and avoiding cellular damage than the antioxidant enzymes.

Consequently, administration of mannitol and 7.5% HS to reduce intracranial pressure by drawing water from interstitial and intracellular areas to an intravascular area also increases the levels of catalase and GSH-Px enzymes. It has been suggested that these enzymes would reduce the production of MDA, which is a harmful substance for cells and would thereby reduce cellular damage.

4. Experimental procedure

All experimental procedures and animal care were in accordance with the guidelines of Yuzuncu Yil University, Faculty of Medicine, Neuroscience Study Center. The experimental protocols used in this study were also approved by the Animal Experimental Ethic Committee of Yuzuncu Yil University (No: B.30.2.YYU.0.00.00.00/300-0087). All efforts were made to minimize animal suffering and to reduce the number of animals used.

In the present study, 24 New Zealand rats (160–250 g weight) were used. The rats were monitored at a suitable temperature (21±2 °C) and moist environment (60±5% humidity). The body weight for each rat was calculated before the surgery A heating pad and a heating lamp were used to maintain the rectal temperature between 36.5 °C and 37.5 °C. Rats were intubated, and respiration was maintained with a small animal respirator (Harvard Apparatus).

We preferred in this experimental study a model of head trauma which consisted of a combination of all injury models (contusion, diffuse axonal injury, edema). Rats were subject to closed head trauma according to the experimental head trauma method reported by Foda and Marmarou (1994) and Marmarou et al. (1994). Ether was used as an anesthetic and applied to the rats prior the trauma. After anesthesia, to avoid cranial fractures, a stainless steel disk made from stainless steel was mounted on the heads of the rats. The rats were then placed on a foam-supported soft bed and subjected to trauma by free fall of an object weighing 500 g from a height of 45 cm.

The animals included in the study were divided into four groups. Group I (n=10) was the control group. Rats were sacrificed and a tissue sample was obtained from their right hemisphere. Group II (n=8) rats underwent trauma by the right craniotomy procedure described above and a tissue sample was then obtained from the right hemisphere. Group III (n=9) rats underwent a trauma over the dura by the right craniotomy procedure described above and mannitol 20% 2.5 g/kg was administered 1 h later by the intraperitoneal route. Group IV (n=10) rats underwent trauma over the dura by the right craniotomy procedure and 7.5% HS 2 g/kg was administered 1 h later intraperitoneally. Rats in Groups I, II, III and IV were sacrificed 4 h after the trauma and a tissue sample was obtained from the right hemisphere.

4.1. *Biochemical analysis*

Right hemisphere was evacuated and homogenized with phosphate buffer [Na_2HPO_4 (Sigma S-0786, Steinheim, Germany), KH_2PO_4 (Merck A802771 426 Darmstadt, Germany)] at a ratio of 1/4 (weight/volume) (Ultra-Turrax T25, Janke-Kunkel GmbH and Co.KG IKA, Germany). Homogenized material was then centrifuged at 4 °C, 14000 rpm for 20 min (Allegra ZRR centrifuge Beckman Coulter, Germany). Supernatant for MDA study was removed and the residue was stored at −80 °C (Sanyo Lfc free, Japan).

The MDA levels and GSH-Px and CAT activities in the tissue samples were measured. MDA levels were determined by the fluorometric method described by Wasowich based on thiobarbituric acid reactivity (Wasowicz et al., 1993). GSH-Px activities were measured using a photometric (Santos et al., 2005). Catalase activities were measured by the method previously described by Goth (1991).

4.2. *Statistical analysis*

Control and trauma groups were compared. Means and standard error of means were calculated, and differences between

means were assessed by one-way analysis of variance (ANOVA). Differences among groups were measured by the post hoc Tukey test.

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·药效与毒理学研究·

甘草酸对 H_2O_2 诱导小肠上皮细胞 DNA 损伤的保护作用

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摘要：目的 初步研究甘草酸（Glycyrrhizic acid，GA）对氧化损伤小肠上皮细胞IEC-6的保护作用及可能机制。**方法** 实验设空白对照组，H_2O_2模型组，GA低、中、高剂量组（50，100，200 μg/mL）。GA各组加入相应浓度的药物作用48 h后，与模型组一起加入200 μmol/L的H_2O_2作用2 h，空白对照组正常培养。用单细胞凝胶电泳检测DNA损伤，RT-PCR检测p53，Gadd45α mRNA表达，Western blot 检测p53，Gadd45α蛋白表达。**结果** 与空白对照组比较，模型组DNA损伤严重，Gadd45α mRNA和蛋白表达增高；与H_2O_2模型组比较，GA各组DNA损伤明显减轻，GA100 μg/mL和200 μg/mL组p53 mRNA及其蛋白表达明显升高，同时Gadd45α mRNA及其蛋白表达明显升高。**结论** GA在100~200 μg/mL可以保护H_2O_2诱导的小肠上皮细胞DNA损伤，其机制可能与p53和Gaad45α的表达增高相关。

关键词： 甘草酸；DNA损伤；小肠上皮细胞；p53，Gaad45α

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Effect of Glycyrrhizic Acid on Protecting Instestinal Epithelial Cells from H_2O_2- induced DNA Damage

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Abstract：Objective To observe the protective effect of glycyrrhizic acid（GA）on DNA damage of intestinal epithelial cells（IEC-6）induced by H_2O_2，and to investigate the potential mechanism. **Methods** Five groups including blank control，H_2O_2 model，GA50 μg/mL，GA100 μg/mL，GA200 μg/mL groups were set up in this study. Cells were cultured with blank control medium or GA of the fixed dosages for 48h，and then were stimulated with 200 μmol/L H_2O_2 for 2 h. DNA damage was analyzed by single cell gel electrophoresis assay. P53，Gadd45α mRNA was evaluated by RT-PCR. Expression of p53 and Gadd45α protein was detected by western blot. **Results** Compared with the blank control group，DNA of IEC-6 in the the H_2O_2 model group was severely damage，and Gaad45α mRNA and protein expression was increased. After treatment with GA of 100 μg/mL or 200 μg/mL for 48 h，DNA damage reduced significantly，expression of p53 mRNA and protein both increased. Meanwhile，Gadd45α mRNA and protein expression was also increased. **Conclusion** The above results indicated GA of 100~200 μg/mL protects IEC-6 from DNA damage induced by H_2O_2，which mechanism is probably related with the increase of p53 and Gadd45α expression.

Keywords： Glycyrrhizic acid；DNA damage；Intestinal epithelial cells；p53；Gadd45α

甘草为豆科植物甘草的干燥根茎，是临床常用中药，尤其常用于治疗消化道疾病，其中三萜皂苷类成分包括甘草酸、甘草次酸等具有多种生物活性，除了消炎、抗菌、抗变态反应等作用外，在抗氧化、抗肿瘤方面也有明显的作用[1]，但其抗氧化的确切作用及分子作用机制研究较少。多种病理因素如炎症、缺

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血缺氧等均可导致小肠上皮细胞氧化应激，使细胞DNA损伤，细胞凋亡[2]。本文以小肠上皮细胞IEC-6为研究对象，并用强氧化剂H_2O_2诱导IEC-6 DNA损伤，观察甘草酸对上皮细胞DNA损伤的保护作用，初步探讨其作用机制。

1 材料和方法

1.1 药物与试剂　甘草酸，Fluka公司，批号：A0267975；DMEM培养基(批号：8111113)、胎牛血清（批号：8206513）、0.25 %胰酶消化液（批号：884758），美国Invitrogen公司。逆转录试剂盒、荧光定量PCR试剂盒，Takara公司。RIPA缓冲液、蛋白酶抑制剂，德国Roche公司，批号：60992921；p53抗体、Gadd45α抗体、HRP标记羊抗鼠抗体，美国CST公司。Western blot所用试剂及分子标准，美国Bio-Rad公司。30 %H_2O_2，广州化学试剂厂，批号：20110201-1。

1.2 细胞　大鼠小肠上皮细胞IEC-6，美国标准生物品收藏中心(ATCC)。

1.3 仪器　酶标仪；美国Perkin Elmer公司；FC500流式细胞仪，美国Beckman Coulter公司；7500实时荧光定量基因扩增系统，美国ABI公司；电泳及转印系统、凝胶成像系统，美国Bio-Rad公司。

1.4 细胞培养及分组　IEC-6用含10 %FBS，0.1 U/mL胰岛素的DMEM，37 ℃，5 %CO_2恒温培养箱培养，0.25 %胰酶消化传代。实验设立空白对照组，模型组，GA低、中、高剂量组（50，100，200 μg/mL）。GA各组加入相应浓度的药物作用48 h后，与模型组一起加入200 μmol/L的H_2O_2作用2 h，空白对照组正常培养，细胞处理后进行后续实验。

1.5 单细胞凝胶电泳分析　①铺胶：细胞同上处理后消化，用PBS制备成细胞悬液，加热熔解0.5 %正常熔点琼脂糖(NMA)，冷至60 ℃，取100 μL铺于防脱载玻片，用盖玻片压平，4 ℃下凝固10 min，移去盖玻片，加热熔解0.5 %低熔点琼脂糖(LMA)，后37 ℃水浴，取20 μL的细胞悬液与80 μL LMA混合均匀，铺于NMA上，用盖玻片压平，4 ℃下凝固10 min，移去盖玻片，再铺上100 μL LMA，盖玻片压平，4 ℃凝固后移去盖玻片。②细胞裂解：将玻片置平皿中，轻轻加入预冷细胞裂解液(2.5 mol/L NaCl，100 mmol/L Na_2EDTA，10 mmol/L Tris，1 %肌氨酸钠，临用前加10 %DMSO，1 % Triton X-100)，4 ℃下裂解1 h。取出载玻片用蒸馏水漂洗2次。③细胞电泳：电压25 V，在碱性电泳液(1 mmol/L Na_2EDTA，300 mmol/L NaOH，Tris·Cl，pH13.0)中电泳30 min。电泳结束后用预冷的0.4 mol/L Tris·Cl(PH7.5)缓冲液中和15 min。④染色和观察：取出载玻片，在胶上滴加5 μg/ml的PI，避光染色30 min，PBS洗3次，后在荧光显微镜下观察拍照。

1.6 逆转录定量PCR(RT-qPCR)　细胞分组处理后，收集细胞用Trizol提取总RNA，测定浓度后逆转录合成cDNA，用SYBR Green染料法荧光定量PCR检测，用内参基因GAPDH进行校正分析p53、Gadd45α mRNA表达含量。引物序列：p53前向引物为5'-ATGATATTCTGCCCACCACA-3'，逆向引物为5'-GGGCCTTCTAACAACTCTGC-3'；Gadd45α前向引物：5'-CGGCAAGAGCAGAGACGCGA-3'，逆向引物：5'-TCGCCCACCGTGTCCATCCTTT-3'；GAPDH前向引物：5'-GGGCTCTCTGCTC CTCCCTGTTC-3'，逆向引物：5'- CGTCCGATACGGCCAAATCCGTT-3'。

1.7 Western blot检测蛋白表达　收集细胞后用RIPA提取细胞总蛋白，用BCA法测定蛋白浓度，100 ℃变性。用10 %SDS-PAGE胶分离蛋白转膜，后用1∶1000抗Gadd45α或p53一抗4 ℃孵育过夜，TBS-T缓冲液洗涤3次，二抗室温孵育2 h，TBS-T缓冲液洗涤3次，ECL作用后，暗室冲印胶片，扫描分析。

1.8 统计分析方法　数据以均数±标准差($\bar{x}\pm s$)表示，用SPSS13.0统计软件进行单因素方差分析，检验水平为α=0.05。

2 结果

2.1 GA对H_2O_2诱导小肠上皮细胞DNA损伤的保护作用　单细胞凝胶电泳可以检测细胞DNA的损伤程度。如细胞未受损伤，核DNA因其分子量大而与基质成一整体，电泳及荧光染色后出现细胞核形荧光团，无脱尾现象；若细胞受损，DNA链断裂，电泳时断裂片断向阳极迁移，染色后形成荧光拖尾现象，形似彗星。细胞DNA受损愈重，产生断链愈多，电泳时迁移的DNA量多，距离长，彗尾长增加及其荧光强度增强。图1所示，在200 μmol/L H_2O_2作用2 h后，细胞DNA受损严重，细胞核头部明显减小，彗

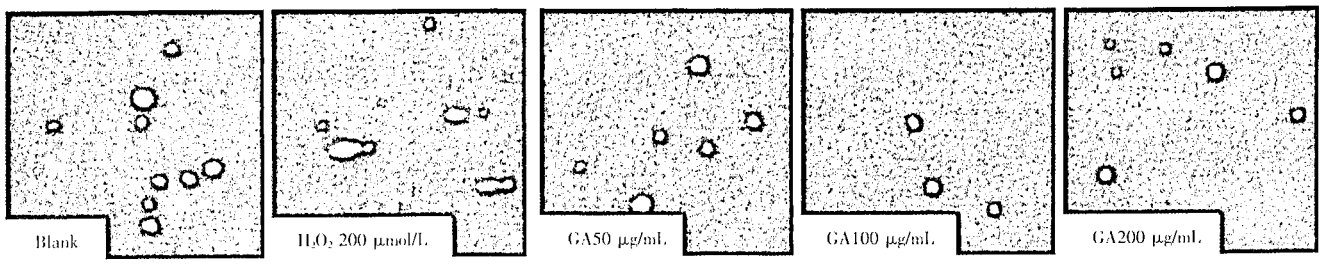
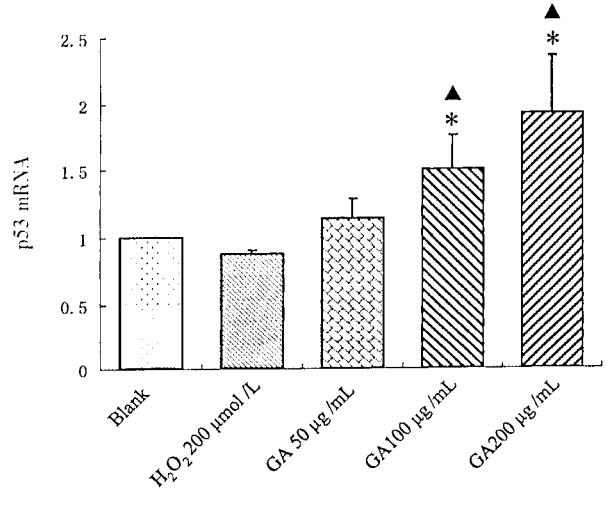


图 1 甘草酸对 H_2O_2 诱导 DNA 损伤的保护作用

Figure 1 Effect of glycyrrhizic acid on H_2O_2-induced DNA damage

星尾明显加长，荧光强度增高。用 GA 50，100，200 μg/mL 预处理 48 h 后再加入 200 μmol/L H_2O_2 刺激，结果显示，与 H_2O_2 模型组比较，仅出现微弱彗星尾，迁移距离明显缩短，提示细胞 DNA 受损程度明显减轻。

2.2 GA 对 H_2O_2 损伤小肠上皮细胞的 p53、Gadd45α mRNA 及其蛋白的表达影响 RT-qPCR 结果显示，与 H_2O_2 模型组比较，GA 50，100，200 μg/mL 各组 p53 mRNA 表达增高，差异有统计学意义，见图 2。与空白对照组比较，H_2O_2 刺激后 Gadd45α mRNA 表达增高，在 GA 预处理后，Gadd45α mRNA 进一步增高，差异有统计学意义，见图 3。

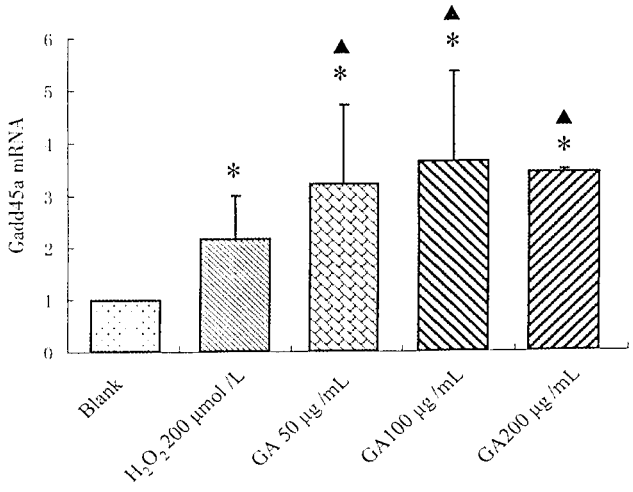


注：与空白对照组比较，$^*P < 0.05$；与 H_2O_2 200 μmol/L 组比较，$^▲P < 0.05$。

图 2 GA 对 H_2O_2 损伤细胞的 p53mRNA 表达影响

Figure 2 Effect of glycyrrhizic acid on p53 mRNA expression in H_2O_2-induced IEC-6 cells

2.3 GA 对 H_2O_2 损伤小肠上皮细胞的 p53、Gadd45α 蛋白表达的影响 与空白对照组比较，H_2O_2 刺激后 p53 和 Gadd45α 蛋白表达均增高。与 H_2O_2 模型组比较，GA100，200 μg/mL 预处理 48 h 后，p53 和 Gadd45α 蛋白表达进一步增高，见图 4、图 5。



注：与空白对照组比较，$^*P < 0.05$；与 H_2O_2 200 μmol/L 组比较，$^▲P < 0.05$。

图 3 GA 对 H_2O_2 损伤细胞 Gadd45α mRNA 表达影响

Figure 3 Effect of glycyrrhizic acid on Gadd45α mRNA expression in H_2O_2-induced IEC-6 cells

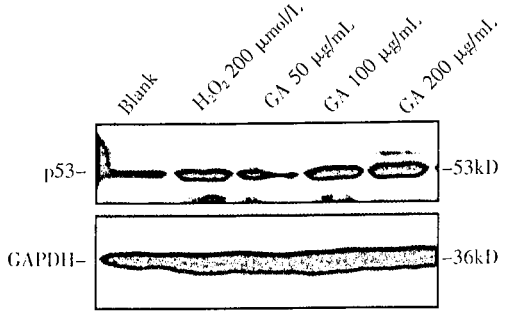


图 4 GA 对 H_2O_2 损伤细胞的 p53 蛋白表达影响

Figure 4 Effect of glycyrrhizic acid on p53 protein expression in H_2O_2-induced IEC-6 cells

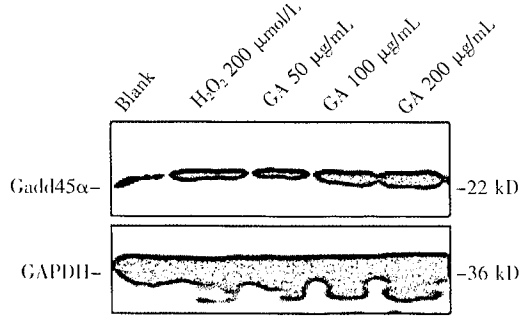


图 5　GA 对 H_2O_2 损伤细胞的 Gadd45α 蛋白表达影响

Figure 5　Effect of glycyrrhizic acid on Gadd45α protein expression in H_2O_2-induced IEC-6 cells

3 讨论

多种环境因素，化学、物理等因素均可导致哺乳动物细胞的 DNA 受损，在 DNA 受损后，细胞会启动一系列信号传导通路进行 DNA 修复，或促进细胞凋亡，从而维持细胞和组织稳态[3-4]。前期的研究证明甘草酸对氧化损伤及 UV 照射损伤细胞有保护作用[5-6]，该作用可能与稳定细胞的 DNA 有关。多种因素和疾病如烧伤、炎症、多脏器功能衰竭等均可导致小肠上皮细胞氧化和 DNA 损伤[2]。临床应用和研究均证明甘草及其有效成分对胃肠黏膜有保护作用[7]，本文选用小肠上皮细胞 IEC-6，用 H_2O_2 诱导 DNA 损伤，并观察甘草酸对 H_2O_2 诱导小肠上皮细胞 DNA 损伤的作用和可能机制。在 200 μmol/L H_2O_2 刺激 2 h 后，用单细胞凝胶电泳进行分析后发现，IEC-6 细胞 DNA 明显受损，而用甘草酸 100，200 μg/mL 浓度预处理 48 h 后，对 DNA 损伤有明显的保护作用。

在此基础上，本文检测相关基因 p53，Gadd45α 的表达变化，以分析其作用机制。研究表明，p53 基因与 DNA 损伤修复密切相关，其主要通过调控下游基因的表达应答 DNA 损伤，p53 能活化 p21waf1/cip1、Gadd45α 等基因的转录表达[8-9]。Gadd45α 基因是生长阻滞和 DNA 损伤基因(growth arrest and DNA damage，GADD)，也是 p53 的下游靶基因，是 DNA 损伤后诱导表达的基因之一。Gadd45α 表达使细胞发生 G2/M 期阻滞，细胞进入 DNA 修复，并调控细胞凋亡和存活等多条信号传导通路[10]。Gupta 研究发现在遗传毒性因素刺激下，与正常造血细胞比较，Gadd45α 基因表达缺陷细胞凋亡明显增加，同时 Gadd45α 可以通过激活 p38-NF-κB 介导的生存信号通路，从而提高细胞抵抗 UV 诱导的凋亡作用[11-12]。

本研究结果表明，在 H_2O_2 刺激后，Gadd45α mRNA，p53 及 Gadd45α 的蛋白表达均增高，在 GA100，200 μg/mL 预处理后，p53 及 Gadd45α mRNA 及其蛋白表达均进一步增高，结合 DNA 损伤程度分析，提示甘草酸可能是通过激活 p53 和 Gadd45α 的表达，从而维持细胞 DNA 稳定性，避免或减轻 H_2O_2 诱导的小肠上皮细胞 DNA 损伤。而且，p53 与 Gadd45α 的表达趋势一致，提示 Gadd45α 的表达可能与 p53 相关。

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青心酮对 ApoE(-/-)小鼠动脉粥样硬化斑块中巨噬细胞 5-脂氧合酶的影响

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摘要：目的 观察青心酮防治 ApoE(-/-)小鼠主动脉粥样硬化病变(atherosclerosis，AS)与 5-脂氧合酶的关系 **方法** 传代培养小鼠巨噬细胞株 RAW264.7，分成 4 组：正常对照组，模型组，青心酮组，辛伐他汀组。收集各组细胞蛋白，应用 Western-blot 检测 5-脂氧合酶含量；收集上清液，用 ELISA 检测白三烯 B4(leukotriene B4，LTB4)。以 C57BL/6 小鼠作正常对照组，24 只雄性 ApoE(-/-)小鼠随机分成 3 组：模型组(等量乙醇)，青心酮治疗组(20 mg·kg^{-1}·d^{-1})，辛伐他汀治疗组(20 mg·kg^{-1}·d^{-1})，给药 8 周。所有实验小鼠饲以普通饲料至 16 周。取血检测 LTB4，剪取主动脉根部行组织切片，HE 染色观察主动脉粥样硬化病变情况；剪取主动脉根部斑块组织，Western blot 法测定斑块中 5-脂氧合酶水平。**结果** 青心酮处理后 LTB4 降低，5-脂氧合酶含量减少，AS 病灶形成数目、面积减少。**结论** 青心酮能减轻 ApoE(-/-)小鼠动脉粥样硬化病变，可能与其抑制 5-脂氧合酶，减少 LTB4 有关。

关键词： 青心酮；5-脂氧合酶；动脉粥样硬化；ApoE(-/-)小鼠；巨噬细胞
中图分类号： R285.5 **文献标志码：** A **文章编号：** 1003-9783(2012)03-0243-04
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Effects of 3,4-dihydroxyacetophenone on 5-lipoxygenase in Macrophages of Atherosclerosis Plaque

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Abstract: Objective To observe the effects of 3,4-dihydroxyacetophenone(DHAP) on 5-lipoxygenase in macrophages (MC) of ApoE(-/-) atherosclerosis(AS) mice. **Methods** The subcultured MC(RAW264.7 cell lines) were divided into four groups, normal control group, model group, DHAP group and Simvastatin group. The content of 5-lipoxygenase in cell protein was detected with Western blot method, and leukotriene B4 was analyzed by ELISA. C57BL/6 mice served as the normal control group, and 24 male ApoE(-/-) mice were equally randomized into 3 groups, model group(ip., 2 mg·kg^{-1}·d^{-1} of ethylalcohol), DHAP group(ip., 20 mg·kg^{-1}·d^{-1} of DHAP), simvastatin treatment group (ip., 20 mg·kg^{-1}·d^{-1} of simvastatin). All mice were fed with a common diet for 16 weeks, and the medication lasted 8 weeks. TB4 in the blood was collected. Sections of aortic root were stained by HE, and the content of 5-lipoxygenase in atherosclerotic plaques at the aortic root was examined by Western blot. **Results** The concentration of LTB4 was decreased, the formation of AS plaque was reduced, 5-lipoxygenase content at atherosclerosis plaque was also decreased, and the number and area of AS focus was reduced in DHAP- treated group. **Conclusion** It suggested that DHAP could be effective for the prevention and treatment of AS, and its possible mechanism is related with the inhibition of 5-lipoxygenase and the decrease of TB4.

Keywords: 3,4-Dihydroxyacetophenone; 5-Lipoxygenase; Atherosclerosis; ApoE(-/-) mice; Macrophages

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异甘草酸镁治疗慢性乙型肝炎疗效观察

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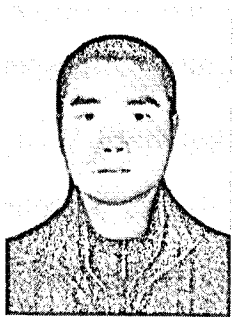

[摘要] **目的** 观察异甘草酸镁治疗慢性乙型病毒性肝炎的疗效。**方法** 将244例慢性乙型肝炎患者随机分为2组:对照组采用B族维生素、维生素C、复方丹参注射液、肌苷注射液等静脉滴注。治疗组在此基础上予异甘草酸镁100 mg静脉滴注,每日1次,疗程30 d。观察症状、体征及治疗前后肝功能改善情况。**结果** 治疗组中、重度患者肝功能指标改善情况优于对照组,治疗总有效率高于对照组。**结论** 异甘草酸镁可明显改善慢性乙型肝炎患者的临床症状及肝功能指标。

[关键词] 异甘草酸镁;慢性乙型肝炎;肌苷注射液
[中图分类号] R0512.62 [文献标识码] A [文章编号] 1008-8849(2010)28-3566-02

Observation of curative effect of magnesium isoglycyrrhizinate on chronic hepatitis B

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Abstract: Objective It is to observe the curative effect of magnesium isoglycyrrhizinate on chronic hepatitis B. **Methods** 244 cases of patients with chronic hepatitis B were randomly divided into 2 group. The control group was treated with Vitamin B, Vitamin C, salvia miltiorrhiza and inosine by intravenous drip. The treatment group was treated magnesium isoglycyrrhizinate 100mg by intravenous drip once a day and for 30 days on the basis of above. The improvement of clinical symptoms, signs and liver function after treatment were observed. **Results** The improvement of liver function indexes in moderate and severe patients of treatment group were better than that in control group, and compared with the control group the total effective rate was also higher. **Conclusion** Magnesium isoglycyrrhizinate can obviously improve clinical symptom, sign and index of liver function in chronic hepatitis patients.

Keywords: magnesium isoglycyrrhizinate, chronic hepatitis B; inosine injection

慢性乙型肝炎在我国发病率高,目前临床还缺乏可靠的特效治疗药物。异甘草酸镁是一种肝细胞保护剂,具有抗炎、保护肝细胞膜及改善肝功能的作用。2008年4月—2009年12月,笔者采用异甘草酸镁治疗慢性乙型肝炎患者124例,效果较好,现报道如下。

1 临床资料

1.1 一般资料 选择符合2000年(西安)第6次全国传染病寄生虫病学术会议讨论修订标准的慢性乙型病毒性肝炎患者244例,排除重型肝炎及肝硬化者。随机分为2组:治疗组124例,男90例,女34例;年龄18~64(36.4±11.5)岁;病情轻度24例,中度68例,重度32例。对照组120例,男85例,女35例;年龄17~61(35.6±10.9)岁;病情轻度20例,中度72例,重度28例。2组患者一般情况具有可比性。

1.2 治疗方法 2组均给予B族维生素、维生素C、肌苷注射液、复方丹参注射液等静脉滴注,不使用其他降酶退黄药物。在此基础上,治疗组予异甘草酸镁(商品名:天晴甘美,江苏正大天晴制药股份有限公司生产,每支50 mg)100 mg加入5%葡萄糖液250 mL中静脉滴注,每天1次,疗程30 d。

1.3 观察指标 观察患者症状体征(乏力、纳差、腹胀、恶心、呕吐、肝区疼痛、腹泻、肝脾肿大、黄疸等)改善情况。治疗前及治疗后每2周复查1次肝功能、血常规、乙肝血清标志物。

1.4 疗效判定标准 显效:自觉症状消失,肝脾肿大回缩,肝功能检查丙氨酸氨基转移酶(ALT)、天冬氨酸氨基转移酶(AST)、总胆红素(TBil)恢复正常或接近正常;有效:自觉症状改善,肝脾肿大略有回缩,肝功能检查较前下降≥50%以上;无效:达不到以上标准或病情进展恶化。

1.5 统计学处理 计数资料采用χ^2检验,计量资料采用t检验。

2 结 果

2.1 临床疗效 见表1。

2.2 肝功能指标变化 治疗30 d,2组ALT、AST、TBil均有改善,治疗组中、重度者改善情况优于对照组,见表2。

2.3 乙肝血清标志物检测结果 2组均无阴转患者。

2.4 不良反应 用药过程中,观察组4例出现一过性恶心、上腹部不适,未予药物治疗而自行缓解,未中断治疗。2组治疗后肾功能、心电图均正常,血常规无明显变化。

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表1 2组治疗效果比较 例

组别	病情	n	显效	有效	无效	总有效率/%
治疗组	轻度	24	21	2	1	96
	中度	68	52	12	4	94①
	重度	32	23	5	4	88①
对照组	轻度	20	16	2	2	90
	中度	72	41	18	13	82
	重度	28	12	6	10	64

注:①与对照组比较,$P<0.05$。

表2 2组治疗前后肝功能比较($\bar{x}\pm s$)

组别	病情	n	ALT/(IU/L)		AST/(IU/L)		TBil/(μmol/L)	
			治疗前	治疗后	治疗前	治疗后	治疗前	治疗后
治疗组	轻度	24	66.23±9.01	25.52±12.62	61.39±8.26	24.14±7.91	28.51±6.95	16.13±4.72
	中度	68	140.38±20.58	41.49±13.18②	132.73±18.42	38.41±11.56①	59.72±17.28	22.57±6.77①
	重度	32	672.54±134.65	66.48±21.72①	708.42±126.87	60.24±18.36①	132.66±32.78	39.72±14.04①
对照组	轻度	20	64.36±6.73	28.95±10.33	60.38±9.52	26.79±7.01	26.90±5.13	19.08±5.16
	中度	72	145.23±22.39	50.42±16.11	135.42±19.30	47.01±13.31	58.60±15.38	30.04±7.18
	重度	28	667.36±118.48	84.74±24.12	703.98±130.34	78.32±22.12	129.75±29.14	66.98±15.81

注:①与对照组比较,$P<0.001$;②与对照组比较,$P<0.005$。

3 讨 论

慢性乙型肝炎患者病情常反复发作,而肝组织的炎症反复活动易导致肝纤维化,最终使病肝向肝硬化甚至肝癌方向发展。抗炎保肝治疗是肝炎综合治疗方案中的一个重要组成部分,也是阻止肝纤维化和癌变的重要措施。甘草酸具有肾上腺素皮质激素样作用,可通过作用于激素受体,减轻炎症细胞浸润,抑制多种炎症递质释放,故能起到明显的抗炎作用,可广泛应用于多种疾病的治疗。尤其在治疗肝脏疾病方面,近来的研究显示它还具有明显的抗纤维化、抗氧化等功效[1]。异甘草酸镁是甘草酸制剂中首个优势的单一异构体构型药物,是单一的α体甘草酸构型的镁盐制剂,是近年研究出来的甘草酸系列药物中的第4代制剂,具有较强的抗炎、保护肝细胞膜、抗生物氧化等多种药理作用,是多功能肝细胞保护剂[2]。动物试验表明,一定浓度范围的异甘草酸镁对大鼠肝星状细胞(HSC)的增殖具有显著的抑制作用。在次氮基三乙酸铁(Fe-NTA)诱导的氧应激中,异甘草酸镁可有效提高HSC的超氧化物歧化酶活性和降低丙二醛含量,从而对Fe-NTA诱导的氧应激具有保护作用[3]。异甘草酸镁还能抑制线粒体跨膜极化,降低线粒体细胞色素C的释放和活性氧的产生,减少线粒体的损害,从而有效保护细胞和显著降低脂肪酸诱导的细胞凋亡和脂质积聚[4]。此外异甘草酸镁还可显著抑制大鼠成纤维细胞Ⅰ、Ⅲ型前胶原mRNA的表达,使Ⅰ、Ⅲ型胶原的合成减少,从而减轻肝组织炎症活动度及纤维化程度[5]。在针对异甘草酸镁的Ⅱ期、Ⅲ期临床试验中,异甘草酸镁的使用者均未出现假性醛固酮症和血压升高等不良反应,而异甘草酸镁较以往的甘草酸制剂有更好的疗效和安全性,具有更强的抗炎、抗氧化、解毒和稳定细胞膜、抗肝纤维化功能,显示出良好的应用前景。同时异甘草酸镁作为新一代的甘草酸制剂,药物清除半衰期可长达19~31 h[6],能够满足每天1次给药,适用于各种肝病的治疗。本研究结果显示,异甘草酸镁可明显改善慢性乙型肝炎患者的临床症状和肝功能指标,不良反应少且较轻微,临床应用前景广泛。

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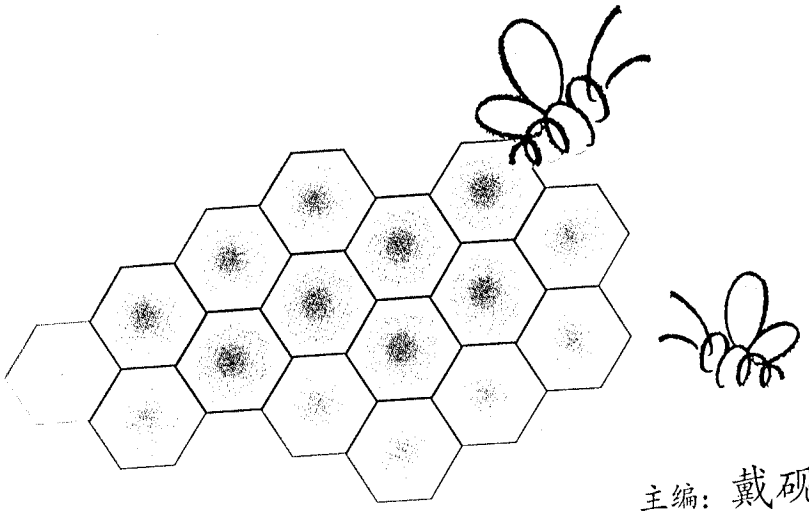

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Exhibit 5

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November 1, 2016

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Regulations and Rulings Division
Alcohol, Tobacco, Tax and Trade Bureau
1310 G Street, N.W., Box 12
Washington, D.C. 20005

Re: Supplement to Petition for Health Related Statements for Bellion Vodka with NTX®

Dear Ms. Greenberg and Ms. Gesser:

Please find enclosed two (2) hardcopies of Bellion Spirts, L.L.C.'s and Chigurupati Technologies Private Ltd.'s supplement to their April 12, 2016 petition for health-related statements concerning the effect of NTX® in distilled spirits and/or other alcoholic beverages ("Petition"). This supplement includes five (5) Exhibits,[1] described below. The five exhibits are relevant to Sections IV.C and IV.D of the Petition. In sum, the exhibits provide additional and compelling evidence, including a peer-reviewed article and two new human clinical studies, confirming the hepatoprotective and DNA protective effects of NTX®.

Exhibit A is the peer-reviewed Article entitled *Hepatoprotective Effects of a Proprietary Glycyrrhizin Product during Alcohol Consumption: A Randomized, Double-Blind, Place-Controlled, Crossover Study*, authored by Harsha Chigurupati, Biswajit Auddy, M. Biyani, and Sidney J. Stohs, and published in PHOTOTHERAPY RESEARCH (2016). We previously provided you with the Clinical Study Report underlying this peer-reviewed article. *See* Petition at Exh. 5,

[1] Bellion submits its confidential and proprietary scientific data "Confidential" under 5 U.S.C. § 552(B)(4).

at pp. 860–965. The study was accepted for publication in the peer-reviewed journal on July 22, 2016.

Exhibit B is a White Paper entitled *NTX®---DNA and Tissue Protective Effects in Association with Alcohol Consumption*, authored by Dr. Sidney J. Stohs. Dr. Stohs's White Paper discusses three clinical trials which demonstrate the protective effects of NTX®. The first such study, which was a randomized, double-blind, placebo-controlled, and crossover study, forms the basis for the peer-reviewed article attached as Exhibit A. *See also* Petition at Exh. 5, at pp. 860–965 (Clinical Study Report forming the basis for the peer-reviewed article). The second study is a randomized, double-blind, placebo-controlled, crossover study involving 50 healthy subjects and evaluates the effect of NTX® in modulating alcohol-induced oxidative stress on the liver. The underlying Final Report documenting that study is attached as **Exhibit C**. The third study, involving 25 human subjects, is a crossover, double-blind, randomized, two-group study that assessed the effects of NTX® on alcohol-induced DNA damage. The underlying Final Report documenting that study is attached as **Exhibit D**.

Dr. Stohs's curriculum vitae is attached as **Exhibit E**.

Your letter dated October 7, 2016 promised a decision by November 10, 2016. We expect that decision to encompass the supplemental exhibits attached hereto because they are relevant and supportive of Bellion's pending petition and, as such, should be included within the administrative record. *See Larita-Martinez v. I.N.S.*, 220 F.3d 1092, 1095 (9th Cir. 2000) *(*due process requires that the agency "review all relevant evidence"). Supplementing the record with timely and relevant information is necessary to ensure a full and complete record for decision at the administrative level.

Please do not hesitate to contact us with any questions.

Sincerely,

Jonathan W. Emord
Peter A. Arhangelsky
Bethany R. Kennedy
Eric J. Awerbuch

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Hepatoprotective Effects of a Proprietary Glycyrrhizin Product during Alcohol Consumption: A Randomized, Double-Blind, Placebo-Controlled, Crossover Study

Harsha Chigurupati,[1] Biswajit Auddy,[1] M. Biyani[1] and Sidney J. Stohs[2]*

[1]Chigurupati Technologies Private Limited, Hyderabad, India
[2]Creighton University, 7068 Maumee Valley Court, Frisco, TX 75034, USA

Traditionally, licorice has been used to treat liver problems. Glycyrrhizin, the primary active compound, has been shown to suppress elevations in liver enzymes that occur when the liver becomes diseased or damaged. This randomized, double-blind, placebo-controlled, crossover study evaluated the hepatoprotective effects of a proprietary glycyrrhizin product during alcohol consumption. Twelve healthy individuals (six male and six female subjects) in a clinic setting consumed vodka nightly for 12 days with the glycyrrhizin product or placebo (blank control), achieving a blood alcohol level of 0.12%. Liver function enzymes including alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and alkaline phosphatase and serum reduced glutathione were measured at overnight visits 1, 6, and 12. In the alcohol only group, AST, ALT, and GGT significantly increased from baseline (overnight visit 1) to overnight visit 12. In the active group, no statistically significant increases were observed for AST, ALT, and GGT, while alkaline phosphatase significantly decreased and plasma glutathione decreased relative to the alcohol control group. These results suggest that consumption of the proprietary glycyrrhizin study product during alcohol consumption may support improved liver health compared with drinking alcohol alone. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: alcohol; glycyrrhizin; D-mannitol; hepatoprotection; glutathione; AST, ALT; GGT.

INTRODUCTION

The detrimental effects of chronic and/or heavy alcohol consumption are well established, and alcohol is considered a risk factor or cause of numerous diseases. The US Center for Disease Control estimates that there are approximately 88,000 alcohol related deaths annually (www.cdc.gov/alcohol/onlinetools.htm, 2015). The most common problem associated with chronic alcohol consumption is alcoholic liver disease, which is defined by histological lesions on the liver that range from hepatic steatosis to more advanced stages as alcoholic steatohepatitis, cirrhosis, hepatocellular carcinoma, and liver failure due to necrosis or apoptosis (programmed cell death) (O'Shea *et al*., 2010; Kim *et al*., 2016).

The mechanisms associated with alcohol toxicity and the induction of alcoholic liver disease can be summarized as follows: metabolism of alcohol to highly toxic acetaldehyde and free radical species, production of reactive oxygen species (ROS) and reactive nitrogen species with resulting oxidative stress, release of inflammatory cytokines as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), abnormal lipid metabolism including formation of the lipid peroxidation product malondialdehyde (MDA), oxidative DNA damage, formation of protein and DNA adducts with metabolites of alcohol and acetaldehyde, and ultimately induction of apoptosis or necrosis with subsequent multisystem organ failure (Bruha *et al*., 2012; Lierena *et al*., 2015; Dhanda and Collins, 2015; Li *et al*., 2015; Han *et al*., 2016).

Biomarkers are biological indicators of the state of health of an organ or tissue. Various common biomarkers useful in assessing the existence, progression, and pathogenesis of alcoholic liver disease include measurements of the blood serum levels of the enzymes aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) (Deshpande *et al*., 2014; Li *et al*., 2015; Kim *et al*., 2015; Qureshi *et al*., 2015). Elevations of the serum levels of these enzymes occur when the liver is damaged as is the case with alcoholic liver disease. Conversely, decreases in the serum levels of these enzymes occur as the liver begins to heal.

Glutathione (GSH) is one of the primary intracellular antioxidants and chemoprotectants in human tissues. Decreases in GSH content occur in blood and tissues as it reacts with and neutralizes ROS and free radicals. Therefore, a decrease in hepatic GSH is associated with an increase in oxidative stress as occurs in the case of alcoholic liver disease (Deshpande *et al*., 2014; Li *et al*., 2015; Kim *et al*., 2015; Qureshi *et al*., 2015; Han *et al*., 2016).

In individuals with alcohol-associated steatosis, studies indicate that approximately 20% will develop cirrhosis within 10 years (Lierena *et al*., 2015), and it is

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estimated that 20% of heavy drinkers will develop acute alcoholic hepatitis (Mathurin and Lucey, 2012). The two primary approaches for successful prevention and reversal of liver damage are abstinence from alcohol and appropriate nutritional support. Realistically, one cannot expect the vast majority of alcohol consumers to abstain. Therefore, there is a pressing need to develop safer strategies for alcohol consumption and treatment in order to reduce alcohol-associated morbidity and mortality, and the high cost to society because of health care costs and lost productivity.

The high morbidity and mortality rate of alcoholic liver disease is attributed to the sequence of events described previously, which can ultimately culminate in multisystem organ failure and death (Mathurin and Lucey, 2012; Li *et al*., 2015). Systems designed to prevent and treat alcohol-induced liver toxicity and alcoholic liver disease must therefore focus on disrupting, inhibiting, and/or reversing these pathological pathways. As a consequence, a new category of alcoholic beverages called Functional Spirits has been created, and a product has been patented (US Patent 9,149,491 B1) that prevents and/or ameliorates the hepatotoxic effects of alcohol while retaining its desired characteristics.

Han *et al*. (2016) have reviewed the relationships between alcoholic liver disease, antioxidants, and antioxidant enzymes. Glycyrrhizin (glycyrrhizic acid; glycyrrhizinic acid; 18β-glycyrrhizin) is a triterpenoid saponin glycoside ($C_{42}H_{61}0_{16}NH_4$) derived from *Glycyrrhiza glabra* L. (licorice) root. It is Generally Recognized as Safe by the US Food and Drug Administration. Various studies have demonstrated the hepatoprotective, antioxidant, and antiinflammatory effects of glycyrrhizin (Hou *et al*., 2014, Hsiang *et al*., 2015; Gong *et al*., 2014; Gu *et al*., 2014; Sil *et al*., 2015). The ability of glycyrrhizin to protect against the hepatotoxic effects of alcohol in humans has not been previously studied.

In an animal study, a licorice extract predominantly containing glycyrrhizin has been shown to inhibit alcohol induced increases in TNF-α, lipid accumulation in liver (steatosis), and decreases in the antioxidant GSH (Jung *et al*., 2016). The hepatoprotective effect was confirmed histologically. In animals, glycyrrhizin also inhibits alcohol-induced increases in the inflammatory cytokine NF-kB and increases in MDA as well as serum lipids while preventing liver injury and cirrhosis (Wang *et al*., 1999; Quan *et al*., 2009).

In a number of animal experiments, glycyrrhizin has been shown to decrease the production of the inflammatory cytokines TNF-α, IL-1 and IL-6 (Gong *et al*., 2012; Gong *et al*., 2014; Gu *et al*., 2014; Sil *et al*., 2015), decrease lipid peroxidation (MDA) (Jeong and Kim, 2003; Gong *et al*., 2014; Sil *et al*., 2015; Liang *et al*., 2015), and increase hepatic GSH content (Jeong and Kim, 2003; Lee *et al*., 2007). Glycyrrhizin provides protection against acetaminophen-induced liver damage by reversing fatty acid metabolism (Yu *et al*., 2014). Glycyrrhizin also provides protection against nephrotoxic drugs (Wu *et al*., 2015) as well as experimental acute pancreatitis in rats (Yildirim *et al*., 2013). In the latter case, glycyrrhizin decreased concentrations of the inflammatory cytokines TNF-α, IL-1 and IL-6 and also decreased MDA production. The protective effects of glycyrrhizin in rats were corroborated histologically (Yildirim *et al*., 2013).

Glycyrrhizin has been shown to ameliorate liver damage associated with high fructose intake in rats (Sil *et al*., 2015) which is characterized by oxidative tissue damage and inflammation. Glycyrrhizin administration significantly decreased levels of AST, ALT, ROS, carbonyl protein, TNF-α, lipid peroxidation (MDA), and apoptosis, all characteristic of liver damage. The antioxidant, antiinflammatory, and hepatoprotective effects of the glycyrrhizin in rats were affirmed histologically (Sil *et al*., 2015).

Various *in vitro* studies have demonstrated the antioxidant and free radical scavenging ability of glycyrrhizin (Ablise *et al*., 2004; Gandhi *et al*., 2004; Cheel *et al*., 2010; Gong *et al*., 2012; Kaur *et al*., 2012). In cell culture experiments, glycyrrhizin has been shown to inhibit inflammatory TNF-α secretion (Ishida *et al*., 2012; Wang *et al*., 2013) and MDA production (lipid peroxidation) (Lecomte *et al*., 1994; Hu *et al*., 2001; Tripathi *et al*., 2009; Hou *et al*., 2014), while enhancing the antioxidant and tissue protectant GSH (Tripathi *et al*., 2009; Hou *et al*., 2014; Hsiang *et al*., 2015). A licorice triterpene has also been shown to induce autophagy activation and exhibit antiviral activity (Laconi *et al*., 2014).

The immunomodulatory activity of glycyrrhizin has been studied in mice with allergic rhinitis (Li and Zhou, 2012). Glycyrrhizin in a dose-dependent manner significantly reduced blood immunoglobulin E (IgE), interleukin-4 (IL-4), interleukin-5 (IL-5), IL-6, nitrous oxide, TNF-α and nitrous oxide synthase, while concurrently enhancing blood levels of immunoglobulin A (IgA), immunoglobulin M (IgM), immunoglobulin G (IgG), interleukin-2 (IL-2), and interleukin-12 (IL-12). The authors concluded that glycyrrhizin improved immune function in this animal model.

Another study has looked at the effect of glycyrrhizin injection on liver function and cellular immunity in children with infectious mononucleosis complicated liver impairment (Cao *et al*., 2006). After 2 weeks of treatment with the glycyrrhizin product, cellular immune function as determined by measuring T lymphocyte subsets significantly improved relative to untreated control children. In addition, liver function also improved as determined by significant decreases in serum levels of AST, ALT and total bilirubin. These results indicate that glycyrrhizin improved immune function and promoted recovery of liver function in children with infectious mononucleosis.

The second of the two primary components of the study product is D-mannitol. D-Mannitol is a sugar alcohol found in many plants (Patel and Williamson, 2016). The amount of D-mannitol naturally present in licorice has not been reported. Its antioxidant and free radical scavenging abilities are well known (Patel and Williamson, 2016). Several human studies have demonstrated the antioxidant and tissue protective effects of D-mannitol. D-Mannitol has been shown to exhibit tissue protective effects in acute ischemic-reperfusion injuries (Shah *et al*., 1996) and antioxidant and free radical scavenging properties in conjunction with cardiopulmonary bypass (England *et al*., 1996). D-Mannitol is used as an antiinflammatory and antioxidant in tissue baths associated with kidney transplants (Cosetino *et al*., 2013).

In a study in rats, D-mannitol provided protection against alcohol-induced gastric mucosal damage because of its antioxidant and antiinflammatory properties (Gharzouli *et al*., 2001). D-Mannitol has also been shown

to exhibit antiinflammatory and antioxidant activity in rats after traumatic brain injury (Yilmaz *et al*., 2007). D-Mannitol decreased production of MDA and normalized the levels of antioxidant enzymes.

NTX® is a novel patented product composed of the primary ingredients glycyrrhizin (glycyrrhizic acid) and D-mannitol. It also contains potassium sorbate as an antioxidant, preservative and antimicrobial. This study was designed to assess the hepatoprotective effects of the combination of glycyrrhizin and D-mannitol (NTX®) in alcohol-consuming human subjects.

METHODS

Background. The primary objective was to evaluate the hepatoprotective properties of the study product during alcohol consumption. Endpoints included liver function tests involving the enzymes GGT, ALT, AST, and ALP, and changes in GSH serum levels.

The study product (NTX®) was a proprietary blend of glycyrrhizin (licorice) and D-mannitol with and potassium sorbate as a product stabilizer. The study product was provided by Chigurupati Technologies Private Limited, Hyderabad, India, and was Good Manufacturing Practice (GMP) certified. Based on the US Patent (9,149,491 B1), the product contains glycyrrhizin preferably in the range of 0.1–0.3% and D-mannitol preferably in the range of 1.0–2.5%. The concentrations of glycyrrhizin and D-mannitol were based on animal studies described in the patent. The glycyrrhizin was derived from the plant source, *Glycyrrhiza glabra* L. The study product was pre-mixed with vodka (40% ethanol) and was consumed in the clinic. The blank control group consumed the alcohol without any additive. Because this was a crossover study, all subjects who completed the study participated in both arms of the study. All subjects consumed for 12 consecutive nights an amount of alcohol with or without the study product to achieve a blood alcohol level of 0.12%. The amount of glycyrrhizin/D-mannitol product consumed was proportional to the amount of alcohol consumed.

Subjects. Twelve healthy subjects between 21 and 50 years of age, with a body mass index between 23 and 29 kg/m^2, and who were social drinkers consuming alcohol at least 1 to 2 occasions per week, completed the study. Subjects were recruited from the general population by online recruiting, advertising, and available clinical trial databases. Subjects were screened by telephone prior to scheduling a screening visit. Subjects had to be willing not to change their current exercise and diet programs throughout the study period. Subjects who were pregnant, had any liver condition including hepatitis, fatty liver or liver disease, and who had a history or record of aggressive or violent behavior were excluded from the study.

Study design. This study was a randomized, double-blind, placebo-controlled, crossover study (Fig. 1). Group allocations were made by a statistician and

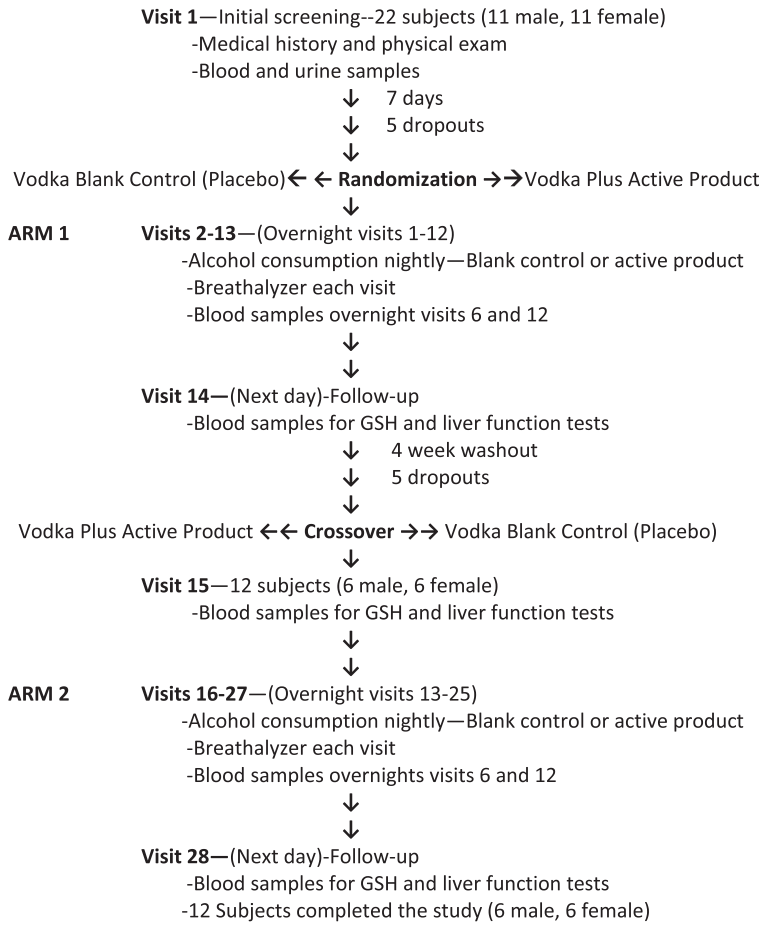

Figure 1. Flow Chart.

placed in individually numbered envelopes to maintain blinding of all individuals. The subjects as well as the clinical staff, data management staff, and statistical analysis staff were unaware of the study groups. Medicus Research was the contract research organization for this study. Institutional review board approval was received from MaGil institutional review board (Rockville, MD) prior to the initiation of any study-related activities.

An in-clinic screening visit was conducted 7 days prior to the baseline overnight visit (visit 1) where subjects underwent the informed consent process and were screened for the presence of all inclusion criteria and the absence of all exclusion criteria. In addition, the screening process included a detailed medical history interview, physical examination, vital signs, and anthropometric measurements, as well as urine collection for pregnancy tests and blood collection for liver function tests.

Subjects returned to the clinic at overnight visit 1 (visit 2, baseline) for a review of medication history, adverse events reporting, and vital signs assessments. Subjects were also provided with a low-fat dinner and a standardized amount of snacks and fluid. Vodka alcohol with juice or soda chaser was offered to each subject and based on preference was provided during both arms of the study. At the onset of alcohol consumption, subjects either consumed the study product in the commercially available vodka alcohol drink or the control alcohol drink, which contained no study product according to the randomization assignment.

Analytical. Blood samples were drawn at −0.5, 0.5, 1, 1.5, 2 and 10 h for assessments of serum GSH levels, and at −0.5 and 10 h for liver function tests (serum AST, ALT, GGT and ALP). Subjects were also administered a breathalyzer test at 0, 0.5, 1, 1.5, 2, and 10 h to estimate blood alcohol concentrations each night.

The subjects consumed the alcohol for 12 consecutive nights with and without the study product (Fig. 1). Blood samples were drawn on overnight visits 6 (V7) and 12 (V13) according to the same schedule as overnight visit 1. At overnight visits 2, 3, 4, 5, 7, 8, 9, 10, and 11 no blood samples were drawn. After a total of 12 overnight visits, subjects returned to the clinic for follow-up visit on day 13 to measure serum GSH and liver function enzymes. All subjects underwent a 4-week washout period between ARM 1 and ARM 2 of this double-blind crossover study. At the conclusion of the study, subjects could not identify which vodka was the blank control and which contained the glycyrrhizin/D-mannitol study product (Fig. 1).

Serum GSH was estimated using commercial ELISA kits (NeoScience, NeoBioLab, Woburn, MA, USA), while AST, ATL, GGT, and ALP were determined using standard commercially available kits (Sigma-Aldrich, St. Louis, MO USA) (Deshpande *et al*., 2014).

Data analysis. Parallel dual data entry was performed by data management personnel across all endpoints. Data validation and reconciliation of parallel entry occurred after the dual data entry process. The database was formally locked after all suspicious entries in the database were resolved. The product assignments were then distinguished from the randomization or blinding codes and merged into the database and data tables.

Descriptive measures such as *N*, means, standard deviations, and standard errors of means were processed for each numeric endpoint on all visits. Percentage changes were used to quantify increase or decrease of endpoints from baseline for each arm.

All efficacy endpoints were analyzed depending on the level of measurement of the endpoint. For each endpoint in ordinal scale, the differences in the medians within time periods for each arm were tested for nominal significance using a nonparametric test (Wilcoxon Signed Rank Test). For each endpoint in the interval/ratio scale within and across, overnight visits for each arm was tested for nominal significance using the paired Student's *t*-test. For continuous variable at each time point, the difference between means of different arms was assessed for significance either using the paired Student's *t*-test or the nonparametric Wilcoxon Signed Ranks Test. Lastly, with respect to longitudinal data, the dependent variables were measured at the selected time points for each subject and were analyzed by the Linear Mixed Model. All tests of hypotheses were done at $\alpha = 0.05$.

RESULTS

A total of 22 subjects were enrolled in the study, with 12 subjects (six male and six female subjects) completing the study. Because this was a crossover study with all 12 subjects completing both arms of the study, the effective sample size was $n = 24$. The primary objective of this study was to evaluate the hepatoprotective properties of the glycyrrhizin/D-mannitol product during alcohol consumption by assessing blood liver function enzymes and serum GSH content. Breathalyzer tests were conducted during each overnight visit to ensure that all subjects had consumed the alcohol and had achieved a blood level of 0.12%. The 12-day average daily vodka consumption to achieve this blood alcohol level for male subjects was 366 mL and 245 mL for female subjects in the blank control (alcohol only) arm of the study and 339 mL for male subjects and 247 mL for female subjects in the active glycyrrhizin/D-mannitol arm of the study.

In the blank control (alcohol only) group, alcohol (vodka) without the addition of the glycyrrhizin/D-mannitol was consumed daily for 12 days. In this arm of the study, increases occurred in the liver biomarker function test enzymes AST (Table 1), GGT (Table 2), and ALT (Table 3). In particular, AST statistically significantly increased by 30.75 U/L (135.16%, $p = 0.012$) from baseline (overnight visit 1, hour −0.5) to overnight visit 12 (hour 10) (Table 1). GGT significantly increased by 10.67 U/L (43.99%, $p = 0.039$) (Table 2), while ALT non-significantly increased by 14.58 U/L (62.06%, $p = 0.227$) from baseline (overnight visit 1) to overnight visit 12 (hour 10) (Table 3).

In the blank control (alcohol only) group, ALP did not change significantly (1.54%, $p = 0.802$) between baseline (overnight visit 1, hour −0.50) and overnight visit 12 (hour 10), or between baseline and the follow-up visit on day 13 (−1.42 U/L; $p = .0826$) (Table 4). However, noteworthy is the observation that AST (Table 1) and ALT (Table 3) were significantly elevated by 19.75

Table 1. Changes in aspartate aminotransferase—comparison between active and blank control

Serum aspartate aminotransaminase		Product	*N*	Mean (U/L)	Standard deviation	Standard error of the mean	Significance
Overnight 1 (visits 2 and 16)	0 h	Active	12	21.67	6.140	1.772	0.539
		Control	12	22.75	5.545	1.601	
	10 h	Active	12	21.33	4.868	1.405	0.667
		Control	12	22.00	5.752	1.661	
Overnight 6 (visits 7 and 21)	0 h	Active	12	21.33	4.097	1.183	0.826
		Control	12	21.67	5.598	1.616	
	10 h	Active	12	20.42	3.605	1.041	0.135
		Control	12	22.00	4.411	1.273	
Overnight 12 (visits 13 and 27)	0 h	Active	12	20.58	4.944	1.427	0.039*
		Control	12	55.00	100.908	29.130	
	10 h	Active	12	21.08	4.641	1.340	0.039*
		Control	12	53.50	90.100	26.010	
Follow-up visit (visits 14 and 28)	—	Active	12	24.67	3.822	1.103	0.109
		Control	12	42.50	53.057	15.316	

N represents the number of subjects having complete pairs for both time points in comparison.Significance testing was performed using paired sample *t*-test except for baseline and 10 h at Overnight 12, which used Sign Test.
*Significant at alpha level = 0.05.

Table 2. Gamma-glutamyl transpeptidase—comparison between active and blank control

Gamma-glutamyl transpeptidase		Product	*N*	Mean (U/L)	Standard deviation	Standard error of the mean	Significance
Overnight 1 (visits 2 and 16)	Baseline	Active	12	22.08	14.519	4.191	0.065
		Control	12	24.25	16.543	4.775	
	10 h	Active	12	22.25	14.827	4.280	0.344
		Control	12	23.75	15.743	4.545	
Overnight 6 (visit 7 and 21)	Baseline	Active	12	21.25	11.725	3.385	0.774
		Control	12	25.50	17.516	5.056	
	10 h	Active	12	20.58	12.102	3.493	0.146
		Control	12	24.92	16.412	4.738	
Overnight 12 (visits 13 and 27)	Baseline	Active	12	23.83	11.700	3.377	0.039*
		Control	12	35.92	36.973	10.673	
	10 h	Active	12	24.17	12.684	3.661	0.065
		Control	12	34.92	32.284	9.320	
Follow-up visit (visits 14 and 28)	—	Active	12	24.83	14.077	4.064	0.021*
		Control	12	39.42	36.926	10.660	

N represents the number of subjects having complete pairs for both time points in comparison. Significance testing was performed using paired sample *t*-test.
*Significant at alpha level = 0.05.

Table 3. Alanine transaminase—comparison between active and blank control

Serum alanine transaminase			*N*	Mean (U/L)	Standard deviation	Standard error of the mean	Significance
Overnight 1 (visits 2 and 16)	0	Active	12	20.58	6.598	1.905	0.309
		Control	12	23.50	9.596	2.770	
	10 h	Active	12	20.08	7.366	2.127	0.230
		Control	12	23.42	8.723	2.518	
Overnight 6 (visits 7 and 21)	0 h	Active	12	21.17	6.631	1.914	0.234
		Control	12	22.75	6.398	1.847	
	10 h	Active	12	21.50	6.446	1.861	0.594
		Control	12	22.08	7.342	2.119	
Overnight 12 (visits 13 and 27)	0 h	Active	12	20.75	6.877	1.985	0.146
		Control	12	36.00	36.947	10.666	
	10 h	Active	12	21.08	6.473	1.869	0.039*
		Control	12	38.08	36.403	10.509	
Follow-up visit (visits 14 and 28)	0 h	Active	12	24.75	8.750	2.526	0.065*
		Control	12	42.58	33.709	9.731	

N represents the number of subjects having complete pairs for both time points in comparison. Significance testing was performed using paired sample *t*-test.
*Significant at alpha level = 0.05.

Table 4. Alkaline phosphatase—comparison between active and blank control

Alkaline phosphatase		Product	*N*	Mean (U/L)	Standard deviation	Standard error of the mean	Significance
Overnight 1 (visit 2 and visit 16)	Baseline	Active	12	68.83	18.542	5.352	0.166
		Control	12	64.83	13.169	3.802	
	10 h	Active	12	64.33	19.171	5.534	0.617
		Control	12	63.00	15.112	4.362	
Overnight 6 (visit 7 and visit 21)	Baseline	Active	12	65.08	23.039	6.651	0.313
		Control	12	68.75	19.056	5.501	
	10 h	Active	12	60.58	19.547	5.643	0.108
		Control	12	64.33	17.890	5.164	
Overnight 12 (visit 13 and visit 27)	Baseline	Active	12	66.33	20.362	5.878	0.167
		Control	12	69.75	20.942	6.045	
	10 h	Active	12	62.75	19.809	5.718	0.296
		Control	12	65.83	21.762	6.282	
Follow-up visit (visit 14 and visit 28)	—	Active	12	63.83	21.829	6.302	0.700
		Control	12	64.92	17.661	5.098	

N represents the number of subjects having complete pairs for both time points in comparison. Significance testing was performed using paired sample *t*-test.

and 19.08 U/L from baseline to the follow-up visit on day 13 (86.81%, $p = 0.006$; 81.21%, $p = 0.065$, respectively). GGT was non-significantly elevated by 15.17 U/L ($p = 0.227$) at this time point relative to baseline at the follow-up visit (Table 2).

In the active group, which received alcohol plus the glycyrrhizin/D-mannitol product (NTX®), there were no statistically significant increases in the liver function enzymes GGT (Table 2) and ALT (Table 3) from baseline to overnight visit 12 (hour 10) and the follow-up visit. In addition, ALP significantly decreased by 8.25 and 6.08 U/L from baseline to overnight visits 6 (11.99%, $p = 0.005$) and 12 (hour 10) (8.84%, $p = 0.035$) (Table 4), and AST non-significantly decreased by 0.58 U/L (2.69%, $p = 0.756$) from baseline to overnight 12 (hour 10) (Table 1).

The changes in the serum GSH content across the overnight visits for the blank control (alcohol only) cohort are presented in Table 5. Alcohol consumption did not alter serum levels of GSH from baseline up to the 2-h time point on overnight visits on days 6 and 12. However, at the 10-h time point, serum GSH demonstrated significant increases of 27.09 and 21.75 μg/mL from baseline on overnight visits 6 and 12, respectively (Table 5), which represented 9.14% ($p = 0.027$) and 7.34% ($p = 0.032$) increases over baseline, respectively.

In the active group receiving alcohol plus the glycyrrhizin/D-mannitol product (NTX®) (Table 5), serum GSH levels demonstrated statistically significant increases from baseline to overnight day 6 at all time points (hour 0.5, hour 1, hour 1.5, hour 2, and hour 10) and from baseline to overnight day 12 at the 1.5-h time point. At the 1.5-h time point on days 6 and 12, the serum GSH levels increased by 10.87% ($p = 0.012$) and 11.57% ($p = 0.007$), respectively, while at the 10-h time point, the increases were 8.08% ($p = 0.021$) and 4.72% ($p = 0.139$), respectively (Table 5).

When comparisons were made between the alcohol only (blank control) group and the alcohol plus glycyrrhizin/D-mannitol product (active) group with respect to serum GSH levels, the GSH levels in the placebo group were approximately 20–30 μg/mL higher than the active group at each point (Table 5). For example, at the 10-h time point for overnight visit 12, the serum GSH level was an average 25 μg/mL higher for the alcohol only (blank control) group as compared with the active group, representing a 7.9% higher level for the control group.

With respect to vital signs, which were assessed daily, no clinically or statistically significant differences were observed between the alcohol only group and the active group, which also received the glycyrrhizin/D-mannitol product with respect to pulse rate, systolic blood pressure, diastolic blood pressure, and body temperature at any time point (data not shown). No observed or reported adverse effects or events occurred in either the blank control group or the active group at any time point, and no effects were observed in the active group that did not occur in the control group.

DISCUSSION

This study evaluated the hepatoprotective effects of a product containing glycyrrhizin and D-mannitol in conjunction with alcohol ingestion. To our knowledge, this study represents the first clinical trial evaluating the hepatoprotective effects of a proprietary product premixed/infused with the alcohol during human consumption. The results of this study found that the concurrent consumption of the glycyrrhizin/D-mannitol product with alcohol during 12 consecutive overnight clinic visits as compared with a control (alcohol only) protected against alcohol-induced liver toxicity based on liver function tests.

When subjects in the blank control (alcohol only) group consumed the alcoholic drink, increases in the liver enzymes AST, ALT, and GGT occurred as compared with baseline (Tables 1–3). Increases in levels of these enzymes serve as biomarkers of liver damage and compromised liver health (Deshpande *et al*., 2014; Li *et al*., 2015; Kim *et al*., 2015; Qureshi *et al*., 2015). However, when the subjects consumed the glycyrrhizin/D-mannitol product in combination with the alcohol (active group), no significant increases in liver enzymes were observed, suggesting protection against alcohol-induced liver damage (Tables 1–3).

Table 5. Glutathione levels—comparison between active and blank control

Glutathione levels		Product	*N*	Mean (μg/mL)	Standard deviation	Standard error of the mean	Significance
Overnight 1 (visit 2 and visit 16)	Baseline	Active	12	268.75	33.770	9.748	0.010*
		Control	12	298.17	30.412	8.779	
	0.5 h	Active	12	267.42	41.322	11.929	0.034*
		Control	12	296.00	29.802	8.603	
	1 h	Active	12	267.08	41.921	12.102	0.003**
		Control	12	299.17	40.152	11.591	
	1.5 h	Active	12	263.00	44.333	12.798	0.039*
		Control	12	293.83	38.395	11.084	
	2 h	Active	12	269.67	34.471	9.951	0.006**
		Control	12	302.00	33.737	9.739	
	10 h	Active	12	270.33	38.679	11.166	0.033*
		Control	12	296.33	33.293	9.611	
Overnight 6 (visit 7 and visit 21)	0 h	Active	12	301.50	31.768	9.171	0.799
		Control	12	304.83	49.763	14.365	
	0.5 h	Active	12	283.75	36.397	10.507	0.019*
		Control	12	316.67	59.370	17.139	
	1 h	Active	12	299.92	42.286	12.207	0.122
		Control	12	316.50	29.789	8.599	
	1.5 h	Active	12	291.58	33.187	9.580	0.052
		Control	12	317.92	46.723	13.488	
	2 h	Active	12	298.25	42.921	12.390	0.066
		Control	12	317.25	35.422	10.226	
	10 h	Active	12	292.17	48.662	14.047	0.009**
		Control	12	323.42	36.405	10.509	
Overnight 12 (visit 13 and visit 27)	0 h	Active	12	295.33	42.656	12.314	0.118
		Control	12	309.00	26.084	7.530	
	0.5 h	Active	12	293.08	43.291	12.497	0.687
		Control	12	286.75	50.582	14.602	
	1 h	Active	12	284.08	40.392	11.660	0.166
		Control	12	298.33	37.845	10.925	
	1.5 h	Active	12	293.42	41.421	11.957	0.825
		Control	12	289.50	49.830	14.385	
	2 h	Active	12	290.75	43.206	12.472	0.180
		Control	12	307.42	46.186	13.333	
	10 h	Active	12	283.08	38.764	11.190	0.007**
		Control	12	318.08	41.489	11.977	
Follow-up visit (visit 14 and visit 28)	—	Active	12	286.00	26.502	7.651	0.195
		Control	12	299.83	47.816	13.803	

N represents the number of subjects having complete pairs for both time points in comparison. Significance testing was performed using paired sample *t*-test except for 1.5 h at overnight 1, which used sign test.

*Significant at alpha level = 0.05.

**Significant at alpha level = 0.01.

The mechanism of alcohol-induced hepatotoxicity involves a cascading series of events that include production of free radicals and ROS as well as release of inflammatory cytokines with resultant tissue damage (Bruha *et al*., 2012; Lierena *et al*., 2015; Dhanda and Collins, 2015; Li *et al*., 2015; Han *et al*., 2016). GSH is a prominent antioxidant in the liver and other tissues and reflects tissue antioxidant status. GSH is a tripeptide that is synthesized in the hepatic cytosol, and alcohol induces loss/efflux of GSH from the hepatocytes (Pierson and Mitchell, 1986; Lauterburg and Velez, 1988; Hansen *et al*., 2009). This efflux of GSH concomitantly decreases intrahepatic GSH content (Adams *et al*., 1983). The restoration of hepatic injuries induced by alcohol consumption is regulated by antioxidant status and GSH content.

In this study, the alcohol only (blank control) group exhibited higher serum levels of GSH than the group receiving alcohol plus the glycyrrhizin/D-mannitol product (Table 5) from baseline to overnight visits 6 and 12. Various other studies in humans have shown that acute alcohol ingestion results in increases in serum GSH (Vendemiale *et al*., 1989; Jerca *et al*., 1993; Michelet *et al*., 1995; Nagamma *et al*., 2012). Alcohol induces hepatic membrane damage with leakage of cellular contents as evidenced by increases in blood levels of AST, ALT, and GGT. Similar efflux of GSH from the liver into the blood can also be expected to occur because of its relatively small size, accounting for the increase in plasma GSH, with a concomitant decrease in hepatic GSH, which is well-documented in animal studies (Wang *et al*., 2016; Jung *et al*., 2016).

The reason for the lower blood levels of GSH (Table 5) as well as AST (Table 1), GGT (Table 2), and ALT (Table 3) associated with coadministration of the glycyrrhizin/D-mannitol product with alcohol

relative to the alcohol only (blank control) group at all day and time points can be attributed to decreased hepatic membrane damage, and therefore less leakage of these constituents from the liver.

It should also be noted that various studies have demonstrated a decrease in blood GSH levels in cases of chronic alcohol consumption and abuse and alcoholic liver disease (Lauterburg *et al*., 1984; Lauterburg and Velez, 1988; Loquercio *et al*., 1997, 1999; Lee *et al*., 2004; Pemberton *et al*., 2005; Sripradha *et al*., 2010). Thus, differences in human blood GSH levels have been demonstrated when comparing acute and chronic alcohol consumption. Long-term alcohol consumption results in a depletion of GSH from liver and blood because of a decrease in GSH synthesis (Lauterburg *et al*., 1984), while transient increases in blood GSH because of the initial GSH efflux from the liver may occur following acute alcohol exposure.

Elevated levels of the liver enzymes GGT, AST, and ALT not only predict disease status but also directly reflect various aspects of liver damage, fatty liver, and oxidative stress. In general, the serum activities of these enzymes increase proportionally to the chronic rate of alcohol consumption (Nyblom *et al*., 2004; Hietala *et al*., 2005; Alatalo *et al*., 2009; Whitfield *et al*., 2013; Danielsson *et al*., 2014; Pisa *et al*., 2015). AST and GGT serum levels increase markedly following acute alcohol consumption, although ALT and ALP activities may not change significantly (Nemesanszky *et al*., 1988; Alatalo *et al*., 2009; Whitfield *et al*., 2013). The results of this study are consistent with these published observations, where AST (Table 1) and GGT (Table 2) were significantly increased by alcohol consumption. ALT (Table 3) was increased but not significantly, and ALP was unchanged (Table 4).

GGT is highly correlated with liver disease (Hietala *et al*., 2005; Thomes *et al*., 2013), is a sensitive marker of hepatobiliary disease, and is also a marker of oxidative stress (Bansal *et al*., 2012) and fatty liver (Cederbaum and Dicker, 1983). Alcohol consumption can increase the ratio of AST to ALT by at least 2:1, measurements that can be used to support a diagnosis of alcohol-induced liver damage and alcoholism (Reinke *et al*., 1987). In a randomized controlled trial, chronic alcohol drinkers had markedly higher AST and GGT levels compared with their nondrinking counterpart (Ikeda *et al*., 1995). Similarly, a study of 100 alcoholics reported that GGT and AST are sensitive markers of hepatotoxicity as these enzymes show progressive increases with increased alcohol consumption (Zelickson *et al*., 2011). ALT levels, on the other hand, may lie within the normal range even in subjects with severe alcoholic liver disease (Reinke *et al*., 1987).

Licorice-derived glycyrrhizin has been shown in animal and human studies to possess antioxidant, antiinflammatory, immunoregulatory, and hepatoprotective properties. Li *et al*. (2015) have provided an excellent literature review of the treatment of liver diseases as chronic hepatitis and viral hepatitis using glycyrrhizin. Thus, glycyrrhizin, one of the active constituents used in the study product, has been shown in a number of clinical studies to significantly reduce and normalize serum enzyme activities as AST and ALT in patients with hepatitis (Li *et al*., 2015).

A study in rats has examined the inhibitory effects of glycyrrhizin on alcohol plus carbon tetrachloride-induced liver cirrhosis (Wang *et al*., 1999). The alcohol plus carbon tetrachloride significantly increased serum ALT and the inflammatory marker NF-kB, and histologically, liver steatosis and fibrosis were severe. Cotreatment with a glycyrrhizin product markedly improved the steatosis and fibrosis, significantly reduced the serum ALT levels, and returned the NF-kB levels to near normal. The authors concluded that the glycyrrhizin protected the liver from hepatotoxin-induced liver injury and cirrhosis (Wang *et al*., 1999).

Glycyrrhizin ameliorates oxidative liver damage associated with high fructose intake in rats (Sil *et al*., 2015). Glycyrrhizin administration significantly normalized levels of AST, ALT, ALP, ROS, carbonyl protein, lipid peroxidation (MDA), and apoptosis, all characteristic of oxidative liver damage. The tissue effects of the glycyrrhizin and normalization of liver function were affirmed histologically (Sil *et al*., 2015).

Glycyrrhizin has also been shown to exhibit protection against acetaminophen-induced liver damage by reversing fatty acid metabolism (Liu *et al*., 1994). Similar findings were observed when licorice root extracts were used to treat cadmium chloride-induced toxicity in rats (Hu *et al*., 2001) and titanium dioxide nanoparticle-induced hepatotoxicity in mice (Orazizadeh *et al*., 2014).

The mechanisms mediating the hepatoprotective effects of glycyrrhizin have been well studied (Li *et al*., 2015). Glycyrrhizin has been shown to act as an antioxidant inhibiting ROS formation and MDA (lipid peroxidation) production (Hu *et al*., 2001; Tripathi *et al*., 2009; Hou *et al*., 2014) and inhibit secretion of cytokines such as TNF-α, IL-1, IL-6, and NF-kB (Ishida *et al*., 2012; Wang *et al*., 2013).

D-Mannitol is a plant-derived sugar alcohol and is the second ingredient in the study product. D-Mannitol has been shown to exhibit tissue protective effects and exhibits antioxidant, free radical scavenging, and antiinflammatory properties (England *et al*., 1996; Cosetino *et al*., 2013). In rats, D-mannitol was shown to protect against alcohol-induced gastric mucosal damage because of its antioxidant and antiinflammatory properties (Gharzouli *et al*., 2001). The effects of D-mannitol are believed to be complementary to those of glycyrrhizin because both exhibit antioxidant and antiinflammatory effects but do so by different mechanisms (Li *et al*., 2015; Patel and Williamson, 2016; US Patent No. 9,149,491 B2).

Natural products used for the prevention and treatment of adverse effects of alcohol have been reviewed by Wang *et al*. (2016). Most of the studies that were reviewed focused on the lowering of blood alcohol levels and suppression of alcohol consumption. Several studies in rats and mice have shown that products derived from the plants *Pueraria lobata* (Willd.), Ohwi (kudzu), *Hovenia dulcis* Thunb., and *Diospyros kaki* L. provided hepatoprotective effects against alcohol. In a recent study (Zhang *et al*., 2016), consumption of juices from fruits of *Averrhoa carambola* Linn., *Citrus limon* L. (yellow), and *Musa nana* Lour. was shown to prevent alcohol-induced elevations of AST in mice, therefore offering hepatoprotection.

Various other studies in rats and mice have demonstrated hepatoprotective effects of plant-derived products against alcohol toxicity including *Garcinia indica* L. fruit rind (Panda *et al*., 2012), noni (*Morinda citrifolia* L.) juice (Chang *et al*., 2013), oils from *Zingiber*

officinale Roscoe. (ginger) and *Curcuma longa* L. (turmeric) (Nwozo *et al*., 2014), *Dendrobium huoshanense* polysaccharide (Wang *et al*., 2015b), and geniposide from *Gardenia jasminoides* Ellis (Wang *et al*., 2015a). However, no similar human studies with these plant products have been conducted.

No adverse effects were reported or observed for either the alcohol only group or the alcohol plus glycyrrhizin/D-mannitol group. Licorice abuse is known to be associated with a cortisol-induced mineralocorticoid effect with a tendency towards an elevation in blood pressure and blood sodium levels and a reduction in potassium levels. These effects are primarily due to the presence of glycyrrhetic acid (glycyrrhetinic acid, enoxolone) (Omar *et al*., 2012), which is not present in the study product, although glycyrrhetic acid is a metabolite of glycyrrhizin. No dropouts from the study were due to these types of effects.

Of the 22 subjects that initially qualified for the study, five subjects elected not to participate following the initial overnight baseline visit and prior to consumption of either alcohol-containing product (Fig. 1). Five additional subjects dropped out of the study after completing the first arm of the study that involved 13 visits with alcohol consumption for 12 consecutive nights. These dropouts were due to the amount of alcohol that was required to be consumed. The data reflect only those subjects that completed both arms of the study.

Approximately 8.3% less alcohol was required to be consumed to achieve a blood alcohol level of 0.12% by the active glycyrrhizin/D-mannitol group than by the blank control alcohol only group for the male subjects, while no difference existed for the female subjects. This minor difference may reflect absorption of alcohol into the blood and tissues as well as the impact of the glycyrrhizin/D-mannitol on the hepatic metabolism of the alcohol in the male subjects (Hurley *et al*., 2002). In general, approximately 30% total (active plus control) less alcohol was consumed by the female participants as compared with the male participants in order to achieve a blood alcohol level of 0.12%, which is a reflection of lower body weight and blood volume as well as decreased gastric oxidation and increased alcohol bioavailability (Frezza *et al*., 1990). For each participant, the amount of alcohol required to reach a blood alcohol level of 0.12% throughout each arm of the study was relatively constant.

The strengths of this study include its placebo-controlled, double-blinded, crossover design. Furthermore, it may represent the first human clinical trial assessing the hepatoprotective properties of a dietary product in conjunction with alcohol consumption. The liver function tests used in the study are widely accepted, and the glycyrrhizin and D-mannitol that comprise the product exhibit a high degree of safety. The weaknesses of the study include the small number of subjects and the use of only a single endpoint of blood alcohol (0.12%) per night. Furthermore, the study involved healthy nonalcoholic subjects who consumed alcohol for 12 days, and as a consequence, the long term effects are not known.

The results of this study provide preliminary evidence regarding the potential protective effects of the proprietary glycyrrhizin/D-mannitol product against ethanol-induced hepatotoxicity. Larger, randomized, placebo-controlled clinical studies are required to further determine the effects of the product on liver protection during acute and chronic alcohol consumption. Such studies should adhere to the CONSORT reporting standards and ensure that appropriate methods of randomization, allocation, blinding and the handling of withdrawals are used as highlighted by Izzo *et al*. (2016).

CONCLUSIONS

The results of this study indicate that the proprietary glycyrrhizin/D-mannitol product may be hepatoprotective during alcohol consumption. Consumption of a prescribed course of alcohol for 12 days resulted in increases in the liver injury markers AST, GGT, and ALT. However, when study participants consumed the proprietary active study product in addition to an identical alcohol course, no alcohol-induced increases in these hepatic biomarkers occurred. These results suggest that consumption of the study product during alcohol consumption may support improved liver health as compared with drinking alcohol alone.

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CONFLICTS OF INTEREST

H. C. is a principal of Chigurupati Technologies, while B. J. and M. B. are employed by this company. S. J. S. serves as a consultant for Chigurupati Technologies.

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WHITE PAPER

NTX®---DNA AND TISSUE PROTECTIVE EFFECTS IN ASSOCIATION WITH ALCOHOL CONSUMPTION

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SUMMARY

Human and animal studies have demonstrated that NTX®, a proprietary combination of glycyrrhizin and D-mannitol, can protect against oxidative stress and tissue damage, including damage to DNA, associated with heavy and chronic alcohol consumption. NTX® is a unique product that can attenuate the adverse effects of alcohol while retaining the desired characteristics. This review summarizes the human clinical as well as animal and *in vitro* studies that demonstrate and support the use and beneficial effects of NTX® when used in conjunction with alcohol consumption.

INTRODUCTION

Heavy alcohol consumption is a major risk factor for numerous diseases. According to the U.S. Center for Disease Control (CDC), approximately 88,000 alcohol related deaths occur annually with alcoholic liver disease being the most commonly observed health-related issue (www.cdc.gov/alcohol/onlinetools.htm, 2015). Alcoholic liver disease is associated with liver histological lesions that range from hepatic steatosis to more advanced stages as cirrhosis, alcoholic steatohepatitis, hepatocellular carcinoma and ultimately liver failure (O'Shea et al., 2010; Kim et al., 2016). Approximately 20% of heavy alcohol drinkers develop acute alcoholic hepatitis (Mathurin & Lucey, 2012) while about 20% will develop cirrhosis within 10 years (Lierena et al., 2015).

Mechanistically, alcohol toxicity and liver disease are associated with the metabolism of alcohol to acetaldehyde and free radical species, production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), release of inflammatory cytokines as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), oxidative DNA damage and lipid metabolism as well as the formation of DNA and protein adducts with metabolites of alcohol (Qureshi et al., 2015). These events ultimately result in tissue damage leading to multi-system organ failure (Bruha et al., 2012; Lierena et al., 2015; Dhanda & Collins, 2015; Li et al., 2015; Han et al., 2016).

Biomarkers are biological indicators that are used in assessing the existence, progression and pathogenesis of physiological and toxicological events, including the effects associated with alcohol consumption. Common biomarkers of oxidative stress include the measurement of serum and tissue levels of ROS (Deshpande et al., 2014; Han et al., 2016) and derivatives of reactive oxidative metabolism (dROM) (Trotti et al., 2001), reduced glutathione (GSH) levels , lipid peroxidation products as malondialdehyde (MDA), oxidative damage to proteins as protein carbonyls (PC), and various indicators of DNA damage (Cederbaum, 2001; Wu & Cederbaum, 2003; Cooke, 2003).

Appropriate nutritional support and abstinence from alcohol are the primary approaches for successful prevention and reversal of the damaging effects of alcohol. However, the vast majority of alcohol consumers does not abstain, and cannot be realistically expected to do so. Thus, there is a pressing need to develop products and strategies to be used in conjunction with alcohol consumption to reduce the morbidity and mortality, and associated high costs to society due to lost productivity and health care.

Therefore, a new category of alcoholic beverages called Functional Spirits has been created and a product (NTX®) has been patented (US Patent 9,149,491 B1) that modulates the effects of alcohol while retaining it desired characteristics (Chigurupati et al., 2015). The primary ingredients in this product are glycyrrhizin and D-mannitol. This review summarizes the human clinical, animal and *in vitro* studies supporting the use and beneficial effects of NTX®.

MECHANISMS

Glycyrrhizin (18β-glycyrrhizin; glycyrrhizic acid; glycyrrhizinic acid) is a triterpenoid saponin glycoside ($C_{42}H_{61}0_{16}NH_4$) from the roots of *Glycyrrhiza glabra* L. (licorice), and is a primary constituent of NTX™. Glycyrrhizin is Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration (FDA). Various animal, human and *in vitro* studies have demonstrated the antioxidant, tissue-protective and anti-inflammatory properties of glycyrrhizin (Ishida et al., 2012; Gong et al., 2012, 2014; Gu et al., 2014; Hou et al.,2014; Hsiang et al., 2015; Sil et al.,

2015). In an animal study, glycyrrhizin, was shown to inhibit alcohol induced increases in the inflammatory cytokine tumor necrosis factor-α (TNF-α), as well as lipid accumulation in liver (steatosis), and the hepatoprotective ability of glycyrrhizin was confirmed histologically (Jung et al., 2016).

Glycyrrhizin has also been shown to inhibit alcohol-induced increases in the inflammatory cytokine NF-κB and increases in lipid peroxidation products as well as serum lipids, while at the same time preventing cirrhosis and liver injury in animals (Wang et al., 1999; Quan et al., 2009). Furthermore, glycyrrhizin was shown in a study in rat hippocampal-entorhinal cortex brain slice cultures to inhibit high-mobility protein B1 (HMGB1), act as a toll-like receptor 4 (TLR4) antagonist, and inhibit microglial activation, all of which are involved in blocking ethanol-induced expression of the pro-inflammatory cytokines IL-1β and TNF-α (Zou & Crews, 2014). Thus, glycyrrhizin exhibits multiple mechanisms of action in its ability to modulate the tissue damaging effects of alcohol.

D-Mannitol is the second of the two primary components of NTX®, and is a sugar alcohol found in many plants. The antioxidant and free radical scavenging abilities of D-mannitol are well known (Patel & Williamson, 2016). D-Mannitol exhibits antioxidant and tissue protective effects in acute ischemic-reperfusion injuries (Shah et al., 1996), and antioxidant and free radical scavenging properties in conjunction with cardiopulmonary bypass (England et al., 1996). D-Mannitol is used as an antioxidant and anti-inflammatory in tissue baths associated with kidney transplants (Cosetino et al., 2013).

D-Mannitol prevents DNA and tissue damage produced by a variety of free radical sources. D-Mannitol provided protection against alcohol-induced gastric mucosal damage due to its antioxidant and anti-inflammatory properties in rats (Gharzouli et al., 2001). D-Mannitol has been shown to prevent oxygen radical (Sarker et al., 1995) and photosensitized tetracycline-copper complex induced DNA strand breaks (Khan et al., 2003) in cell free systems, as well as cadmium (Hsu et al., 2013) and UV radiation induced DNA damage (Lu et al., 2004).

The above studies provide support for the results of the clinical studies whereby the combination of glycyrrhizin and D-mannitol (NTX®) provides protection against the DNA, lipid and protein damaging effects of alcohol.

HUMAN CLINICAL STUDIES

Three separate studies involving human subjects have demonstrated the protective effects of NTX® against the tissue damaging properties of heavy alcohol consumption. A randomized, placebo-controlled, double-blinded, crossover study evaluated the hepato-protective effects of

NTX® during heavy alcohol consumption (Chigurupati et al., 2016a). As per the patent, NTX® contains glycyrrhizin preferably in the range of 0.1-0.3 % and D-mannitol preferably in the range of 1.0-2.5 %.

Twelve healthy individuals (6 males; 6 females) in a clinic setting consumed vodka nightly for 12 days with the NTX® or placebo (blank control), achieving a blood alcohol level of 0.12%. Because of the crossover design, this provided an effective subject sample size of 24. The 12 day average daily vodka consumption to achieve this blood alcohol level for males was 366 mL and 245 mL for females in the blank control (alcohol only) arm of the study, and 339 mL for males and 247 ml for females in the active alcohol plus NTX® arm of the study. The amount of NTX® consumed was proportional to the amount of alcohol consumed. A four week washout was employed between the two arms of the study. No adverse effects were reported or observed regarding NTX® ingestion by any of the study participants.

The liver function enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) as well as alkaline phosphatase (ALP) and serum reduced glutathione (GSH) were measured at overnight visits 1, 6, and 12. In the alcohol only group, AST, ALT and GGT significantly increased from baseline (overnight Visit 1) to overnight visit 12. For example, AST significantly increased by 135 %, GGT increased by 44 %, while ALT increased by 62 % from baseline (overnight visit 1) to overnight visit 12. When the study subjects received NTX® in conjunction with the alcohol, no statistically significant increases were observed for AST, ALT, and GGT while ALP significantly decreased and plasma GSH decreased relative to the alcohol control group (Chigurupati et al., 2016a). The addition of NTX® provided hepato-protection as evidenced by decreases of approximately 90 % in the alcohol induced increases in AST, ALT and GGT. The results strongly indicate that consumption of the NTX® during alcohol consumption may support improved liver health compared to drinking alcohol alone.

A second randomized, double-blind, placebo-controlled, crossover study involving 50 healthy subjects evaluated the ability of NTX® to modulate alcohol-induced oxidative stress and tissue damage (Chigurupati et al., 2016b). Because of the crossover design, the effective subject sample size was 100. Alcohol consumption at 1.58 grams/kg in the form of vodka (40 % alcohol) within an hour caused elevations in serum reactive oxygen species (ROS) and derivatives of reactive oxygen metabolites (dROM), increases in lipid peroxidation and protein carbonyls, and DNA damage in peripheral lymphocytes. A seven day washout was employed between the two arms of the study.

When alcohol containing NTX® was consumed, these biomarkers of oxidative stress and tissue damage resulted in significant decreases in DNA damage as assessed by three assay techniques, and decreases in lipid and protein oxidative damage. In the presence of NTX® the alcohol

induced production of ROS decreased by 76 % ($p<0.001$) and serum protein carbonyl levels decreased by about 51 % ($p<0.022$) at the 60 minute time point. The NTX® decreased the serum levels of the alcohol-induced increases in dROM at the 120 minute time point by 66 % ($p<0.002$). With respect to alcohol-induced serum lipid peroxidation, NTX® decreased alcohol-induced increases by 41 % ($p<0.005$) and 31 % ($p<0.019$) at the 30 and 240 minute time points, respectively (Chigurupati et al., 2016b).

With respect to DNA damage, when NTX® was consumed with the alcohol as compared to alcohol consumption alone, at the 120 minute time point the peripheral lymphocyte DNA comet score decreased by 31 % ($p<0.004$) while the number of micronuclei in the peripheral lymphocytes decreased by about 27 % ($p<0.013$). Furthermore, the consumption of NTX® decreased the serum concentration of the DNA breakdown product 8-hydroxyguanosine by about 38 % at the 240 minute time point ($p<0.001$). Thus, three assay techniques demonstrated the protective effects of NTX® against alcohol induced DNA damage. No adverse effects were reported or observed with respect to NTX® consumption by any of the study participants (Chigurupati et al., 2016b).

A third human crossover, double-blinded, randomized, two-group study has assessed the effects of NTX® on alcohol-induced DNA damage (manuscript in preparation). Because of the crossover design, the effective subject sample size was 50. The study involved 13 male and 12 female subjects ages 21-50 in good general physical health. Alcohol intake was adjusted for each individual based on 1.275 grams/kg body weight for men and 1.020 grams/kg body weight for women to be drunk in the form of vodka in about one hour. NTX® consumption for the active group was proportional to the amount of alcohol consumed.

The study's primary endpoint, the comet DNA assay which measures DNA damage in eukaryotic cells, was determined at 120 minutes after the alcohol consumption in the absence and presence of NTX®. Blood alcohol concentrations were monitored at 15 minute intervals during the hour of drinking and at 20 minute intervals for the next six hours. The consumption of alcohol alone resulted in a significant increase in comet tail DNA, while a small non-significant increase in comet tail DNA occurred in the presence of NTX®. The results demonstrated that the co-consumption of NTX® with alcohol significantly decreased the extent of DNA damage as compared to consumption of alcohol alone by approximately 83 % ($p=0.02$). As with previous human studies, no adverse effects were reported or observed with respect to NTX® consumption by any of the study participants.

The mechanism for DNA damage following exposure to acute or chronic alcohol operates via production of reactive oxygen species (ROS) with a concomitant drop in the level or activity of

antioxidants, as previously observed in the above studies. This oxidative stress exerts a detrimental effect on the repair of damaged complex molecules as DNA, with potential genetic and epigenetic mutations that can increase susceptibility to a number of diseases known to occur in association with heavy and chronic alcohol consumption.

ANIMAL AND *IN VITRO* STUDIES

An extensive series of animal studies have assessed the antioxidant and tissue protective effects of NTX® as well as its individual components glycyrrhizin and D-mannitol (Chigurupati et al., 2015). The hepato-protective effects were investigated and demonstrated in rats that received 2.5 grams of alcohol orally per gram body weight for 28 days. Subacute alcohol toxicity is induced under these conditions. Glycyrrhizin and D-mannitol alone and in various combinations were used in conjunction with the alcohol, and their protective effects assessed.

For example, the results demonstrated that when glycyrrhizin/ D-mannitol were combined to produce a 3.5 % solution in aqueous alcohol which was administered to the rats as compared to alcohol alone, the glycyrrhizin/D-mannitol provided the following approximate degrees of hepato-protection as indicated by various biomarkers: 40 % protection based on decreases in ALT, AST and ALP activity levels; 60 % protection based on increases in superoxide, catalase and glutathione peroxidase activity levels; 48 % protection based on increases in hepatic GSH content; 86 % protection based on decreases in hepatic lipid peroxidation (malondialdehyde content); and 51 % protection based on decreases in the inflammatory cytokine TNF-α.

When the effects of the two individual components were assessed relative to the combination, the 3.5 % combination of glycyrrhizin/D-mannitol (NTX®) clearly provided greater hepato-protective effects than the sum of D-mannitol and glycyrrhizin alone. Thus, synergistic effects were observed with respect to these biomarkers when the two ingredients were used together where the observed effect was greater than the sum of the individual parts. The per cent synegy for the 3.5 % combination of glycyrrhizin and D-mannitol when added to the alcohol with respect to superoxide dismutase was 27 %, with respect to GSH content 38 %, with respect to hepatic lipid peroxidation (MDA) was 28 %, and with respect to TNF-α was 29 %, and with respect to ALT was 11 %. These results clearly demonstrate that the combination of glycyrrhizin and D-mannitol (NTX®) offer greater protection of tissues (synergy) than either ingredient alone or the simple sum of the effects of the two ingredients.

Other combinations of glycyrrhizin and D-mannitol were also used in conjunction with alcohol in these rat studies, and the hepato-protective effects are reported (Chigurupati et al., 2015). The studies demonstrated that selected concentrations of the two ingredients resulted in

optimal results with respect to hepato-protection, and indicated that synergistic effects can be produced.

Various *in vitro* studies have demonstrated the antioxidant and free radical scavenging ability of glycyrrhizin (Ablise et al., 2004; Gandhi et al., 2004; Cheel et al., 2010; Gong et al., 2012; Kaur et al., 2012). For example, in *in vitro* systems, glycyrrhizin has been shown to inhibit the generation of ROS, inhibit lipid peroxidation, inhibit release of the inflammatory cytokine NTF-α, and attenuate apoptosis. In cell culture experiments, glycyrrhizin has been shown to inhibit inflammatory TNF-α secretion (Ishida et al., 2012; Wang et al., 2013) and lipid peroxidation (Lecomte et al., 1994; Hu et al., 2001; Tripathi et al., 2009; Hou et al., 2014), while enhancing the antioxidant and tissue protectant GSH (Tripathi et al., 2009; Hou et al., 2014; Hsiang et al., 2015).

Various *in vitro* studies have also demonstrated the antioxidant, free radical and DNA protective effects of D-mannitol (Hall et al., 1988; Sahu & Washington, 1991; Tsou et al., 1999; Sarker et al., 1995; Kim & Kang 2005). These studies demonstrated that D-mannitol could prevent oxidative damage to DNA associated with iron, chromium (VI) and copper mediated reactive oxygen species generating systems as well as against hydrogen peroxide.

The above animal and *in vitro* studies provide extensive evidence of the free radical scavenging, antioxidant and tissue protective effects of glycyrrhizin and D-mannitol.

DISCUSSION

The results from both human and animal studies have demonstrated that NTX®, a proprietary combination of glycyrrhizin and D-mannitol, can protect against the oxidative stress and tissue damage induced by heavy alcohol consumption. The consumption of ethanol in the form of vodka over a period of approximately one hour by human subjects results in oxidative stress as indicated by various biomarkers in serum. Two human studies have shown that alcohol consumption caused oxidative DNA damage as evidenced by increased DNA comet scores, increased numbers of micronuclei, and increased serum levels of the DNA oxidation product 8-OHdG. Furthermore, alcohol alone (blank control) resulted in time dependent increases in serum reactive oxygen species (ROS), increased derivatives of reactive oxygen metabolites (dROM), increased lipid peroxidation (MDA formation), and increased oxidative protein damage. The alcohol consumption alone also increased serum levels of AST, GGT and ALT, markers of hepatic toxicity and damage.

When the alcohol was pre-mixed with NTX®, all indicators of alcohol induced oxidative stress and tissue damage were significantly modulated toward normal. The results of these studies affirm and support the results of other human and animal studies that have demonstrated individually the hepato-protective, antioxidant, and anti-inflammatory effects of the glycyrrhizin and D-mannitol. The results of the animal studies described above provided the optimal concentrations of glycyrrhizin and D-mannitol to be used, and the amounts that result in synergistic effects, amounts incorporated into NTX® (Chigurupati et al., 2015).

No differences were reported by the study participants with respect to the effects of the alcohol in the presence and absence of NTX®. The subjects were exposed to an acute amount of alcohol with resulting characteristic effects. Furthermore, no long term effects were reported by any of the subjects with respect to NTX® consumption.

In summary, the results of these studies indicate that ingestion of NTX® during alcohol consumption provides protection against alcohol-induced oxidative stress and tissue damage, including damage to DNA.

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Biographical Sketch

Dr. Sidney (Sid) J. Stohs received his B. S. Degree in Pharmacy and M. S. Degree in Pharmacognosy (Natural Medicinal Products) from the University of Nebraska and his Ph. D. Degree in Biochemistry from the University of Wisconsin. He conducted sabbatical studies and research in the Department of Forensic Medicine at Karolinska Institute in Stockholm, Sweden, and the National Institute of Environmental Health, Research Triangle Park, NC, in pharmacology, toxicology and nutrition.

Dr. Stohs served as a faculty member in the College of Pharmacy at the University of Nebraska Medical Center (UNMC) for over 22 years (1967-1989). He rose to the rank of Professor in 1974, one of the youngest in the history of the University. He served as a Department Chair (Biomedicinal Chemistry) for 18 years, and as an Assistant Dean for three years. He was involved in the development of the graduate program, extensive pharmacy curriculum revisions, and the Doctor of Pharmacy degree as the sole professional degree (third in the nation). He also held an appointment as Professor in the Eppley Cancer Institute at UNMC.

Dr. Stohs served as Dean of Pharmacy and Health Professions at Creighton University for 13 years (1990-2003) and was responsible for doctoral programs in pharmacy, physical therapy, and occupational therapy as well as programs in health services administration and emergency medical services, and clinics in pharmacy, physical therapy and occupational therapy. He also maintained a research laboratory in toxicology and nutrition funded through various federal and industrial contracts and foundation support. He was also Professor of Pharmacology in the School of Medicine at Creighton University. He is currently Dean Emeritus of the Creighton University Health Sciences Center School of Pharmacy and Health Professions, Omaha, NE.

At Creighton University, Dr. Stohs lead the development of the Doctor of Pharmacy degree as the sole professional degree, a distance learning Doctor of Pharmacy pathway for practitioners holding the B.S. degree, and the Nation's first web-based entry level Doctor of

Pharmacy pathway. He also lead the development of a M. S. in Pharmaceutical Sciences, a Pharm. D./MBA pathway, a Master of Health Services Administration program, a B.S. in Health Sciences for students in the Doctors of Pharmacy, Physical Therapy and Occupational therapy programs, and the first entry-level Doctor of Physical Therapy and Doctor of Occupational Therapy programs in the country, as well as distance learning pathways for the Doctor of Physical Therapy and Doctor of Occupational Therapy for practitioners holding the B.S. or M. S. degrees.

He served as Sr. Vice President of Research and Development (retired) for AdvoCare International, Carrollton, TX, a nutrition and wellness company (2003-2009), and was responsible for the formulation of over 25 commercial products and reformulation of over 40 products. He was responsible for research studies on a variety of AdvoCare products, and the presentation and publication of the results of these studies. He received two patents for products developed and being marketed. He served as Chair of the AdvoCare Scientific and Medical Advisory Board until 2015. During 2014 he was the Vice President for Scientific Affairs at AdvoCare.

Dr. Stohs has published over 400 peer reviewed articles in scientific and professional journals and books, and has made over 500 presentations at international, national and regional scientific and professional meetings. He was been the recipient of numerous grants and contracts for over 40 years to support his research studies.

He has received various honors and awards, including the American Academy of Pharmaceutical Sciences Research Achievement Award in Pharmacodynamics, the American Association of Colleges of Pharmacy Teacher of the Year Award, the Nebraska Pharmacists Outstanding Service Award, the Sigma Xi Outstanding Research Award, the John Doull Award in Toxicology, the Mark Bieber Industry Award from the American College of Nutrition, and was the holder of the Gilbert F. Taffe Endowed Chair in Pharmaceutical Sciences at Creighton University.

Based on his credentials, he has been elected into fellowship of the American College of Nutrition (FACN), the Academy of Toxicological Sciences (ATS), the Association of Schools of Allied Health Professions (FASAHP) and the American Pharmacists Association (FAPHA). He is also a Certified Nutrition Specialist (CNS), and is the immediate Past-President of the Certification Board for Nutrition Specialists. He has served on a wide range of boards and committees for the National Institutes of Health, Environmental Protection Agency, and the U. S. Pharmacopeial Convention, as well as national, regional and international organizations in pharmacy, nutrition, toxicology, and allied health. He serves on a number of Scientific and

Medical Advisory Boards, as well as Editorial Boards for various scientific journals. He is the past-chair of the Publications Committee for the Journal of the American College of Nutrition.

Since 2009 Dr. Stohs has served as a consultant in association with Kitsto Consulting which specializes in research design, product formulation and development, nutrition and wellness, toxicology and organizational analysis. He has written extensively with respect to book chapters, review articles, scientific research articles, and product and ingredient analysis.

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