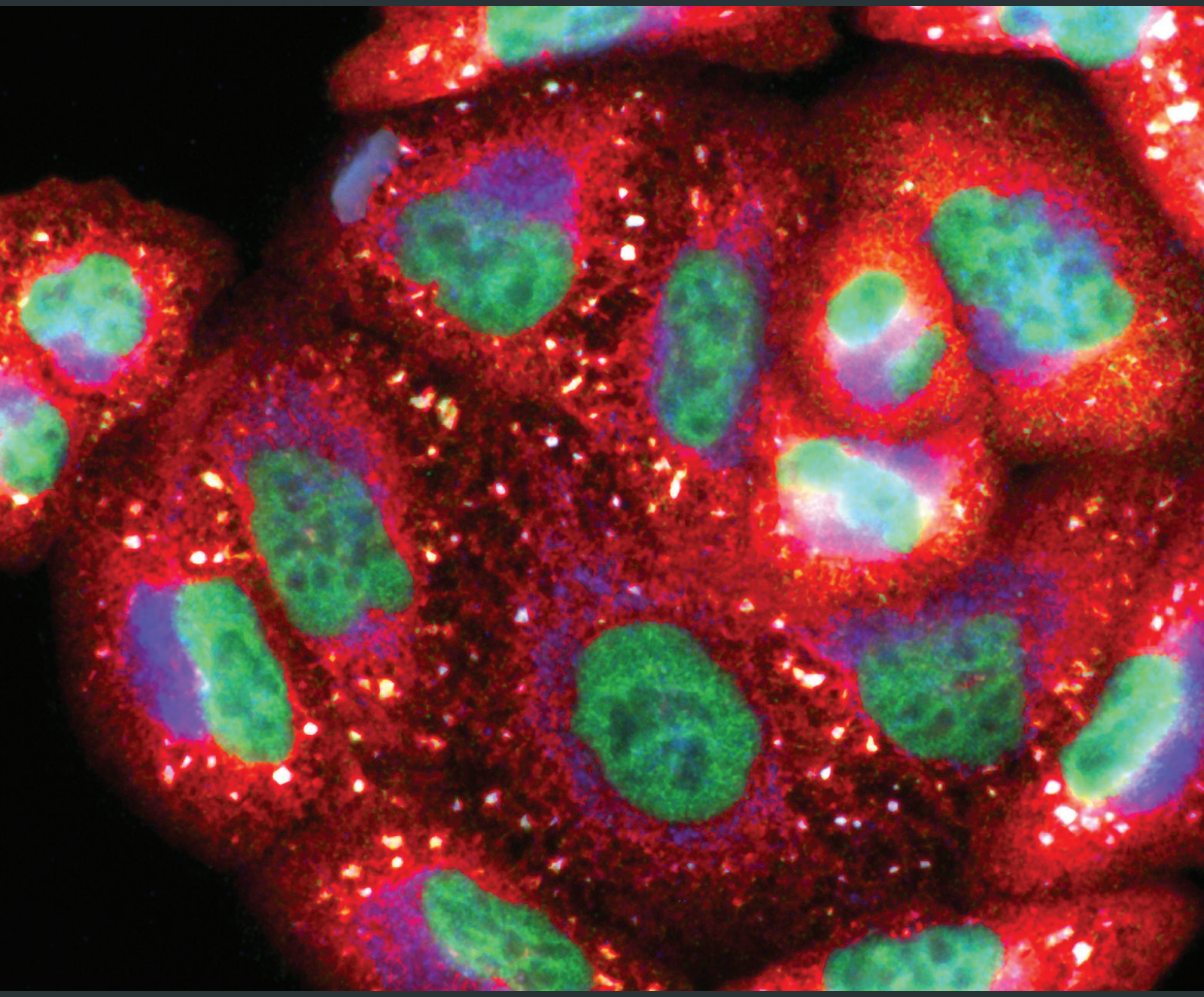
Oxidative Medicine and Cellular Longevity

Oxidative Stress in Liver Diseases: Pathogenesis, Prevention, and Therapeutics

Guest Editors: Ravirajsinh N. Jadeja, Ranjitsinh Devkar, and Srinivas Nammi



Oxidative Stress in Liver Diseases: Pathogenesis, Prevention, and Therapeutics

Oxidative Medicine and Cellular Longevity

Oxidative Stress in Liver Diseases: Pathogenesis, Prevention, and Therapeutics

Guest Editors: Ravirajsinh N. Jadeja, Ranjitsinh Devkar, and Srinivas Nammi

Editorial Board

Contents

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 8341286, 2 pages
https://doi.org/10.1155/2017/8341286

Editorial

Oxidative Stress in Liver Diseases: Pathogenesis, Prevention, and Therapeutics

Ravirajsinh N. Jadeja,[1] Ranjitsinh V. Devkar,[2] and Srinivas Nammi[3,4]

[1]*Division of Gastroenterology/Hepatology, Department of Medicine, Medical College of Georgia, Augusta University, Augusta, GA 30912, USA*
[2]*Division of Phytotherapeutics and Metabolic Endocrinology, Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat 390002, India*
[3]*School of Science and Health, Western Sydney University, Penrith, NSW 2751, Australia*
[4]*National Institute of Complementary Medicine (NICM), Western Sydney University, Penrith, NSW 2751, Australia*

Correspondence should be addressed to Ravirajsinh N. Jadeja; rjadeja@augusta.edu

Received 28 March 2017; Accepted 29 March 2017; Published 25 April 2017

Reactive oxygen species- (ROS-) induced oxidative stress has been implicated in various forms of diseases. Excessive ROS generation depletes the endogenous antioxidants that subsequently fail to counteract all the ROS leading to cellular injury. Alcohol consumption, high-calorie diet, drug overdose, environmental pollutants, heavy metals, and so forth have been implicated towards manifestation of liver injury via generation of ROS. Hepatocyte mitochondria and endoplasmic reticulum are the major site for ROS generation in various forms of liver diseases. Hence, antioxidants are frequently used to treat oxidative liver injury. Although preclinical results are promising for antioxidant therapy in treating liver diseases, evidences obtained from clinical studies are unclear. Further, detailed investigation on the mechanism of ROS-mediated hepatocyte injury and protective role of antioxidants could provide insight into pathogenesis of hepatocyte injury and open new avenues for diagnosis and development of biomarkers and pharmacological therapies. This special issue is a complication of five preclinical research articles and a review accepted from many submissions following a peer review process.

Cholesterol has emerged as a critical player in liver injury. N. Nuño-Lámbarri et al. evaluated the impact of liver cholesterol overload on the progression of the obstructive cholestasis in mice induced by bile duct ligation (BDL) surgery. The results showed that high cholesterol- (HC-) fed mice exhibited augmented oxidative stress and apoptosis along with reduced proliferation of hepatocytes. Further, mortality following BDL was higher in HC-fed mice compared to sham-operated mice. Authors concluded that hepatic cholesterol accumulation impairs hepatocyte regeneration during obstructive cholestasis and aggravates the disease with early fatal consequences. In a study by F. Li et al., aged male *Sprague Dawley* rats were exposed to treadmill training with medium intensity and preatomic analysis of liver extract was carried out. Results obtained from two-dimensional gel electrophoresis followed by MS/MS identification indicated changes in several antioxidants. The expression of several proteins involved in key liver metabolic pathways including mitochondrial sulfur, glycolysis, methionine, and protein metabolism was altered compared to that of control rats. These findings indicate that exercise may be beneficial to aged rats through modulation of hepatic protein expression profiles. A beneficial effect of n-3 polyunsaturated fatty acids (PUFAs) in a model of liver disease was evaluated by R. Feng et al. They used fat-1 transgenic mice that synthesize endogenous n-3 from n-6 PUFA. Fat-1 and their WT littermates were exposed to a modified AIN93 diet containing 10% corn oil followed by intraperitoneal injection of a single dose of carbon tetrachloride. Their results showed that severe liver injury in WT mice was remarkably ameliorated in fat-1 mice. They attributed these effects to the activation of Nrf2/keap1 pathway. These findings indicate that n-3 PUFA has potent

protective effects against acute liver injury and can be explored for other forms of liver diseases.

The fungus *Antrodia cinnamomea* (AC) is traditionally been used in China for various health benefits. A study by Y. Liu et al. not only systematically analyzed the components of fermented AC but also demonstrated its protective effect against alcohol-induced hepatocyte injury. Treatment of acute ethanol-intoxicated mice with AC extract significantly improved levels of AST and ALT activity, oxidation-related enzymes, inflammatory cytokines, and caspases. They also concluded that these effects might be via Akt/NF-κB signaling pathway. Garlic is well known for its beneficial effects against many diseases, including liver ailments. A study by F. Zhang et al. evaluated the underlying mechanism for reduced live fibrosis by diallyl trisulfide (DATS), the primary organosulfur compound in garlic. The primary rat hepatic stellate cells (HSCs) were treated with hydrogen peroxide (H_2O_2), and profibrogenic and oxidative stress parameters were evaluated. The results showed that DATS reduced fibrotic marker expression in HSCs. Further, DATS arrested cell cycle at G2/M checkpoint associated with downregulation of cyclin B1 and cyclin-dependent kinase 1, induced caspase-dependent apoptosis, and reduced migration in HSCs. Moreover, intracellular levels of reactive oxygen species and lipid peroxide were decreased by DATS. Authors concluded that DATS mediated its effects via H_2S generation. In hepatocytes, oxidative stress frequently triggers antioxidant response by activating nuclear erythroid 2-related factor 2 (Nrf2), a transcription factor, which upregulates various cytoprotective genes. Many studies have highlighted Nrf2 as a potential therapeutic target to treat liver diseases. A review article by R. N. Jadeja et al. comprehensively appraises various phytochemicals that have been assessed for their potential to halt acute and chronic liver injury by enhancing the activation of Nrf2 and have the potential for use in humans.

Acknowledgments

We express our sincere thanks and gratitude to the Editorial Board of Oxidative Medicine and Cellular Longevity for their approval on this concept and continuous help in the successful publication of this special issue. We would also like to thank the contributors of this special issue for their scientifically sound paper. With great pleasure and respect, we extend our thanks to the reviewers for the critical assessment of each paper, their constructive criticisms, and timely response that made this special issue possible.

Ravirajsinh N. Jadeja
Ranjitsinh V. Devkar
Srinivas Nammi

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 7841823, 12 pages
https://doi.org/10.1155/2017/7841823

Research Article

Hepatoprotective Effects of *Antrodia cinnamomea*: The Modulation of Oxidative Stress Signaling in a Mouse Model of Alcohol-Induced Acute Liver Injury

Yange Liu,[1] Juan Wang,[1] Lanzhou Li,[1] Wenji Hu,[1] Yidi Qu,[1] Yipei Ding,[1] Lina Meng,[1] Lirong Teng,[1,2] and Di Wang[1,2]

[1] *School of Life Sciences, Jilin University, Changchun 130012, China*
[2] *Zhuhai College of Jilin University, Jilin University, Zhuhai 519000, China*

Correspondence should be addressed to Lirong Teng; tenglr@jlu.edu.cn and Di Wang; jluwangdi@outlook.com

Received 21 October 2016; Accepted 15 January 2017; Published 27 February 2017

Academic Editor: Ranjitsinh Devkar

In the present study, the components of *A. cinnamomea* (AC) mycelia were systematically analyzed. Subsequently, its hepatoprotective effects and the underlying mechanisms were explored using a mouse model of acute alcohol-induced liver injury. AC contained 25 types of fatty acid, 16 types of amino acid, 3 types of nucleotide, and 8 types of mineral. The hepatoprotective effects were observed after 2 weeks of AC treatment at doses of 75 mg/kg, 225 mg/kg, and 675 mg/kg in the mouse model. These effects were indicated by the changes in the levels of aspartate aminotransferase, alanine aminotransferase, several oxidation-related factors, and inflammatory cytokines in serum and/or liver samples. AC reduced the incidence rate of necrosis, inflammatory infiltration, fatty droplets formation, and cell apoptosis in liver detecting via histological and TUNEL assay. In addition, AC reduced the expression of cleaved caspase-3, -8, and -9 and the levels of phosphor-protein kinase B (Akt) and phosphor-nuclear factor-κB (NF-κB) in the liver samples. Collectively, AC-mediated hepatoprotective effects in a mouse model of acute alcohol-induced liver injury are the result of reduction in oxidative stress. This may be associated with Akt/NF-κB signaling. These results provide valuable evidence to support the use of *A. cinnamomea* as a functional food and/or medicine.

1. Introduction

Alcohol metabolism and the associated oxidative stress and proinflammatory milieu in the liver can lead to hepatocellular injury [1, 2]. Alcoholic liver disease (ALD) develops in approximately 20% of alcoholics [1]. It is primarily caused by the byproducts of alcohol metabolism that promote the development of steatosis, which can lead to steatohepatitis, fibrosis, cirrhosis, and/or hepatocellular carcinoma [3]. ALD is a major health problem in the United States, accounting for 15% of the total healthcare costs, and it is associated with a mortality rate of 20% [4].

Under physiological conditions, reactive oxygen species (ROS) are efficiently eliminated by antioxidant defense systems, which involve enzymes that detoxify oxygen free radicals, such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) [5]. However, under pathological conditions, the overproduction of ROS induces apoptosis by activating proteins in the cysteine-dependent aspartate-directed protease (caspase) family and other signaling molecules such as nuclear factor-κB (NF-κB) and tumor necrosis factor-α (TNF-α) [6]. Interestingly, the overexpression of anti-inflammatory cytokines further accelerates cell damage [7].

Along with ROS, nitric oxide (NO) is involved in a wide range of toxic oxidative reactions [8]; therefore, inhibiting the release of NO from macrophages is a potential method of controlling inflammation [9, 10]. It has been demonstrated that oxidative stress is involved in the pathogenesis of ALD, as ROS generation has been observed in alcohol-exposed cultured cells and in alcohol-exposed mouse embryos [11]. It has also been shown that curcumin reduces inflammation by inhibiting NF-κB in alcohol-exposed rats [3].

The most common medications for ALD are classified into three categories: supplemental raw materials for liver cell metabolism [12], opioid receptor antagonists [12], and agents that manage and improve alcohol metabolism [13, 14]. However, drug dependence, vomiting, dermatitis, dizziness, and leukopenia have been observed in ALD patients after long-term treatment with these agents [15]. Thus, there are currently no highly satisfactory therapeutic options for ALD. Many herbs and fungi have biological effects in humans and so they have been used as functional foods and medicines for centuries. In more recent years, many of these natural products have been used as the basis for the development of new biopharmaceuticals [16]. For example, genistein ameliorates alcohol-induced liver injury by reducing oxidative stress [17].

Another example is *Antrodia cinnamomea*, which is a basidiomycete that is found throughout Taiwan. It has multiple bioactive effects and it has traditionally been used as a health food [18]. One of the most effective ways to produce *A. cinnamomea* fruit bodies is to carry out submerged fermentation with the fungus. In total, 70 compounds have been isolated from *A. cinnamomea*, including polysaccharides, diterpenes, triterpenoids, fatty acids, amino acids, and steroids [19]. *A. cinnamomea* has been shown to have hepatoprotective activity (as a result of its inhibition of free radical generation) in a rat model of liver injury [20]. In addition, it has also been shown to demonstrate hepatoprotective activity in rats with carbon tetrachloride-induced hepatotoxicity [21]. Although previous research has suggested that *A. cinnamomea* may act as a hepatoprotective agent, studies on the effects of *A. cinnamomea* mycelia (AC) on alcohol-induced liver injury (and the underlying mechanisms) remain rare.

First, we systematically analyzed the components of AC obtained from submerged fermentation. Subsequently, its hepatoprotective effects and the underlying mechanisms (related to the modulation of oxidative stress signaling) were explored in a mouse model of acute alcohol-induced hepatotoxicity. Our data provide valuable evidence to support the use of *A. cinnamomea* as a functional food and/or medicine.

2. Material and Methods

2.1. AC Culture and Sample Preparation. A. cinnamomea (ATCC 200183) was cultured in a liquid medium comprising 20 g/L glucose, 10 g/L yeast extract powder, 10 g/L tryptone, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, and 0.1 g/L vitamin B_1 with an initial pH ranging from 5.5 to 6.5. The AC were then collected and lyophilized for later use.

2.2. Measurement of the AC Components

2.2.1. Main Components. The quantities of the main AC components—total protein, total sugar, reducing sugar, crude fat, triterpenoids, flavonoids, mannitol, adenosine, and total ash—were measured. The quantities were assessed using the Kjeldahl method [22], 3,5-dinitrosalicylic acid colorimetric estimation [23], phenol-sulfuric acid determination [23], the petroleum benzine extraction method, vanillin-glacial acetic acid and perchloric acid colorimetric spectrophotometry [24], the periodate oxidation method [25], an erinitrit-aluminium trichloride assay [23], a high-performance liquid chromatography analysis [23], and the ashing method [26], respectively.

2.2.2. Amino Acids. The AC was hydrolyzed using 6 mol/L HCl at 110°C for 22 h. After vacuum drying, the samples were dissolved in 1 mL of a buffer with a pH of 2.2. A quantitative analysis of the amino acids was carried out using an automatic amino acid analyzer (L-8900, Hitachi, Japan).

2.2.3. Nucleotides. The components of the AC were extracted using double distilled water at 50°C for 3 h and they were then centrifuged at 3500 rpm for 10 min. The nucleotides were analyzed using high-performance liquid chromatography with a C18 column (4.6 mm × 250 mm; 880975-902, Agilent, USA) and a UV detector (LC-20AD, Shimadzu, Japan) at 30°C. The mobile phase consisted of 5% methanol and 95% (50 mM) NaH_2PO_4. Adenosine monophosphate and uridine monophosphate were detected at 254 nm, hypoxanthine nucleotide was detected at 250 nm, and guanosine monophosphate was detected at 280 nm [27].

2.2.4. Minerals. The AC was pretreated with hydrogen nitrate at a temperature of 110°C and an atmospheric pressure of 30 atm for 30 min. Subsequently, the levels of minerals, potassium (K), sodium (Na), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), selenium (Se), manganese (Mn), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), arsenic (As), and cadmium (Cd), were detected by inductively coupled plasma optical emission spectrometry [28].

2.2.5. Fatty Acids. The components of the AC were extracted using a ratio of ether : petroleum ether of 1 : 1 (V : V). They were then mixed with 0.5 M NaOH in a methanol solution at 60°C for 30 min. A 25% BF_3 solution was added to the samples and then they were incubated at 60°C for another 20 min. The samples were then mixed with a saturated solution of NaCl and hexane and the levels of fatty acids were analyzed using a gas chromatography-mass spectrometer (QP2010, Shimadzu, Japan) [29].

2.3. Animals and Experimental Design. The experimental protocol was approved by the Institutional Animal Ethics Committee of Jilin University. Sixty Kunming mice (8 weeks old; 18–22 g) (SCXK (JI)-2015-0047) were kept in an environmentally controlled room (at a temperature of 23°C ± 1°C and a relative humidity of 50% ± 10%) with a 12 h light-dark cycle and free access to water and food (except at night during the 2-week treatment period). The mice were acclimatized for 7 days and then they were randomly separated into six groups (with 10 mice in each group). Three of these groups were control groups (an alcohol-only group, a no-alcohol group, and a positive control group) and the other three were treated with AC.

The process of model development and drug treatment was similar to previous studies with some modification [30–32]. During the treatment period, after overnight fasting, all

the mice except for those in the no-alcohol control group were orally given 9.52 g/kg white wine (Beijing Shunxin Agricultural Co. Ltd, China) with an alcohol degree of 56° once a day at 9:00 A.M. Once a day at 4:00 P.M., the mice in the positive control group were orally treated with 63 mg/kg silymarin (Sil; Madaus AG, Germany), which is a putative hepatoprotective agent that is extracted from the seeds of *Silybum marianum*. The mice in the three AC-treated groups were orally given 75 mg/kg, 225 mg/kg, and 675 mg/kg of AC, respectively, once a day at 4:00 P.M. The mice in the alcohol-only control group were orally given an equal volume of physiological saline once a day at 4:00 P.M. and the mice in the no-alcohol control group were orally given an equal volume of physiological saline twice a day at 9:00 A.M. and 4:00 P.M. During the 2-week treatment period, the behavior and bodyweight of each mouse were monitored daily.

2.4. Collection of Serum and Liver Samples. After the last treatment, each mouse was fasted overnight and a sample of blood was taken from its caudal vein. Each mouse was then sacrificed using an injection of 200 mg/kg pentobarbital and a sample of liver tissue was immediately collected and stored at −80°C.

2.5. Biochemical Assays. The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum and the levels of SOD, GSH-Px, ROS, and NO in the livers were measured using commercial diagnostic kits purchased from the Nanjing Jiancheng Institute of Biotechnology Co. Ltd. (Nanjing, China) in accordance with the instruction manuals. In addition, the levels of TNF-α and interleukin-10 (IL-10) in the serum and livers were measured using enzyme-linked immunosorbent assay (ELISA) kits obtained from Shanghai Yuanye Bio-Technology Co. Ltd. (Shanghai, China).

2.6. Histological Evaluation and TUNEL Assay. Following as previous description [33], liver tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, dehydrated in graded alcohol, and embedded in paraffin and 5 μm sections were prepared. Sections were stained with hematoxylin and eosin (H&E) for histological evaluation. All stained slides were visualized using an IX73 inverted microscope (40x; Olympus, Japan)

Cell apoptosis in liver tissues was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) kit (Life Technologies, USA) following the manufacturer's protocol. The changes of green fluorescence were determined by a fluorescent microscope (20x; CCD camera, TE2000, Nikon, Japan).

2.7. Western Blotting. For each mouse, a portion of the liver sample was homogenized in a lysis buffer, 1% of which consisted of a protease inhibitor cocktail (Sigma-Aldrich, USA), 2% of which was phenylmethanesulfonyl fluoride (Sigma-Aldrich, USA), and 97% of which consisted of the components of a radio-immunoprecipitation assay (Sigma-Aldrich, USA). The total protein concentration was measured using a bicinchoninic acid protein assay kit (Merck Millipore, Germany).

Table 1: Main components of AC.

Compounds	Contents (%)
Total sugar	11.74
Reducing sugar	2.20
Triterpenoids	8.05
Flavonoids	0.35
Mannitol	5.37
Crude fat	30.01
Total ash	6.00
Total protein	30.60
Adenosine	0.16

AC: *A. cinnamomea* mycelia.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins in 40 μg of each liver sample. The SDS-PAGE was carried out using 12% polyacrylamide gel slabs and minivertical electrophoresis equipment (Bio-Rad, USA) and the proteins were electrotransferred onto 0.45 μm nitrocellulose membranes (Millipore, USA). The membranes were blocked using 5% bovine serum albumin (BSA)/tris-buffered saline (TBS) at room temperature for 4 h. The blocked membranes were incubated in a 1000–2000-fold diluted solution of primary antibodies against phosphor (P)-Akt (07-1398; Merck Millipore, Germany), total (T)-Akt (ab131443), P-NF-κB (ab25901), T-NF-κB (ab7970), cleaved caspase-3 (ab13847; Abcam, USA), cleaved caspase-8 (ab25901), cleaved caspase-9 (ab25758), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-25778; Santa Cruz Biotechnology, USA) at 4°C overnight.

Each membrane was washed five times with TBS plus Tween 20 and 5% BSA and it was then incubated with a 1500-fold diluted horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (sc-3836; Santa Cruz Biotechnology, USA) at 4°C for 4 h. The proteins were visualized using a gel imaging system (UVP, USA). The intensity of each band was quantified using densitometric scanning with ImageJ software (National Institutes of Health, USA).

2.8. Statistical Analysis. The data were analyzed using SPSS 16.0 software (IBM corporation, USA). The results were presented as means ± standard errors of the mean (SEMs) and the statistical significance of each difference was determined using a one-way analysis of variance (ANOVA) followed by Dunn's test. P values of < 0.05 were considered to indicate statistically significant differences.

3. Results

3.1. Composition of AC. Of the constituents of AC, 11.7% was total sugar, 2.2% was reducing sugar, 8.05% was triterpenoids, 0.35% was flavonoids, 5.4% was mannitol, 30.01% was crude fat, 30.6% was total protein, and 0.16% was adenosine (Table 1).

A total of 35 types of fatty acid were detected but octanoic acid, tridecanoic acid, myristoleic acid, pentadecenoic acid, elaidic acid, linoleic acid, α-linolenic acid, docosadienoic

Table 2: The composition and percentage content of fatty acids.

Compounds	Contents (‰)
Octanoic acid (C8:0)	ND①
Capric acid (C10:0)	0.014
Undecanoic acid (C11:0)	0.052
Lauric acid (C12:0)	0.170
Tridecanoic acid (C13:0)	ND①
Myristic acid (C14:0)	6.541
Myristoleic acid (C14:1n5)	ND①
Pentadecanoic acid (C15:0)	1.616
Pentadecenoic acid (C15:1n5)	ND①
Hexadecanoic acid (C16:0)	66.599
Palmitoleic acid (C16:1n7)	0.300
Heptadecanoic acid (C17:0)	2.586
Heptadecenoic acid (C17:1n7)	1.704
Stearic acid (C18:0)	31.015
Elaidic acid (C18:1n9t)	ND①
Oleic acid (C18:1n9)	59.524
Translinoleic acid (C18:2n6t)	132.489
Linoleic acid (C18:2n6c)	ND①
Arachidic acid (C20:0)	0.535
γ-Linolenic acid (C18:3n6)	0.091
Paullinic acid (C20:1)	0.826
α-linolenic acid (C18:3n3)	ND①
Heneicosanoic acid (C21:0)	0.065
Eicosadienoic acid (C20:2)	0.642
Docosanoic acid (C22:0)	1.099
Dihomo-gamma-linolenic acid (C20:3n6)	0.018
Erucic acid (C22:1n9)	0.322
Eicosatrienoic acid (C20:3n3)	0.464
Arachidonic acid (C20:4n6)	0.098
Tricosanoic acid (C23:0)	0.091
Docosadienoic acid (C22:2n6)	ND①
Tetracosanoic acid (C24:0)	3.624
Eicosapentaenoic acid (C20:5n3)	0.303
Nervonic acid (C24:1n9)	ND①
Docosahexaenoic acid (C22:6n3)	ND①

①ND: not detected (the detection limit was 0.05 mg/kg).

Table 3: Percentage composition of amino acids in AC.

Compounds	Contents (%)
Aspartic acid (Asp)	2.86
L-Threonine (Thr)	1.53
Serine (Ser)	1.51
Glutamic acid (Glu)	4.44
Glycine (Gly)	2.25
Alanine (Ala)	2.34
Valine (Val)	1.66
DL-Methionine (Met)	1.01
Isoleucine (Iso)	1.23
Leucine (Leu)	2.42
Tyrosine (Tyr)	1.05
Phenylalanine (Phe)	1.38
Lysine (Lys)	2.3
Histidine (His)	0.89
Arginine (Arg)	3.19
Proline (Pro)	1.37

AC: *A. cinnamomea* mycelia.

Table 4: Percentage composition of nucleotides and minerals (including heavy metals) in AC.

Compounds	Contents
(‰)	
Kalium (K)	77.16
Natrium (Na)	21.29
Calcium (Ca)	5.82
Magnesium (Mg)	16.75
Ferrum (Fe)	0.67
Zinc (Zn)	0.62
Selenium (Se)	ND②
Manganese (Mn)	0.06
Chromium (Cr)	0.10
(mg/kg)	
Cuprum (Cu)	6.63
Lead (Pb)	ND③
Mercury (Hg)	ND④
Arsenic (As)	ND⑤
Cadmium (Cd)	ND⑤
Guanylic acid (GMP)	70000
Uridylic acid (UMP)	460.2
Adenylic acid (AMP)	1142.1

AC: *A. cinnamomea* mycelia.
②ND: not detected (the detection limit was 5 mg/kg).
③ND: not detected (the detection limit was 2 mg/kg).
④ND: not detected (the detection limit was 3 mg/kg).
⑤ND: not detected (the detection limit was 1 mg/kg).

acid, nervonic acid, and docosahexaenoic acid were not detected (Table 2). In addition, 16 types of amino acid were detected; the three most common ones were glutamic acid, arginine, and aspartic acid (Table 3). Furthermore, eight minerals and three nucleotides were observed in the following proportions: 77.2‰ K, 21.3‰ Na, 5.8‰ Ca, 16.8‰ Mg, 0.7‰ Fe, 0.6‰ Zn, 0.06‰ Mn, 0.1‰ Cr, 70000 mg/kg guanylic acid (GMP), 460.2 mg/kg uridylic acid (UMP), and 1142.1 mg/kg adenylic acid (AMP) (Table 4). Regarding the heavy metals, Pb, Hg, As, and Cd were not detected, and Cu was detected at a concentration of less than 20 parts per million, which is much lower than the official safety limits for humans (Table 4).

3.2. Hepatoprotective Effects of AC. During the 2-week treatment period, the bodyweight of the no-alcohol control mice

Table 5: Effects of two-week AC treatment on the bodyweight of mice with acute alcohol-induced liver injury.

Days	CTRL	Model	Sil (mg/kg)	AC (mg/kg)		
			63	75	225	675
1st day	25.06 ± 0.51	25.87 ± 0.38	25.09 ± 0.39	25.90 ± 0.43	25.69 ± 0.29	26.30 ± 0.19
4th day	29.57 ± 0.53	27.55 ± 0.81	28.43 ± 0.61	29.77 ± 0.56	29.34 ± 0.52	28.31 ± 0.63
7th day	33.00 ± 0.58	27.77 ± 0.63$^{\#}$	29.04 ± 1.31	28.98 ± 0.70	29.85 ± 0.64*	29.06 ± 0.59
10th day	33.90 ± 0.50	26.14 ± 0.94$^{\#\#}$	28.11 ± 1.00	29.15 ± 1.14*	30.11 ± 0.78**	29.00 ± 0.99*
13th day	36.11 ± 0.36	27.22 ± 0.88$^{\#\#\#}$	29.53 ± 0.95	31.06 ± 1.02*	29.94 ± 0.76*	29.40 ± 0.91

The data were analyzed using a one-way ANOVA and they are expressed as means SEMs ($n = 10$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ in a comparison with the no-alcohol control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ in a comparison with the alcohol-only control group. AC: *A. cinnamomea* mycelia; Sil: silymarin.

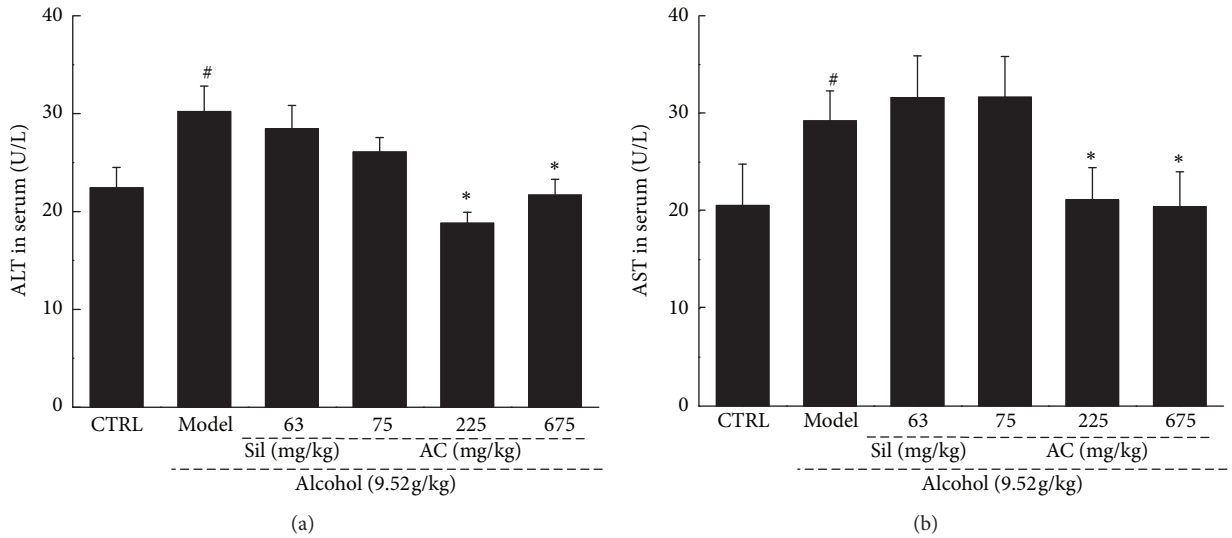


Figure 1: Two-week AC and Sil treatment reduced the levels of (a) ALT and (b) AST in the serum of mice with acute alcohol-induced liver injury. The data were analyzed using a one-way ANOVA and they are expressed as means SEMs ($n = 10$). $^{\#}P < 0.05$ in a comparison with the no-alcohol control group; $^{*}P < 0.05$ in a comparison with the alcohol-only control group. AC: *A. cinnamomea* mycelia; Sil: silymarin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ANOVA: analysis of variance; SEM: standard error of the mean.

increased by 44.1% (Table 5). In contrast, 2 weeks of alcohol consumption caused a 24.6% decrease in the bodyweight of the alcohol-only control mice, which began on the seventh day ($P < 0.001$; Table 5). Unlike the mice in the Sil-treated group, those in the AC-treated groups had bodyweight increases of up to 10% (beginning on the tenth day) compared to those in the alcohol-only control group ($P < 0.05$; Table 5).

The levels of ALT and AST activity in the serum, which are biochemical markers for assessing early-stage liver injuries, were examined to explore the effect of AC on acute alcohol-induced liver injury [34]. Compared to the no-alcohol control mice, the alcohol-only mice had strikingly increased levels of ALT and AST ($P < 0.05$; Figure 1), which were suppressed back to their normal levels by AC treatment at doses of 225 mg/kg and 675 mg/kg ($P < 0.05$; Figure 1).

3.3. Antioxidative Effects of AC. ROS and NO levels can be used as markers of peroxidation and inflammation [9]. To assess the effects of AC on acute alcohol-induced hepatic oxidative stress, the levels of ROS and NO and the activities of SOD and GSH-Px were assessed. Extremely high ROS and NO levels and low SOD and GSH-Px activities were noted in the liver samples of mice with alcohol-induced hepatotoxicity ($P < 0.05$; Figure 2). Treatment with AC reversed these pathological changes and it even improved the levels of ROS, NO, GSH-Px activity, and SOD activity in the livers of the alcohol-only mice compared to those of the no-alcohol control mice ($P < 0.05$; Figure 2). In comparison, Sil only had beneficial effects on the levels of NO, GSH-Px activity, and SOD activity (not ROS) in the livers of alcohol-exposed mice ($P < 0.05$; Figure 2). These results demonstrated that AC may protect against alcohol-induced hepatic oxidative stress.

3.4. Effects of AC on Inflammatory Cytokines. To prove whether AC has anti-inflammatory effects, the levels of two important cytokines, IL-10 and TNF-α, in the serum and liver samples were assessed.

Acute alcohol exposure induced a dramatic increase in TNF-α levels in the serum and liver samples, and these increased levels were significantly suppressed by 2 weeks of treatment with AC ($P < 0.05$; Figures 3(a) and 3(b)). However, Sil only suppressed the increased levels of TNF-α in the liver samples and not in the serum samples ($P < 0.05$; Figure 3(b)).

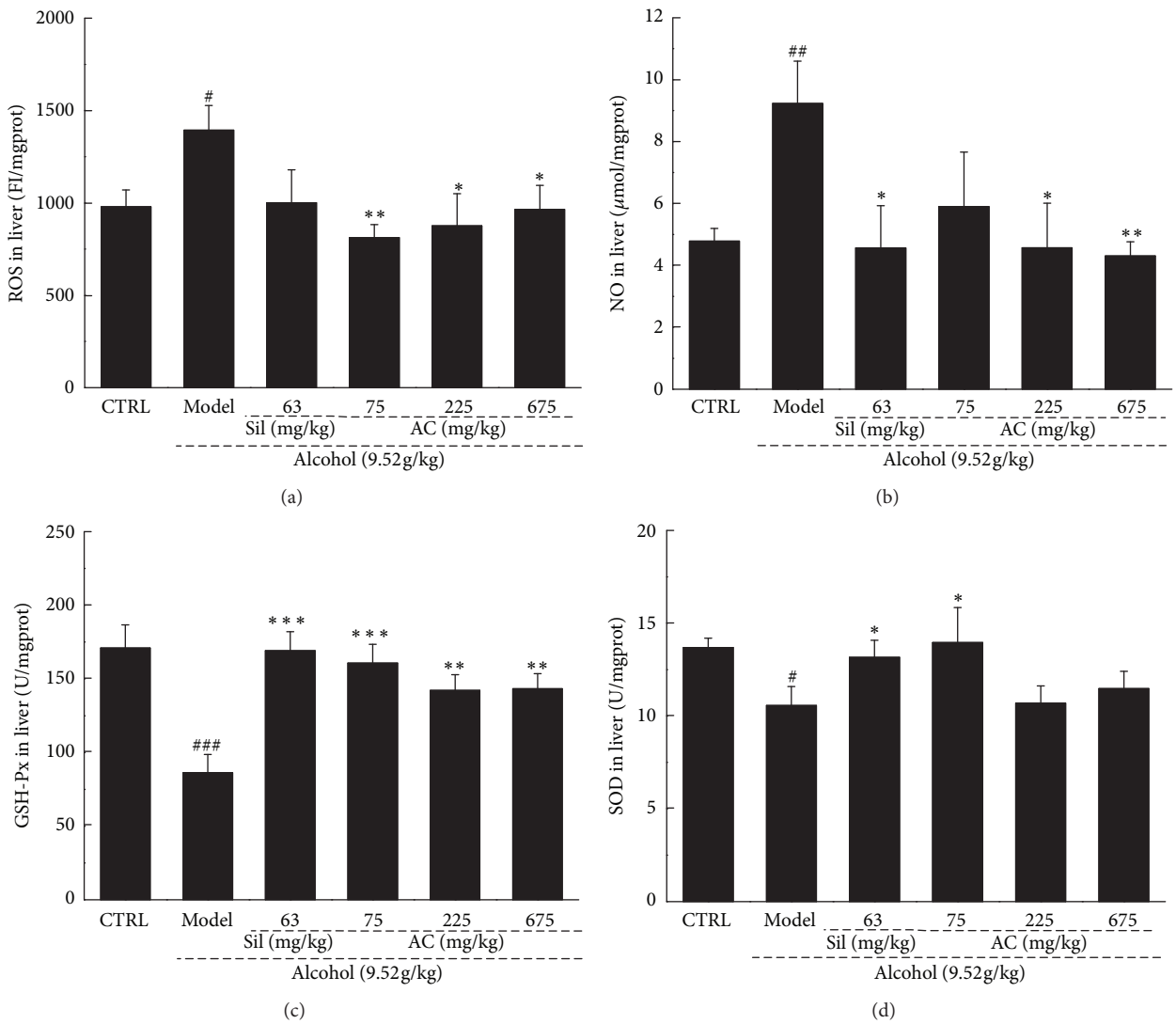


Figure 2: Two-week AC and Sil treatments affected the levels of (a) ROS, (b) NO, (c) GSH-Px, and (d) SOD in the livers of mice with acute alcohol-induced liver injury. The data were analyzed using a one-way ANOVA and they are expressed as means SEMs ($n = 10$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ in a comparison with the no-alcohol control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in a comparison with the alcohol-only control group. AC: *A. cinnamomea* mycelia; Sil: silymarin; ANOVA: analysis of variance; GSH-Px: glutathione peroxidase; NO: nitric oxide; ROS: reactive oxygen species; SOD: superoxide dismutase; SEM: standard error of the mean.

Acute alcohol exposure significantly suppressed the levels of IL-10 in both the serum and liver samples ($P < 0.05$; Figures 3(c) and 3(d)). Treatment with AC increased the IL-10 levels in a dose-dependent manner in the serum and liver samples of mice with alcohol-induced liver damage compared with the alcohol-only control group, at doses ranging from 75 mg/kg to 675 mg/kg ($P < 0.05$; Figures 3(c) and 3(d)). However, treatment with Sil for 2 weeks failed to influence the IL-10 levels in the alcohol-exposed mice (Figures 3(c) and 3(d)).

3.5. Effect of AC on Histopathological Changes and Alcohol-Induced Apoptosis. H&E stain is the most fundamental and universal method for the histologic and pathologic examination [35]. Compared with control mice, alcohol-induced typical pathological characteristics in liver including necrosis, inflammatory infiltration of lymphocytes, and an increased number of fat droplets were noted (Figure 4(a)). In contrast, AC and Sil exhibited significant liver protection indicated by the reduced occurrence rate of necrosis, inflammatory infiltration, and fat droplets in liver of treated mice (Figure 4(a)).

TUNEL assay is performed to evaluate cell apoptosis condition in liver tissues. Large apoptotic cells with high intensity green fluorescence were noted in liver of alcohol-exposed mice (Figure 4(b)). Compared with model group, low cell apoptosis rate was observed in liver of AC- and Sil-treated mice (Figure 4(b)).

3.6. Effects of AC on the Activation of Akt and NF-κB. NF-κB activation is involved in the positive regulation of TNF-α

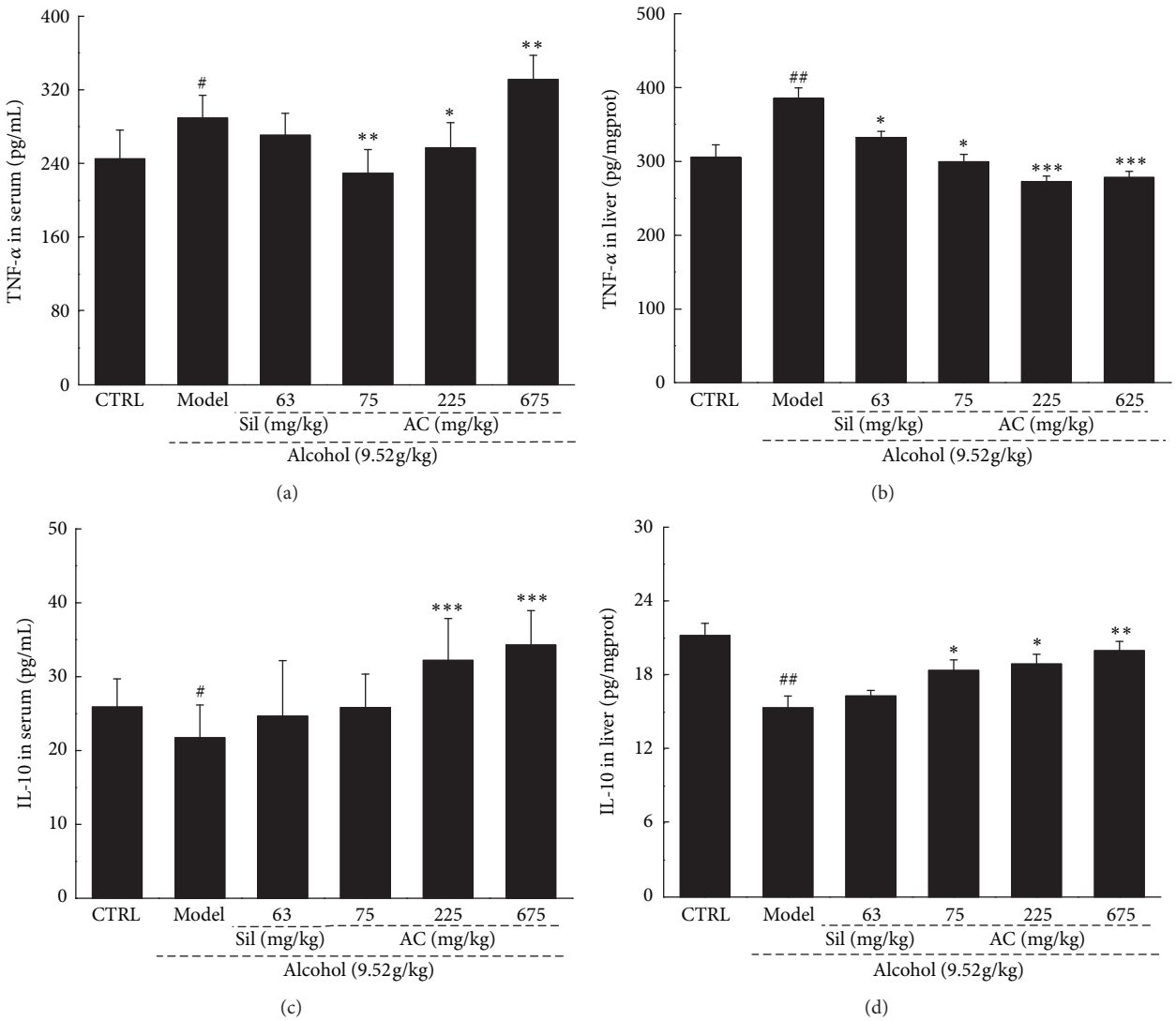


Figure 3: Two-week AC and Sil treatment (a and b) reduced the levels of TNF-α and (c and d) increased the levels of IL-10 in the serum and liver of mice with acute alcohol-induced liver injury. The data were analyzed using a one-way ANOVA and they are expressed as means SEMs ($n = 10$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ in a comparison with the no-alcohol control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ in a comparison with the alcohol-only control group. AC: *A. cinnamomea* mycelia; Sil: silymarin; ANOVA: analysis of variance; SEM: standard error of the mean; IL-10: interleukin-10; TNF-α: tumor necrosis factor-α.

stimuli, and Akt is an important upstream activator in the inflammatory response involving NF-κB and the generation of ROS [36]. The phosphorylation of both NF-κB and Akt was remarkably increased in mice with alcohol-induced liver damage compared to the no-alcohol mice ($P < 0.01$; Figure 5). Unlike Sil, AC at doses from 75 mg/kg to 675 mg/kg resulted in a significant reduction in the expression of both phosphor-Akt and phosphor-NF-κB in the livers of the alcohol-exposed mice ($P < 0.05$; Figure 5).

3.7. Effects of AC on the Expression of Caspases. Oxidative stress is known to activate caspases which, in turn, cause cell damage [37]. Thus, to understand the mechanisms underlying the protective effect of AC against alcohol-induced oxidative stress in the liver, we assessed the expression of cleaved caspases-3, -8, and -9 in the liver samples. The expression of each of the three cleaved caspases was greatly increased in the livers of the alcohol-only mice compared to those of the no-alcohol mice ($P < 0.01$; Figure 6). Sil only reduced the expressions of cleaved caspases-3 (not cleaved caspases-8 and -9) in the alcohol-exposed mice ($P < 0.05$; Figure 6). Unlike Sil, treating with all doses of AC, there was significant depression in protein expression of cleaved caspase-3, -8, and -9 ($P < 0.05$; Figure 6). The effect of AC on caspases may be involved in its hepatoprotective effects against oxidative stress.

4. Discussion

We confirmed the hepatoprotective effects of AC in mice with acute alcohol-induced hepatotoxicity and clarified the underlying mechanisms of action, which are associated with

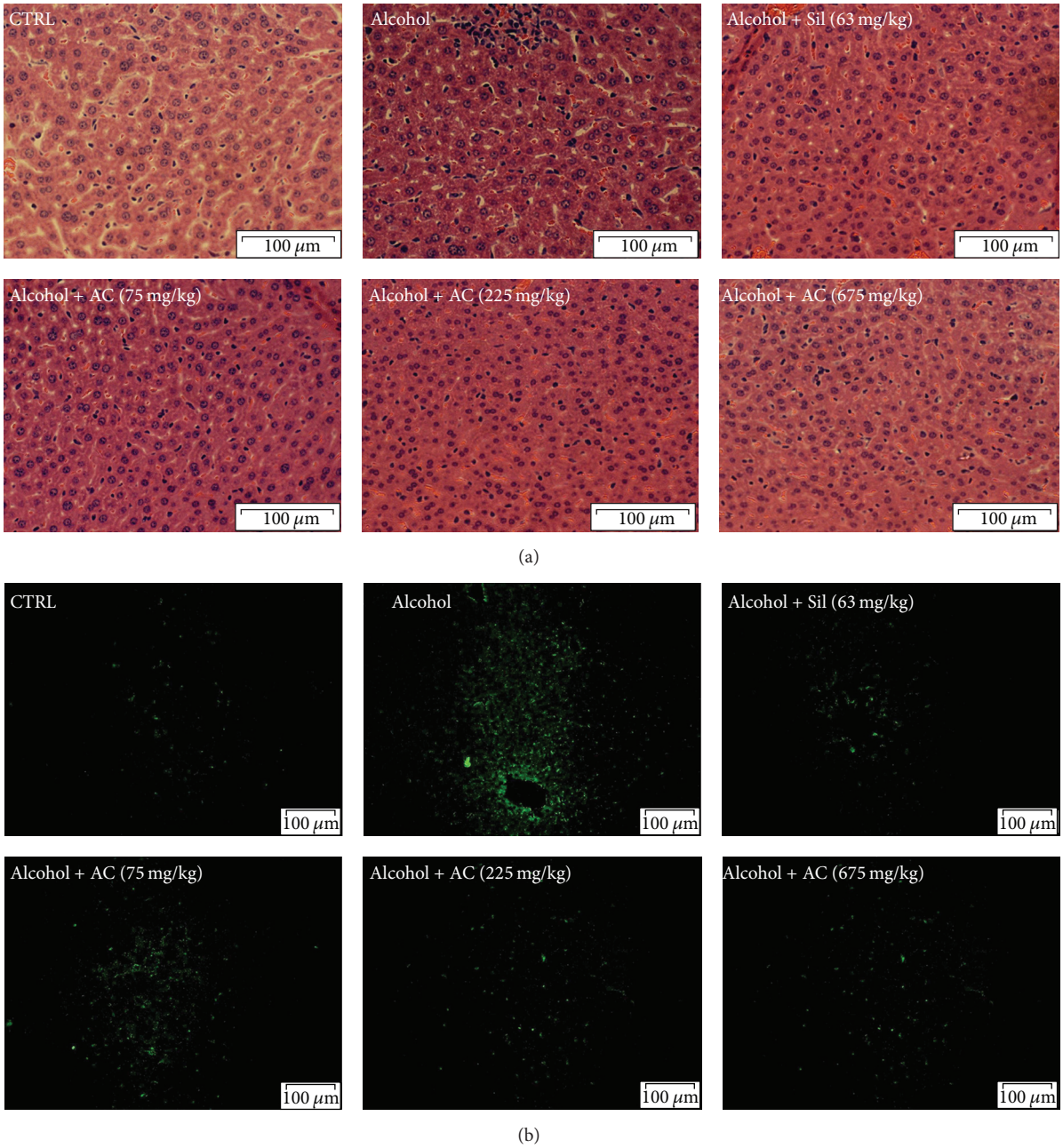


Figure 4: (a) Histopathological analysis in liver shown by H&E staining (scale bar: 100 μm; magnification: 40x). (b) Apoptosis rate detection shown by TUNEL-positive cells with green fluorescence (scale bar: 100 μm; magnification: 20x). AC: *A. cinnamomea* mycelia; Sil: silymarin; H&E: Hematoxylin and eosin; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

the modulation of oxidative stress signaling. In contrast to other putative hepatoprotective agents (such as Sil), which are often extracted from plants, *A. cinnamomea* is an edible fungus that contains pharmacologically active ingredients [38]. In this study, 25 types of fatty acid, 16 types of amino acid, 3 types of nucleotide, and 8 types of mineral were detected in AC obtained from submerged fermentation.

Terpenoids, such as oleanolic acid, can increase the glutathione content of the liver, suppress lipid peroxidation, eliminate oxygen free radicals, and stimulate endogenous liver regeneration [39]. Flavonoids have protective effects against nonalcoholic steatohepatitis [40]. Our ongoing experiments focus on the hepatoprotective effects of purified terpenoids and flavonoids in alcohol-exposed mouse models similar to the one used in this study. The levels of the fatty acids oleic acid and translinoleic acid, which help to soften blood vessels and cannot be synthesized by the human body, were very high in the AC [29]. Furthermore, eicosapentaenoic acid, which was also detected in the AC, can reduce inflammatory immune responses and cure autoimmune diseases [41]. Together, these findings suggest that AC contains multiple active ingredients that can target various processes

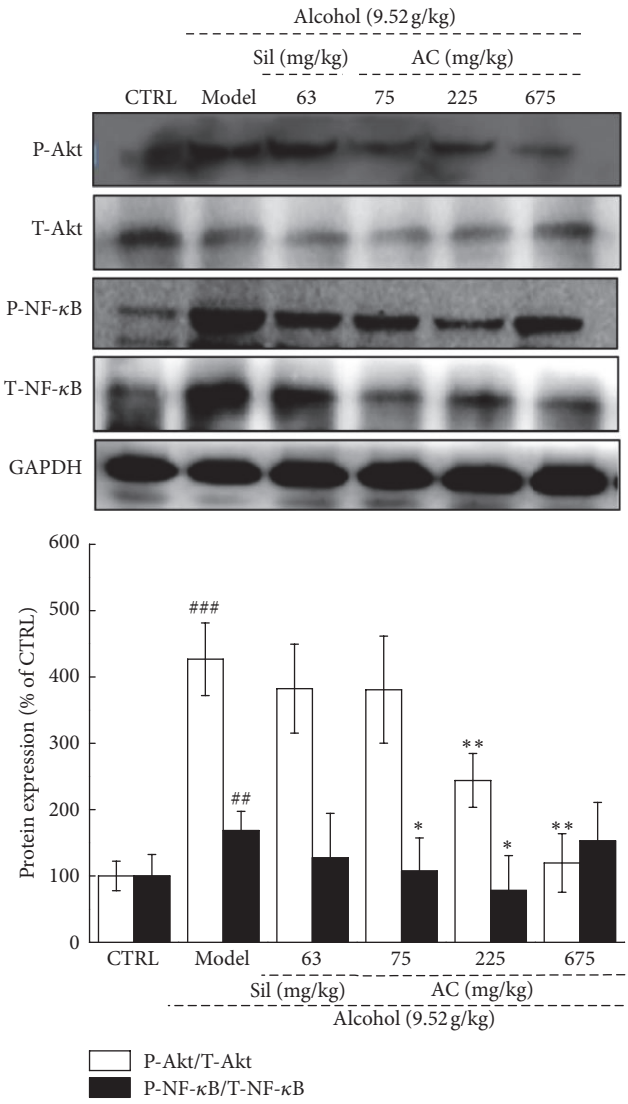


Figure 5: Two-week AC and Sil treatment suppressed the levels of phosphor-NF-κB and phosphor-Akt in the livers of mice with acute alcohol-induced liver injury. The data on quantified protein expression were normalized to the levels of GAPDH. The data were analyzed using a one-way ANOVA and they are expressed as means SEMs ($n = 10$). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ in a comparison with the no-alcohol control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ in a comparison with the alcohol-only control group. AC: *A. cinnamomea* mycelia; Sil: silymarin; Akt: protein kinase B; ANOVA: analysis of variance; SEM: standard error of the mean; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; NF-κB: nuclear factor-κB.

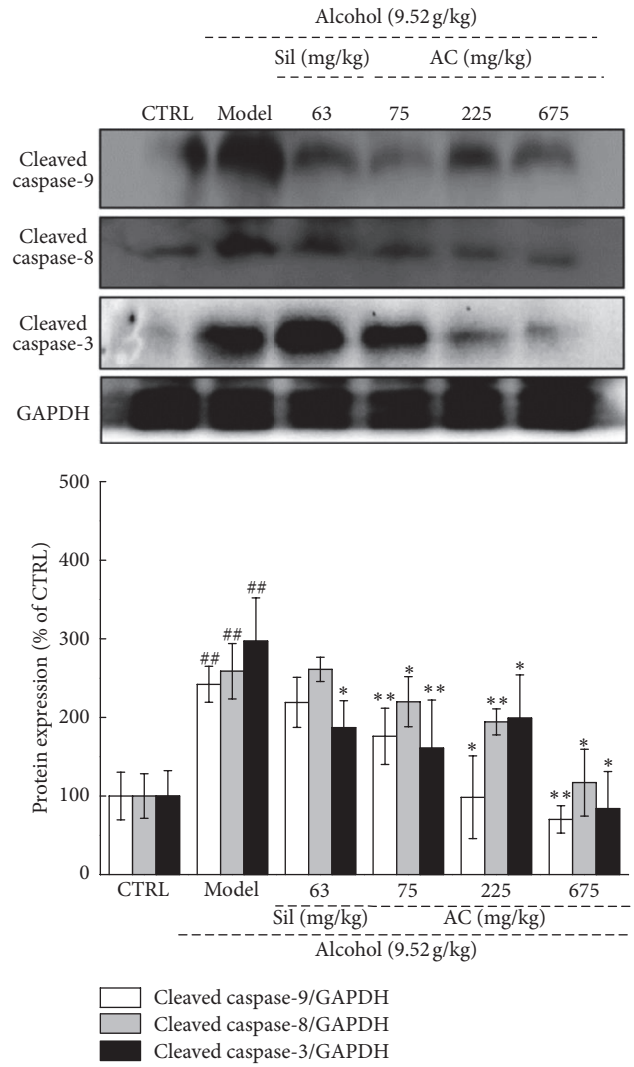


Figure 6: Two-week AC and Sil treatments suppressed the expression of cleaved caspase-3, -8, and -9 in the livers of mice with acute alcohol-induced liver injury. The data on quantified protein expression were normalized to the levels of GAPDH. The data were analyzed using a one-way ANOVA and they are expressed as means SEMs ($n = 10$). $^{\#\#}P < 0.01$ in a comparison with the no-alcohol control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ in a comparison with the alcohol-only control group. AC: *A. cinnamomea* mycelia; Sil: silymarin; ANOVA: analysis of variance; SEM: standard error of the mean; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

in the liver and can thereby prevent alcohol-induced liver damage. The presence of multiple active ingredients in AC may explain the fact that there was no dose-dependent response in our experiment. The absence of a dose-dependent response is a common feature of pharmaceutically active natural products [42].

The safety of *A. cinnamomea* has been verified by its use as a traditional functional food over several centuries. According to our data, many nonheavy metals were detected in AC. However, the heavy metals Pb, Hg, As, and Cd were not detected and Cu was present at a concentration of less than 20 parts per million, which further indicates the safety of AC. Encouragingly, in our study, the AC had no effect on the behavior of the mice (data not shown).

ALD is one of the most serious complications of heavy drinking. Liver serves as the main target organ of alcohol metabolism. Alcohol can cause several types of liver damage, including alcoholic hepatic steatosis, alcoholic hepatitis, fibrosis, and cirrhosis [43]. Severe liver damage occurred as a result of 2 weeks of alcohol administration, which was

highlighted by large increases in the levels of AST and ALT activity, prooxidation enzymes, inflammatory cytokines, and caspases. These increased levels were all greatly decreased by AC. Additionally, the observed necrosis accumulation, inflammatory infiltration, fatty droplets formation, and cell apoptosis in liver of model mice provided clear evidence for alcohol-induced liver damage. The hepatoprotective effects of AC against alcohol-induced damage have been directly certified by reducing necrosis, inflammatory infiltration, fatty droplets formation, and apoptosis rate in liver.

Oxidative stress is a crucial causal factor of acute alcohol-induced liver injury. This is especially the case when the liver has lower levels of antioxidant protection to cope with the generation of ROS [44], which react with macromolecules including deoxyribonucleic acid (DNA), proteins, and the components of cellular membranes [45]. NO, another factor that is involved in oxidative stress, induces the formation of hydroxyl radicals, which are involved in alcohol-induced liver damage [46]. Targeting oxidative stress may prevent alcohol-induced liver injury.

AC not only suppressed the overproduction of ROS and NO, but also enhanced the activities of SOD and GSH-Px. The restoration of SOD and GSH-Px activity ameliorates alcohol-induced hepatotoxicity by reducing oxidative stress. A high level of SOD activity in liver cells diminishes oxygen free radicals and thereby reduces alcohol-induced hepatotoxicity [47]. Our data on a mouse model of alcohol-induced liver damage suggest that the antioxidative activities of AC are involved in its hepatoprotective effect.

Evidence has indicated that alcohol ingestion activates the innate immune system by changing the levels of inflammatory cytokines [48]. As a representative inflammatory cytokine with pleiotropic functions, TNF-α is closely associated with the progression of many inflammatory disorders [49]. Alcohol stimulates the production of TNF-α, and IL-10, a potent anti-inflammatory cytokine, can inhibit the production of TNF-α [50]. Mice injected with high doses of TNF can develop hepatocellular dysfunction with an elevation of serum levels of ALT and AST [51]. Previous studies have shown that oxidative stress leads to chronic inflammation [52]. However, during an inflammatory reaction, an increased oxygen uptake at the site of injury leads to a "respiratory burst" which, in turn, leads to an increased production and accumulation of ROS [53]. The generation of ROS is inhibited by TNF-α, which mediates a mild uncoupling of the mitochondrial respiratory chain in liver cells. The modulation of inflammatory cytokines in a mouse model of alcohol-induced liver damage by antioxidation factors therefore plays an important role in AC-mediated hepatoprotection.

The data revealed that AC strongly inhibits the phosphorylation of NF-κB by inhibiting the phosphorylation of Akt, which controls NF-κB activation via the activation of the IκB kinase complex [54]. NF-κB, a central transcription mediator, regulates the generation of inflammatory cytokines. The suppression of NF-κB activation greatly decreases the levels of proinflammatory cytokines [55]. According to previous findings, ROS serves as a secondary messenger molecule between NF-κB activation and TNF production [56]. TNF-α can influence cell activity by activating caspase-8 and -10 [57]. Moreover, ROS activates caspase-3, -8, and -9, which are cysteine-dependent proteases that mediate cell death [58]. Caspase-9 directly influences caspase-3 activation, usually by interacting with caspase-8 [58]. All the data suggest that the regulation of Akt/NF-κB signaling (which is normally induced by oxidative stress) plays a central role in the hepatoprotective effect of AC against acute alcohol-induced liver damage.

In conclusion, AC, a nutritious natural product, has significant hepatoprotective effects on acute alcohol-induced hepatotoxicity in mice. It does this by regulating the levels of AST and ALT activity, oxidation-related enzymes, inflammatory cytokines, caspases; it may therefore influence Akt/NF-κB signaling. Although the study may not provide sufficient evidence on the hepatoprotective effects of AC to support the use of AC as a medicine, it does provide evidence to support the use of AC as a functional natural product that can protect against ALD.

Ethical Approval

The experimental animal protocol was approved by the Animal Ethics Committee of Jilin University.

Competing Interests

The authors have declared that there is no conflict of interests.

Acknowledgments

This work was supported by Science and Technology Key Project in Jilin Province of China (Grants nos. 20140311072YY, 20150203002NY, and 20160204029YY).

References

[1] B. Bakhautdin, D. Das, P. Mandal et al., "Protective role of HO-1 and carbon monoxide in ethanol-induced hepatocyte cell death and liver injury in mice," *Journal of Hepatology*, vol. 61, no. 5, pp. 1029–1037, 2014.

[2] H.-W. Chiu and K.-F. Hua, "Hepatoprotective effect of wheat-based solid-state fermented *antrodia cinnamomea* in carbon tetrachloride-induced liver injury in rat," *PLOS ONE*, vol. 11, no. 4, Article ID e0153087, 2016.

[3] H.-I. Lee, R. A. McGregor, M.-S. Choi et al., "Low doses of curcumin protect alcohol-induced liver damage by modulation of the alcohol metabolic pathway, CYP2E1 and AMPK," *Life Sciences*, vol. 93, no. 18-19, pp. 693–699, 2013.

[4] Y. Tang, L. Zhang, C. B. Forsyth, M. Shaikh, S. Song, and A. Keshavarzian, "The role of miR-212 and iNOS in alcohol-induced intestinal barrier dysfunction and steatohepatitis," *Alcoholism: Clinical and Experimental Research*, vol. 39, no. 9, pp. 1632–1641, 2015.

[5] C. Dai, D. Li, L. Gong, X. Xiao, and S. Tang, "Curcumin ameliorates furazolidone-induced DNA damage and apoptosis in human hepatocyte L02 cells by inhibiting ROS production and mitochondrial pathway," *Molecules*, vol. 21, no. 8, article no. 1061, 2016.

[6] K. Ma, C. Zhang, M.-Y. Huang, Y.-X. Guo, and G.-Q. Hu, "Crosstalk between Beclin-1-dependent autophagy and caspase-dependent apoptosis induced by tanshinone IIA in human

osteosarcoma MG-63 cells," *Oncology Reports*, vol. 36, no. 4, pp. 1807–1818, 2016.

[7] T. Wang, G. Di, L. Yang et al., "Saponins from Panax japonicus attenuate D-galactose-induced cognitive impairment through its anti-oxidative and anti-apoptotic effects in rats," *Journal of Pharmacy and Pharmacology*, vol. 67, no. 9, pp. 1284–1296, 2015.

[8] K. Shibata, Z. Omahdi, and S. Yamasaki, "Necroptosis DAMPens anti-tumor immunity," *Cell Death Discovery*, vol. 2, Article ID 16033, 2016.

[9] M. L. Cho, D.-J. Lee, H.-S. Lee, Y.-J. Lee, and S. G. You, "LPS-induced NO inhibition and antioxidant activities of ethanol extracts and their solvent partitioned fractions from four brown seaweeds," *Ocean Science Journal*, vol. 48, no. 4, pp. 349–359, 2013.

[10] A. Ganguli, D. Choudhury, S. Datta, S. Bhattacharya, and G. Chakrabarti, "Inhibition of autophagy by chloroquine potentiates synergistically anti-cancer property of artemisinin by promoting ROS dependent apoptosis," *Biochimie*, vol. 107, pp. 338–349, 2014.

[11] X. Chen, J. Liu, and S.-Y. Chen, "Sulforaphane protects against ethanol-induced oxidative stress and apoptosis in neural crest cells by the induction of Nrf2-mediated antioxidant response," *British Journal of Pharmacology*, vol. 169, no. 2, pp. 437–448, 2013.

[12] H. Ozkol, G. Bulut, R. Balahoroglu, Y. Tuluce, and H. U. Ozkol, "Protective Effects of selenium, N-acetylcysteine and vitamin E against acute ethanol intoxication in rats," *Biological Trace Element Research*, vol. 175, no. 1, pp. 177–185, 2017.

[13] F. Higuera-de la Tijera, A. I. Servín-Caamaño, A. E. Serralde-Zúñiga et al., "Metadoxine improves the three- and six-month survival rates in patients with severe alcoholic hepatitis," *World Journal of Gastroenterology*, vol. 21, no. 16, pp. 4975–4985, 2015.

[14] N. Tufton, N. Hashim, C. Sze, and M. Waterhouse, "A case of thyroid storm complicated by acute hepatitis due to propylthiouracil treatment," *Endocrinology, Diabetes & Metabolism Case Reports*, Article ID 150052, 2015.

[15] K. Triantafyllou, J. Vlachogiannakos, and S. D. Ladas, "Gastrointestinal and liver side effects of drugs in elderly patients," *Best Practice and Research: Clinical Gastroenterology*, vol. 24, no. 2, pp. 203–215, 2010.

[16] V. Camara-Clayette, Y. Lecluse, C. Schrader et al., "The NF-κB pathway is rarely spontaneously activated in mantle cell lymphoma (MCL) cell lines and patient's samples," *European Journal of Cancer*, vol. 50, no. 1, pp. 159–169, 2014.

[17] L. Zhao, Y. Wang, J. Liu et al., "Protective effects of genistein and puerarin against chronic alcohol-induced liver injury in mice via antioxidant, anti-inflammatory, and anti-apoptotic mechanisms," *Journal of Agricultural and Food Chemistry*, vol. 64, no. 38, pp. 7291–7297, 2016.

[18] C.-Y. Chang, C.-L. Lee, and T.-M. Pan, "Statistical optimization of medium components for the production of Antrodia cinnamomea AC0623 in submerged cultures," *Applied Microbiology and Biotechnology*, vol. 72, no. 4, pp. 654–661, 2006.

[19] X. Qiao, W. Song, Q. Wang et al., "Comprehensive chemical analysis of triterpenoids and polysaccharides in the medicinal mushroom Antrodia cinnamomea," *RSC Advances*, vol. 5, no. 58, pp. 47040–47052, 2015.

[20] Z.-M. Lu, W.-Y. Tao, H.-Y. Xu, Z.-H. Ao, X.-M. Zhang, and Z.-H. Xu, "Further studies on the hepatoprotective effect of Antrodia camphorata in submerged culture on ethanol-induced acute liver injury in rats," *Natural Product Research*, vol. 25, no. 7, pp. 684–695, 2011.

[21] T.-Y. Song and G.-C. Yen, "Protective effects of fermented filtrate from Antrodia camphorata in submerged culture against CCl4-induced hepatic toxicity in rats," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 6, pp. 1571–1577, 2003.

[22] H. Wang, N. Pampati, W. M. McCormick, and L. Bhattacharyya, "Protein nitrogen determination by kjeldahl digestion and ion chromatography," *Journal of Pharmaceutical Sciences*, vol. 105, no. 6, pp. 1851–1857, 2016.

[23] N. Zhang, Q. Li, J. Wang et al., "Screening of Irpex lacteus mutant strains and optimizing fermentation conditions," *Journal of Food, Agriculture & Environment*, vol. 12, no. 2, pp. 1213–1219, 2014.

[24] T.-W. Ma, Y. Lai, and F.-C. Yang, "Enhanced production of triterpenoid in submerged cultures of Antrodia cinnamomea with the addition of citrus peel extract," *Bioprocess and Biosystems Engineering*, vol. 37, no. 11, pp. 2251–2261, 2014.

[25] S. R. Khoigani, A. Rajaei, and S. A. Goli, "Evaluation of antioxidant activity, total phenolics, total flavonoids and LC-MS/MS characterisation of phenolic constituents in Stachys lavandulifolia," *Natural Product Research*, vol. 31, no. 3, pp. 355–358, 2016.

[26] E. Jurak, A. M. Punt, W. Arts, M. A. Kabel, and H. Gruppen, "Fate of carbohydrates and lignin during composting and mycelium growth of Agaricus Bisporus on wheat straw based compost," *PLoS ONE*, vol. 10, no. 10, Article ID e0138909, 2015.

[27] S. Serra and S. Deaglio, "HPLC-based assay to monitor extracellular nucleotide/nucleoside metabolism in human chronic lymphocytic leukemia cells," *Journal of Visualized Experiments*, vol. 2016, no. 113, Article ID e54124, 2016.

[28] C. Hurel, M. Taneez, A. Volpi Ghirardini, and G. Libralato, "Effects of mineral amendments on trace elements leaching from pre-treated marine sediment after simulated rainfall events," *Environmental Pollution*, vol. 220, pp. 364–374, 2017.

[29] M. U. Granata, F. Bracco, L. Gratani et al., "Fatty acid content profile and main constituents of *Corylus avellana* kernel in wild type and cultivars growing in Italy," *Natural Product Research*, vol. 31, no. 2, pp. 204–209, 2016.

[30] J. D. Lim, S. R. Lee, T. Kim et al., "Fucoidan from fucus vesiculosus protects against alcohol-induced liver damage by modulating inflammatory mediators in mice and Hepg2 cells," *Marine Drugs*, vol. 13, no. 2, pp. 1051–1067, 2015.

[31] Y. Han, Q. Xu, J.-N. Hu, X.-Y. Han, W. Li, and L.-C. Zhao, "Maltol, a food flavoring agent, attenuates acute alcohol-induced oxidative damage in mice," *Nutrients*, vol. 7, no. 1, pp. 682–696, 2015.

[32] H.-H. Li, K. Doiron, A. D. Patterson, F. J. Gonzalez, and A. J. Fornace Jr., "Identification of serum insulin-like growth factor binding protein 1 as diagnostic biomarker for early-stage alcohol-induced liver disease," *Journal of Translational Medicine*, vol. 11, no. 1, article no. 266, 2013.

[33] B. Relja, R. Weber, M. Marasliogl et al., "Differential relevance of NF-κB and JNK in the pathophysiology of hemorrhage/resususcitation-induced liver injury after chronic ethanol feeding," *PLoS ONE*, vol. 10, no. 9, Article ID e0137875, 2015.

[34] W. Okiyama, N. Tanaka, T. Nakajima et al., "Polyenephosphatidylcholine prevents alcoholic liver disease in PPARα-null mice through attenuation of increases in oxidative stress," *Journal of Hepatology*, vol. 50, no. 6, pp. 1236–1246, 2009.

[35] S. Pal, A. Bhattacharjee, S. Mukherjee, K. Bhattacharya, S. Mukherjee, and S. Khowala, "Effect of alocasia indica tuber

extract on reducing hepatotoxicity and liver apoptosis in alcohol intoxicated rats," *BioMed Research International*, vol. 2014, Article ID 349074, 10 pages, 2014.

[36] J. Liu and L. Du, "PERK pathway is involved in oxygen-glucose-serum deprivation-induced NF-kB activation via ROS generation in spinal cord astrocytes," *Biochemical and Biophysical Research Communications*, vol. 467, no. 2, pp. 197–203, 2015.

[37] M. Ishaq, M. A. Khan, K. Sharma, G. Sharma, R. K. Dutta, and S. Majumdar, "Gambogic acid induced oxidative stress dependent caspase activation regulates both apoptosis and autophagy by targeting various key molecules (NF-κB, Beclin-1, p62 and NBR1) in human bladder cancer cells," *Biochimica et Biophysica Acta*, vol. 1840, no. 12, pp. 3374–3384, 2014.

[38] K. J. S. Kumar, F.-H. Chu, H.-W. Hsieh et al., "Antroquinonol from ethanolic extract of mycelium of *Antrodia cinnamomea* protects hepatic cells from ethanol-induced oxidative stress through Nrf-2 activation," *Journal of Ethnopharmacology*, vol. 136, no. 1, pp. 168–177, 2011.

[39] F. He, H.-C. Bi, Z.-Y. Xie et al., "Rapid determination of six metabolites from multiple cytochrome P450 probe substrates in human liver microsome by liquid chromatography/mass spectrometry: application to high-throughput inhibition screening of terpenoids," *Rapid Communications in Mass Spectrometry*, vol. 21, no. 5, pp. 635–643, 2007.

[40] J. H. Shin and J. H. Jung, "Non-alcoholic fatty liver disease and flavonoids: current perspectives," *Clinics and Research in Hepatology and Gastroenterology*, vol. 41, no. 1, 2016.

[41] C. Morin, P. U. Blier, and S. Fortin, "Eicosapentaenoic acid and docosapentaenoic acid monoglycerides are more potent than docosahexaenoic acid monoglyceride to resolve inflammation in a rheumatoid arthritis model," *Arthritis Research and Therapy*, vol. 17, no. 1, article no. 142, 2015.

[42] L. Ma, S. Zhang, and M. Du, "Cordycepin from *Cordyceps militaris* prevents hyperglycemia in alloxan-induced diabetic mice," *Nutrition Research*, vol. 35, no. 5, pp. 431–439, 2015.

[43] Y.-W. Cao, Y. Jiang, D.-Y. Zhang et al., "Protective effects of Penthorum chinense Pursh against chronic ethanol-induced liver injury in mice," *Journal of Ethnopharmacology*, vol. 161, pp. 92–98, 2015.

[44] V. Diaz-Aguirre, C. Velez-Pardo, and M. Jimenez-Del-Rio, "Fructose sensitizes Jurkat cells oxidative stress-induced apoptosis via caspase-dependent and caspase-independent mechanisms," *Cell Biology International*, vol. 40, no. 11, pp. 1162–1173, 2016.

[45] Y. Li, B. Liu, F. Yang et al., "Lobaplatin induces BGC-823 human gastric carcinoma cell apoptosis via ROS- mitochondrial apoptotic pathway and impairs cell migration and invasion," *Biomedicine and Pharmacotherapy*, vol. 83, pp. 1239–1246, 2016.

[46] H. Yamasaki, H. Shimoji, Y. Ohshiro, and Y. Sakihama, "Inhibitory effects of nitric oxide on oxidative phosphorylation in plant mitochondria," *Nitric Oxide*, vol. 5, no. 3, pp. 261–270, 2001.

[47] N. Wu, H. Shen, H. Liu, Y. Wang, Y. Bai, and P. Han, "Acute blood glucose fluctuation enhances rat aorta endothelial cell apoptosis, oxidative stress and pro-inflammatory cytokine expression in vivo," *Cardiovascular Diabetology*, vol. 15, no. 1, article no. 109, 2016.

[48] Z. Zhao, S. Gong, S. Wang, and C. Ma, "Effect and mechanism of evodiamine against ethanol-induced gastric ulcer in mice by suppressing Rho/NF-κB pathway," *International Immunopharmacology*, vol. 28, no. 1, pp. 588–595, 2015.

[49] S. Nepal, M. J. Kim, A. Subedi et al., "Globular adiponectin inhibits ethanol-induced apoptosis in HepG2 cells through heme oxygenase-1 induction," *Biochemical Pharmacology*, vol. 84, no. 7, pp. 974–983, 2012.

[50] A. Denys, I. A. Udalova, C. Smith et al., "Evidence for a dual mechanism for IL-10 suppression of TNF-α production that does not involve inhibition of p38 mitogen-activated protein kinase or NF-κB in primary human macrophages," *The Journal of Immunology*, vol. 168, no. 10, pp. 4837–4845, 2002.

[51] M. Buffler, C. Becker, W. Windisch, and K. Schümann, "nflammation neither increases hepatic hepcidin nor affects intestinal ^{59}Fe-absorption in two murine models of bowel inflammation, hemizygous TNF$^{\Delta ARE/+}$ and homozygous IL-10$^{-/-}$ mice," *Journal of Trace Elements in Medicine and Biology*, vol. 32, pp. 162–167, 2015.

[52] A. R. Cannon, N. L. Morris, A. M. Hammer et al., "Alcohol and inflammatory responses: Highlights of the 2015 Alcohol and Immunology Research Interest Group (AIRIG) meeting," *Alcohol*, vol. 54, pp. 73–77, 2016.

[53] B. Melichar, "Biomarkers, inflammation and cancer: where to go?" *Clinical Chemistry and Laboratory Medicine*, vol. 54, no. 12, pp. 1847–1852, 2016.

[54] S. Y. Shin, C. G. Kim, Y. J. Jung, Y. Lim, and Y. H. Lee, "The UPR inducer DPP23 inhibits the metastatic potential of MDA-MB-231 human breast cancer cells by targeting the Akt-IKK-NF-κB–MMP-9 axis," *Scientific Reports*, vol. 6, Article ID 34134, 2016.

[55] T. Jiang, F. Tian, H. Zheng et al., "Nrf2 suppresses lupus nephritis through inhibition of oxidative injury and the NF-κB-mediated inflammatory response," *Kidney International*, vol. 85, no. 2, pp. 333–343, 2014.

[56] D. A. Averill-Bates and P. Pallepati, "Activation of apoptosis by hydrogen peroxide through death receptor signaling is inhibited by mild heat preconditioning at 40°C," *Free Radical Biology and Medicine*, vol. 49, p. S54, 2010.

[57] L. Kastl, S. Sauer, T. Beissbarth, M. Becker, P. Krammer, and K. Gülow, "TNF-a stimulation enhances ROS-dependent cell migration via NF-κB activation in liver cells," *Free Radical Biology and Medicine*, vol. 75, supplement 1, p. S32, 2014.

[58] Q.-Y. Lv, B. Wan, L.-H. Guo, L. Zhao, and Y. Yang, "In vitro immune toxicity of polybrominated diphenyl ethers on murine peritoneal macrophages: apoptosis and immune cell dysfunction," *Chemosphere*, vol. 120, pp. 621–630, 2015.

Hindawi
Oxidative Medicine and Cellular Longevity
Volume 2017, Article ID 1406726, 13 pages
https://doi.org/10.1155/2017/1406726

Research Article

Diallyl Trisulfide Suppresses Oxidative Stress-Induced Activation of Hepatic Stellate Cells through Production of Hydrogen Sulfide

Feng Zhang,[1,2,3] Huanhuan Jin,[1] Li Wu,[1,2,3] Jiangjuan Shao,[2,3] Xiaojing Zhu,[1] Anping Chen,[4] and Shizhong Zheng[1,2,3]

[1] *Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, Nanjing University of Chinese Medicine, Nanjing 210023, China*
[2] *Jiangsu Key Laboratory of Therapeutic Material of Chinese Medicine, Nanjing University of Chinese Medicine, Nanjing 210023, China*
[3] *State Key Laboratory Cultivation Base for TCM Quality and Efficacy, Nanjing University of Chinese Medicine, Nanjing 210023, China*
[4] *Department of Pathology, School of Medicine, Saint Louis University, Saint Louis, MO 63104, USA*

Correspondence should be addressed to Shizhong Zheng; szhengnucm@163.com

Received 31 July 2016; Revised 22 December 2016; Accepted 30 January 2017; Published 20 February 2017

Academic Editor: Ranjitsinh Devkar

Accumulating data reveal that garlic has beneficial effects against chronic liver disease. We previously reported that diallyl trisulfide (DATS), the primary organosulfur compound in garlic, reduced fibrosis and attenuated oxidative stress in rat fibrotic liver. The present study was aimed at elucidating the underlying mechanisms. The primary rat hepatic stellate cells (HSCs) were cultured and stimulated with hydrogen peroxide (H_2O_2) for inducing HSC activation under oxidative stress. We examined the effects of DATS on the profibrogenic properties and oxidative stress in H_2O_2-treated HSCs. The results showed that DATS suppressed and reduced fibrotic marker expression in HSCs. DATS arrested cell cycle at G2/M checkpoint associated with downregulating cyclin B1 and cyclin-dependent kinase 1, induced caspase-dependent apoptosis, and reduced migration in HSCs. Moreover, intracellular levels of reactive oxygen species and lipid peroxide were decreased by DATS, but intracellular levels of glutathione were increased in HSCs. Furthermore, DATS significantly elevated hydrogen sulfide (H_2S) levels within HSCs, but iodoacetamide (IAM) reduced H_2S levels and significantly abrogated DATS production of H_2S within HSCs. IAM also abolished all the inhibitory effects of DATS on the profibrogenic properties and oxidative stress in HSCs. Altogether, we demonstrated an H_2S-associated mechanism underlying DATS inhibition of profibrogenic properties and alleviation of oxidative stress in HSCs. Modulation of H_2S production may represent a therapeutic remedy for liver fibrosis.

1. Introduction

Hepatic fibrosis represents an overactive wound healing process secondary to a variety of chronic liver injuries. During fibrogenesis, excessive connective tissue accumulates in the liver and results in the distortion of hepatic architecture [1]. Chronic liver injury activates and transforms quiescent hepatic stellate cells (HSCs) from vitamin A-storing pericytes to myofibroblast-like cells. Once activated, HSCs become profibrogenic through acquiring proliferative, contractile, and proinflammatory properties as the primary cellular source of extracellular matrix (ECM) components in the liver [2]. Clinical and basic data suggest that oxidative stress critically participates in the progression of fibrosis and acts as mediators of molecular and cellular events implicated in liver fibrosis. Generation of reactive oxygen species (ROS) plays an important role in HSC activation and initiation of hepatic fibrogenesis [3]. Culture of HSCs treated with oxidative stress-related molecules can mimic the HSC activation caused by oxidative stress in liver fibrosis [4].

This cellular model is commonly established for investigating the mechanisms of HSC activation and evaluating the effects of antifibrotic candidates. However, currently there are few breakthroughs in the therapeutic intervention of hepatic fibrosis. Identification of antifibrogenic agents that are innocuous is urgently needed.

Garlic has been recognized for prevention and treatment of various diseases by many different cultures throughout history. Recent studies support the effects of garlic and its functional ingredients in a wide range of applications, including anticancer, antithrombotic, antiatherosclerotic, antidiabetic, and antioxidant properties [5]. It is characterized that diallyl trisulfide (DATS), a major organosulfur compound in garlic, is responsible for the pharmacological efficacy of garlic [6]. We previously demonstrated that DATS reduced carbon tetrachloride (CCl_4)-caused liver injury and fibrogenesis in rats, which was associated with inhibition of HSC activation and attenuation of hepatic oxidative stress [7]. However, the molecular mechanisms underlying DATS's antifibrotic activity are not fully understood. Interestingly, emerging data indicate that drugs that target hydrogen sulfide (H_2S) or generate safe levels of H_2S in vivo may be therapeutic options for chronic liver diseases [8]. It is given that DATS is an exogenous donor of H_2S and the liver has a high hepatic capacity for H_2S metabolism [9]. We hypothesized that DATS exerted its antifibrotic effects associated with generation of H_2S. Experiments were performed to verify this hypothesis.

2. Materials and Methods

2.1. Reagents and Antibodies. The following compounds were used in this study: DATS (purity > 97%; Shenzhen Minn Bolin Biotechnology Co., Ltd., Shenzhen, China), iodoacetamide (IAM; Nanjing Dingguo Changsheng Biotechnology Co., Ltd., Nanjing, China), and mitomycin (Sigma, St Louis, MO, USA). They were dissolved in dimethylsulfoxide (DMSO) for experiments. DMSO at a concentration of 0.02% (w/v) was set as a vehicle control throughout the studies. Analytical grade 30% hydrogen peroxide (H_2O_2; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was diluted with deionized water to indicated concentrations for experiments. The following primary antibodies were used in this study: α-SMA, α1(I) procollagen, and fibronectin (Epitomics, San Francisco, CA, USA); TGF-βRI, TGF-βRII, PDGF-βR, EGF-R, and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); cyclin A, cyclin B1, CDK1, CDK2, Bax, pro-caspase-9, cleaved-caspase-9, pro-caspase-8, cleaved-caspase-8, pro-caspase-7, cleaved-caspase-7, pro-caspase-3, cleaved-caspase-3, full-length PARP-1, cleaved-PARP-1, and β-Actin (Cell Signaling Technology, Danvers, MA, USA).

2.2. Cell Culture. Primary rat HSCs were obtained from Jiangyin CHI Scientific, Inc. (Wuxi, China). HSCs were cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Wisent Biotechnology Co., Ltd., Nanjing, China), 1% antibiotics, and grown in a 5% CO_2 humidified atmosphere at 37°C. Cell morphology was assessed under an inverted microscope (Leica, Germany).

2.3. Cytotoxicity Assay. HSCs were seeded in 24-well plates and cultured for 24 h. HSCs were then treated with DMSO or DATS at indicated concentrations for 24 h. Lactate dehydrogenase (LDH) activity in culture medium was determined using LDH cytotoxicity assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the protocol. Results were from triplicate experiments.

2.4. Cell Viability Assay. HSCs were seeded in 96-well plates and cultured for 24 h. HSCs were then treated with H_2O_2 and/or DATS at indicated concentrations for 24 h. The medium was replaced with 100 μL phosphate buffered saline (PBS) containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and cells were maintained at 37°C for 4 h. Then, the crystals were dissolved with 200 μL DMSO. The spectrophotometric absorbance was measured with a SPECTRAmax™ microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 490 nm. Results were from triplicate experiments.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA) for H_2S. HSCs were seeded in 6-well plates and cultured for 24 h. HSCs were then treated with DATS and/or IAM at indicated concentrations for 24 h. Cells were then lysated with RIPA buffer. Intracellular H_2S concentrations were determined using an ELISA kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the protocol. Experiments were performed in triplicate.

2.6. Cell Cycle Analyses. HSCs were seeded in 6-well plates and cultured for 24 h. HSCs were then treated with various reagents at indicated concentrations for 24 h and harvested and fixed. Cell cycle was analyzed using cellular DNA flow cytometric kits (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the protocol. Percentages of cells within cell cycle compartments were determined by flow cytometry (FACSCalibur; BD, Franklin Lakes, NJ, USA). Results were from triplicate experiments.

2.7. Apoptosis Analyses. HSCs were seeded in 6-well plates and cultured for 24 h. HSCs were then treated with various reagents at indicated concentrations for 24 h. Morphology of apoptotic HSCs was evaluated using Hoechst staining kits (Nanjing KeyGen Biotechnology Co., Ltd., Nanjing, China) according to the protocol. Photographs were taken under a fluorescence microscope (Nikon, Tokyo, Japan). In certain experiments, the apoptotic rates were determined by flow cytometry using Annexin V-FITC apoptosis assay kits (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the protocol. Apoptotic cells were defined as the cells situated in the right two quadrants of each plot and the percentages were determined by flow cytometry (FACSCalibur; BD, Franklin Lakes, NJ, USA). Results were from triplicate experiments.

2.8. Wound Healing Assay. HSCs were seeded in 6-well plates and cultured in DMEM with 10% FBS. Once the cells attached properly, they were treated with mitomycin at 4 μg/mL for

3 h. One linear wound was scraped in each well with a sterile pipette tip, and cells were washed with PBS to remove the unattached cells. Then cells were treated with various reagents at indicated concentrations. Images were taken at 12 h after wound induction at the same field under an inverted microscope (Leica, Germany). Results were from triplicate experiments.

2.9. Boyden Chamber Assay. HSCs were seeded to the upper wells of polycarbonate membrane transwell inserts (8 μm pore-size; Corning, USA) and cultured in DMEM with 10% FBS, and meanwhile they were treated with various reagents at indicated concentrations. The lower chambers were filled with complete medium and H_2O_2 at 5 μM. After 12 h incubation, the polycarbonated filter was removed and the migrated cells on the lower surface were stained with crystal violet. The number of migrated cells at five random fields for each well was counted and normalized to control. Results were from triplicate experiments.

2.10. Measurement of ROS. Analysis of intracellular ROS was determined using dichlorofluorescein diacetate (DCFH-DA) probe (Shanghai Genmed Co., Ltd., Shanghai, China). DCFH-DA is an oxidation-sensitive nonfluorescent precursor dye that can be oxidized by H_2O_2, other ROS, and low molecule weight peroxides to fluorescent DCFH. HSCs were seeded in 6-well plates and cultured for 24 h. HSCs were then treated with various reagents at indicated concentrations for 24 h. In certain experiments, cells were washed with PBS and incubated with DCFH-DA solutions (10 μM) for 20 min. Micrographs were taken under a fluorescence microscope (Nikon, Tokyo, Japan). The excitation and emission wavelengths were set at 488 and 525 nm, respectively. In certain experiments, cells were detached by trypsinization and suspended in DCFH-DA solution (10 μM) for 20 min. The intensity of cellular fluorescence intensity was determined by flow cytometry (FACSCalibur; BD, Franklin Lakes, NJ, USA). Results were from triplicate experiments.

2.11. Measurement of Lipid Peroxide (LPO). HSCs were seeded in 6-well plates and cultured for 24 h. HSCs were then treated with various reagents at indicated concentrations for 24 h and were lysated with RIPA buffer. Intracellular LPO levels were determined using kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the protocol. Experiments were performed in triplicate.

2.12. Measurement of Glutathione (GSH). HSCs were seeded in 6-well plates and cultured for 24 h. HSCs were then treated with various reagents at indicated concentrations for 24 h and were lysated with RIPA buffer. Intracellular GSH levels were determined using kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the protocol. Experiments were performed in triplicate.

2.13. Western Blot Analyses. HSCs were treated with various reagents at indicated concentrations for 24 h. Whole cell protein preparation, detection, and band visualization were performed as we previously described [7]. β-Actin was used as an invariant control for equal loading of total proteins. Representative blots were from three independent experiments.

2.14. Statistical Analyses. Data were presented as mean ± SD and analyzed using SPSS 16.0 software. Histograms were created using GraphPad Prism 5 software (San Diego, CA, USA). The significance of difference was determined by one-way ANOVA with the post hoc Dunnett's test. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. DATS Inhibits Viability and Reduces Fibrotic Protein Expression Associated with H_2S in HSCs. We initially evaluated potential cytotoxicity of DATS on HSCs using LDH assay, showing that DATS at 20 μM or higher concentrations had significant cytotoxic effects on HSCs (Figure 1(a)). We thus investigated DATS effects at nontoxic doses subsequently. We herein used H_2O_2 to stimulate HSC activation mimicking oxidative stress, because H_2O_2 is the most stable ROS and diffuses readily in and out of cells [10]. DATS at doses of 0.1–10 μM inhibited cell viability concentration-dependently in H_2O_2-stimulated HSCs (Figure 1(b)). Moreover, DATS reduced the expression of fibrotic marker proteins α-smooth muscle actin (α-SMA), α1(I) procollagen, and fibronectin and downregulated the type I and II receptors for transforming growth factor-β (TGF-βRI and TGF-βRII), platelet-derived growth factor-β receptor (PDGF-βR), and epidermal growth factor receptor (EGF-R) in H_2O_2-stimulated HSCs (Figure 1(c)). We hypothesized that DATS exerted its effects associated with production of H_2S. The compound IAM has been demonstrated to block both the exofacial and intracellular thiols resulting in elimination of garlic-induced H_2S production [11]. Here, IAM at concentration range within 100 μM did not cause morphological alterations in HSCs, and accordingly we selected 100 μM IAM for subsequent experiments (Figure 1(d)). We found that DATS concentration-dependently increased the H_2S content in HSCs and that IAM at 100 μM significantly abrogated DATS elevation of H_2S production and reduced the base level of H_2S in HSCs (Figure 1(e)), highlighting IAM as a potent blocker of DATS generation of H_2S. Furthermore, IAM effectively rescued DATS downregulation of fibrotic marker molecules in H_2O_2-stimulated HSCs (Figure 1(f)). Taken together, these data suggested that H_2S was involved in DATS suppression of the expression of fibrotic proteins in activated HSCs.

3.2. DATS Induces Cell Cycle Arrest Associated with H_2S in HSCs. We next examined DATS effects on cell fate of HSCs. DATS arrested cell cycle at G2/M checkpoint in H_2O_2-stimulated HSCs (Figure 2(a)). Addition of IAM abrogated DATS-induced G2/M arrest (Figure 2(b)). Cyclin A, cyclin B1, cyclin-dependent kinase (CDK) 1, and CDK2 are four critical regulators at G2/M checkpoint responsible for driving

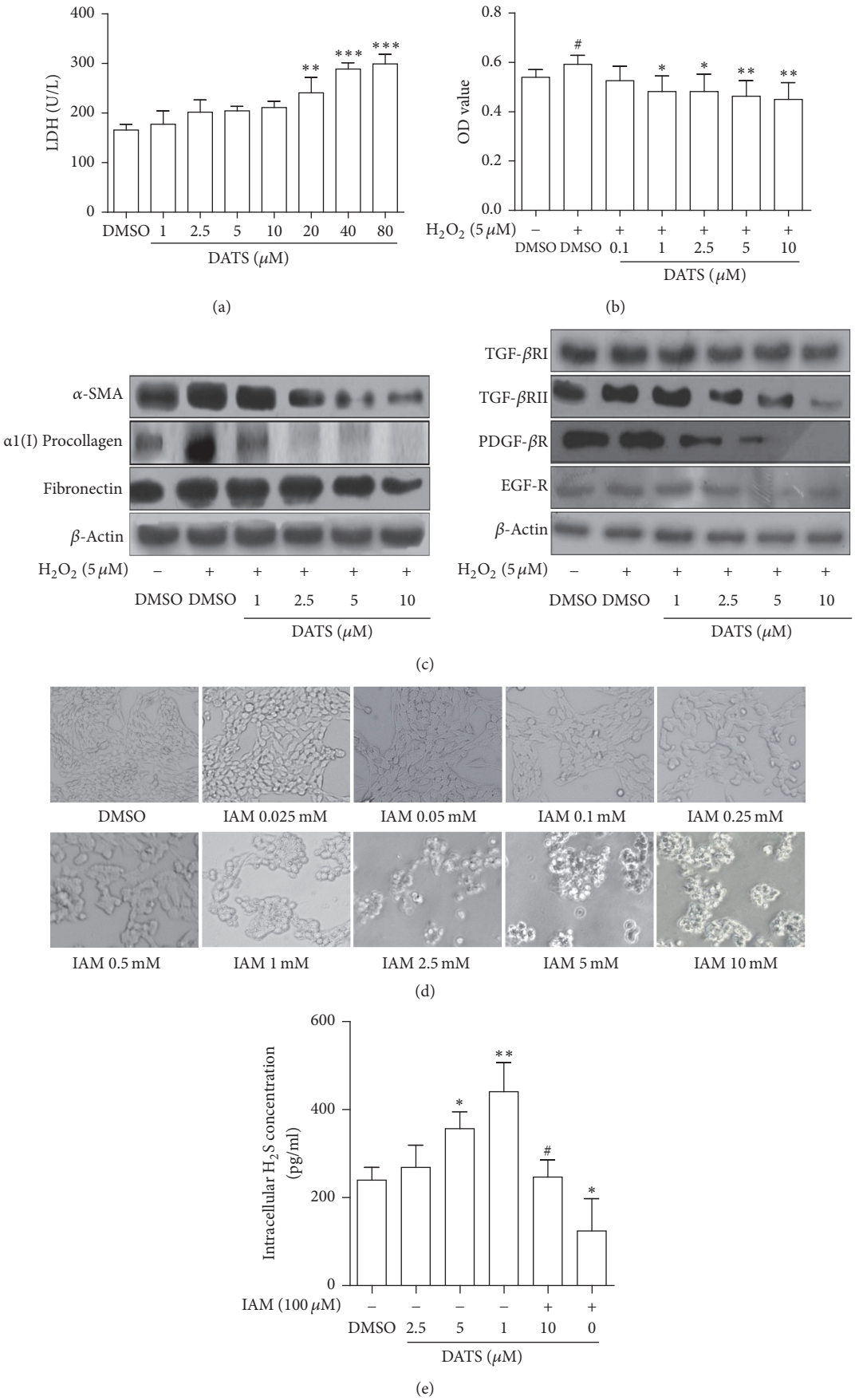
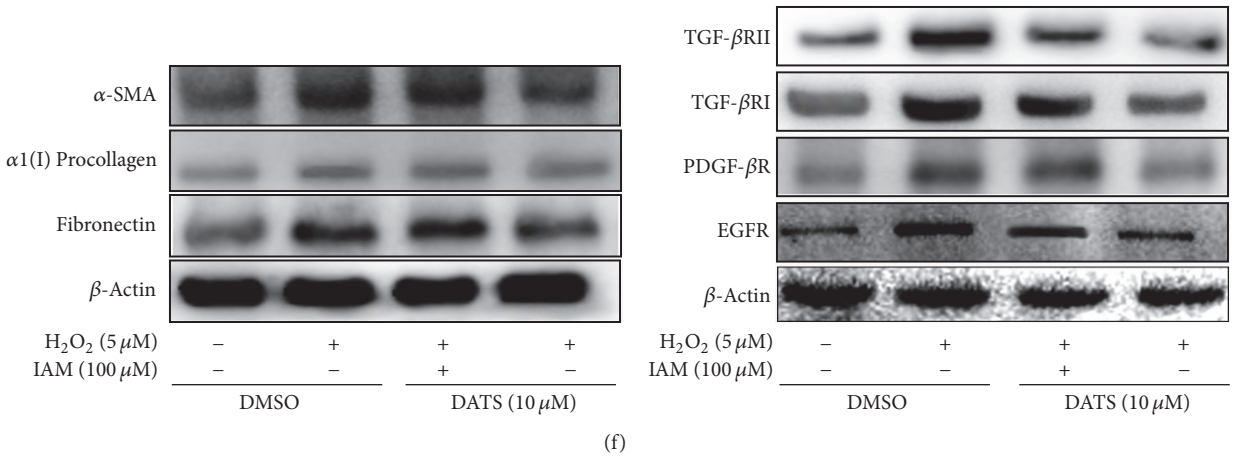


Figure 1: Continued.



(f)

Figure 1: DATS inhibits viability and reduces fibrotic protein expression associated with H_2S in HSCs. (a) LDH release assay. Significance: $^{**}P < 0.01$ versus DMSO and $^{***}P < 0.001$ versus DMSO. (b) MTT assay. Significance: $^{\#}P < 0.05$ versus DMSO, $^{*}P < 0.05$ versus DMSO + H_2O_2, and $^{**}P < 0.01$ versus DMSO + H_2O_2. (c) Western blot analyses of profibrotic marker proteins. (d) Morphology evaluation with light microscope (200x magnification). (e) ELISA for H_2S concentration. Significance: $^{*}P < 0.05$ versus DMSO, $^{**}P < 0.01$ versus DMSO, and $^{\#}P < 0.05$ versus DATS 10 μM. (f) Western blot analyses of profibrotic marker proteins.

cells into the mitosis process [12]. DATS decreased the expression of cyclin B1 and CDK1, while cyclin A and CDK2 were not apparently affected by DATS (Figure 2(c)). Moreover, DATS inhibition of cyclin B1 and CDK1 was abolished by IAM (Figure 2(d)). These data collectively indicated that H_2S was involved in DATS modulation of cell cycle regulators and G2/M checkpoint arrest in HSCs.

3.3. DATS Stimulates Apoptosis Associated with H_2S in HSCs. We subsequently examined DATS effects on HSC apoptosis. HSCs treated with DATS exhibited significant DNA condensation and fragmentation with brilliant blue staining (Figure 3(a)), and DATS increased apoptotic rates concentration-dependently in H_2O_2-treated HSCs (Figure 3(b)). However, the increase in apoptotic rate by DATS was abolished by IAM (Figure 3(c)). Furthermore, the antiapoptotic protein Bcl-2 was diminished by DATS; the proapoptotic molecule Bax was increased by DATS (Figure 3(d)). DATS also activated the caspase cascade in H_2O_2-stimulated HSCs, because the cleaved forms of caspase-9, caspase-8, caspase-7, and caspase-3, and PARP-1 were all increased by DATS (Figure 3(d)). However, DATS effects on these molecules were abrogated by IAM (Figure 3(e)). Altogether, these findings indicated that H_2S was involved in DATS induction of caspase-mediated apoptosis in HSCs.

3.4. DATS Inhibits Migration Associated with H_2S in HSCs. We next examined DATS effects on migration in HSCs. DATS inhibited the lateral migration of H_2O_2-activated HSCs (Figure 4(a)). However, IAM abrogated DATS's inhibitory effects on HSC lateral migration (Figure 4(b)). To confirm the results, we performed transwell migration assay to evaluate the vertical migration of HSCs, showing that H_2O_2-stimulated vertical migration was suppressed by DATS (Figure 4(c)). Similarly, DATS effects were also significantly compensated by IAM (Figure 4(d)). Collectively, these data indicated that DATS inhibition of cell migration was associated with H_2S in HSCs.

3.5. DATS Alleviates Oxidative Stress Associated with H_2S in HSCs. We finally determined DATS effects on oxidative stress in HSCs. Fluorescence microscope analyses and flow cytometry assessments consistently demonstrated that H_2O_2-treated HSCs had significantly high intracellular levels of ROS, but DATS decreased ROS production (Figures 5(a) and 5(b)). However, IAM significantly rescued DATS-caused reduction in ROS levels in H_2O_2-stimulated HSCs (Figure 5(c)). Furthermore, DATS decreased the intracellular levels of LPO and increased the intracellular levels of GSH in H_2O_2-stimulated HSCs (Figure 5(d)). However, DATS inhibition of LPO and elevation of GSH were significantly compensated by IAM (Figure 5(e)). Altogether, these results suggested that DATS attenuated oxidative stress in HSCs, which was relevant to H_2S.

4. Discussion

Many studies demonstrated that garlic extracts elicited therapeutic effects against liver fibrosis. Garlic extracts restored liver histology and accelerated ECM degradation, resulting in regression of liver fibrosis in CCl_4-intoxicated rats [13]. Administration of aqueous garlic extracts alleviated hepatic oxidative injury and reduced collagen content in bile duct ligation-induced fibrosis in rats [14]. However, these investigations did not identify the specific functional ingredients responsible for the antifibrotic efficacy of garlic. Our previous data showed that DATS attenuated collagen deposition, inhibited HSC activation, and ameliorated oxidative stress in rat fibrotic liver, strengthening the therapeutic value of

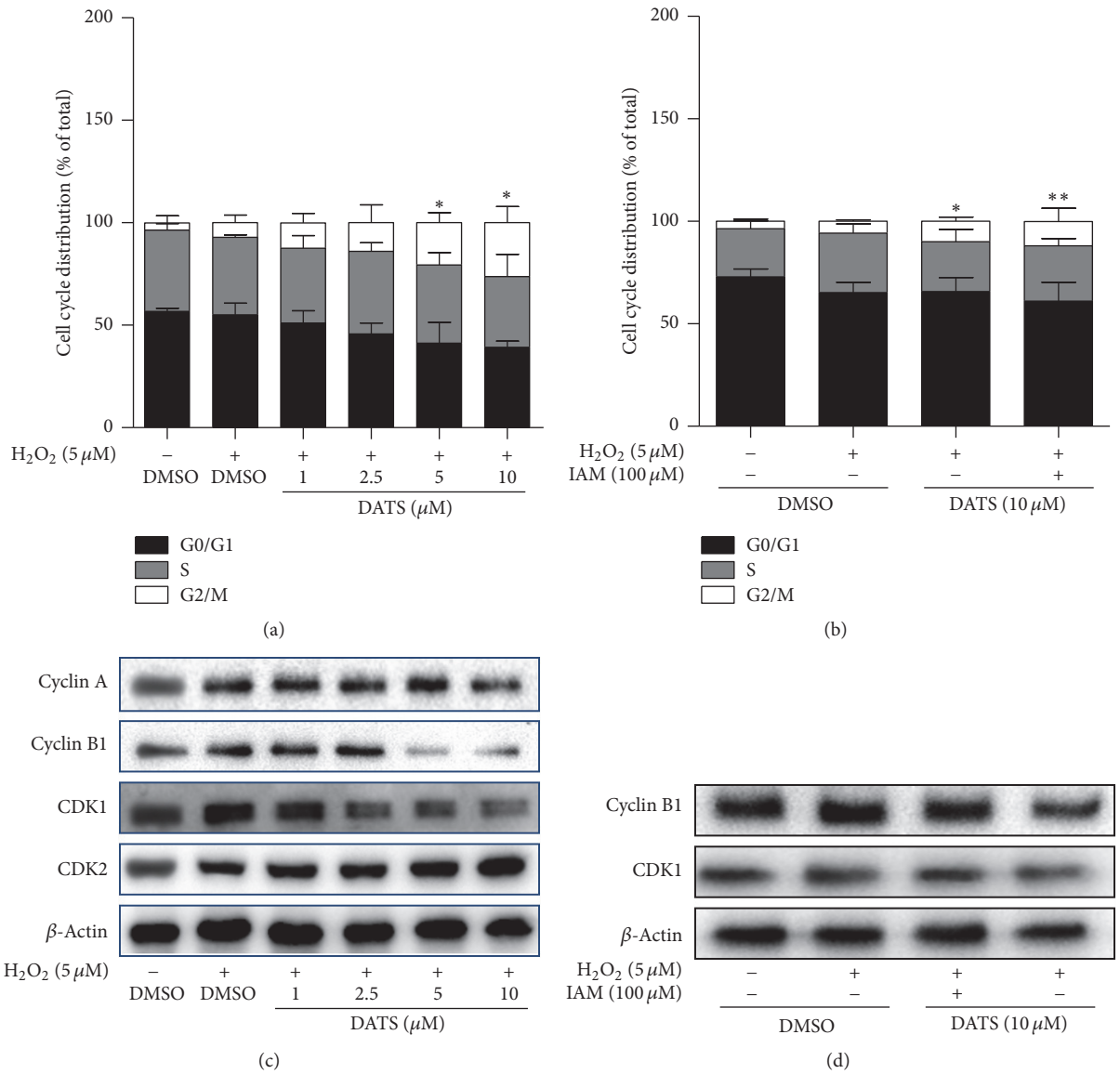


Figure 2: DATS induces cell cycle arrest associated with H_2S in HSCs. (a, b) Percentages of cell cycle distributions were determined by flow cytometry. Significance: $^{*}P < 0.05$ versus DMSO + H_2O_2 and $^{**}P < 0.01$ versus DMSO + H_2O_2. (c, d) Western blot analyses of cell cycle regulatory proteins.

garlic for liver fibrosis [7]. Elucidation of the underlying mechanisms would be essentially important for developing DATS as a promising antifibrotic candidate.

Our current experiments could provide consistent support for the prior in vivo data. We found that DATS inhibited HSC activation in culture, which confirmed that HSCs could be target cells for DATS, because DATS was able to inhibit nearly all aspects of HSC activation. To explore the underlying mechanisms, we concentrated on the potential biotransformation of DATS. A 2007 study firstly described the real-time kinetics of H_2S production of DATS within cells, showing that DATS with allyl moieties and three sulfur atoms reacted with exofacial membrane thiols and cross the cell membrane to react with GSH to produce H_2S, which was a thiol-dependent manner for DATS to produce H_2S [11]. Thereafter, increasing evidence reveals that the beneficial effects of garlic-derived organic polysulfides are mediated by production of H_2S. Herein, we thus strongly speculated that H_2S contributed to DATS inhibition of HSC activation. As expected, DATS could significantly increase H_2S levels concentration-dependently in HSCs in current study. Moreover, we reasoned that IAM could be a proper blocker of DATS production of H_2S, because their thiol-dependent mechanisms of action were exactly matching. Consistently, we here observed that IAM not only abolished DATS elevation of H_2S production, but also reduced basal H_2S synthesis in HSCs. Accordingly, we used IAM to testify the role of H_2S in DATS inhibition of HSC activation properties. The obtained results clearly demonstrated that this H_2S manipulation significantly weakened DATS effects.

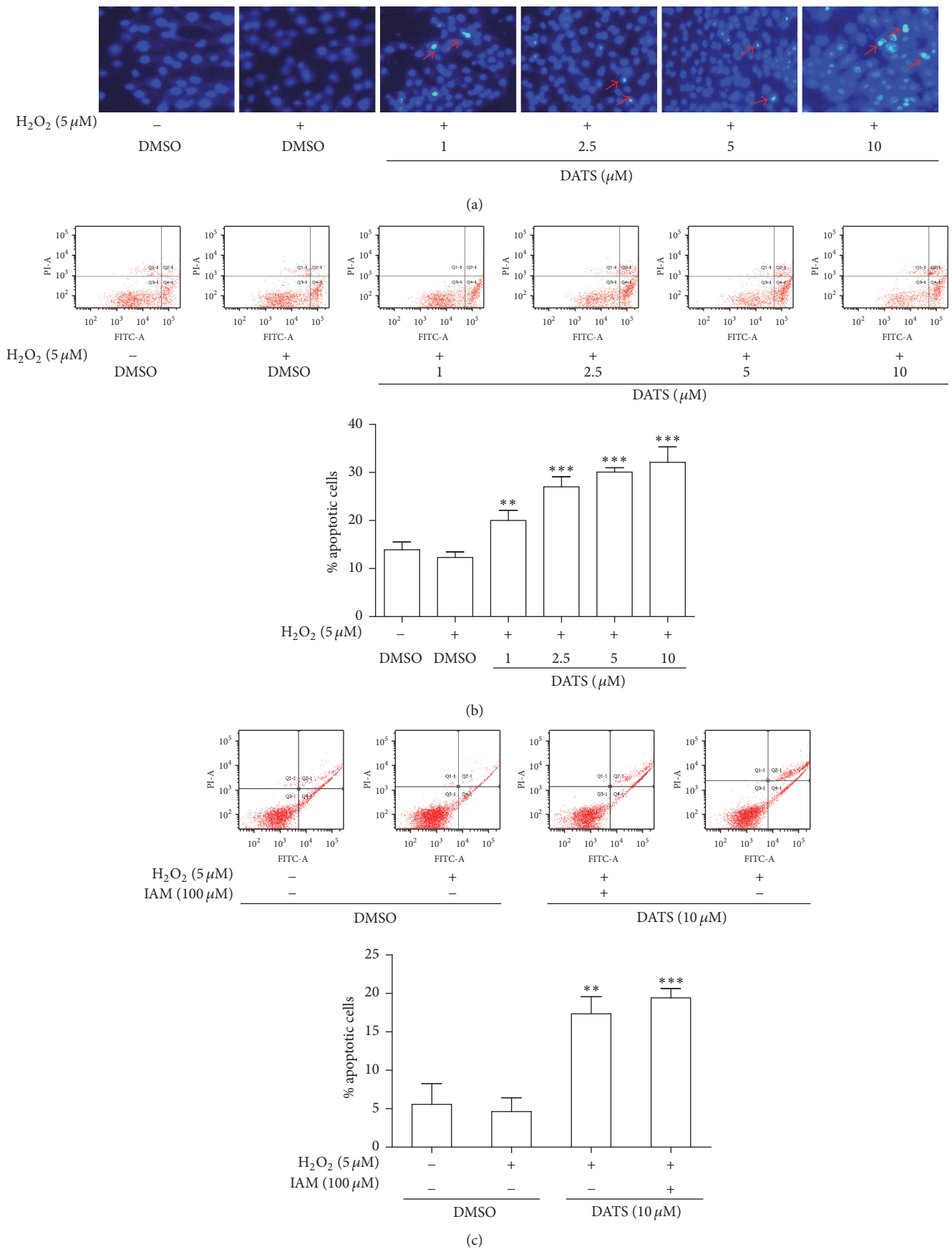
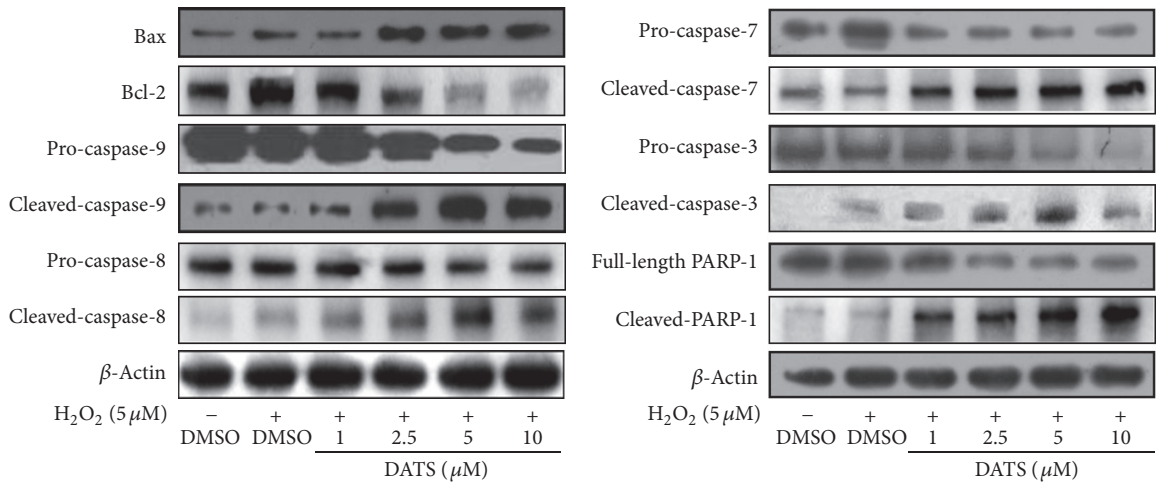
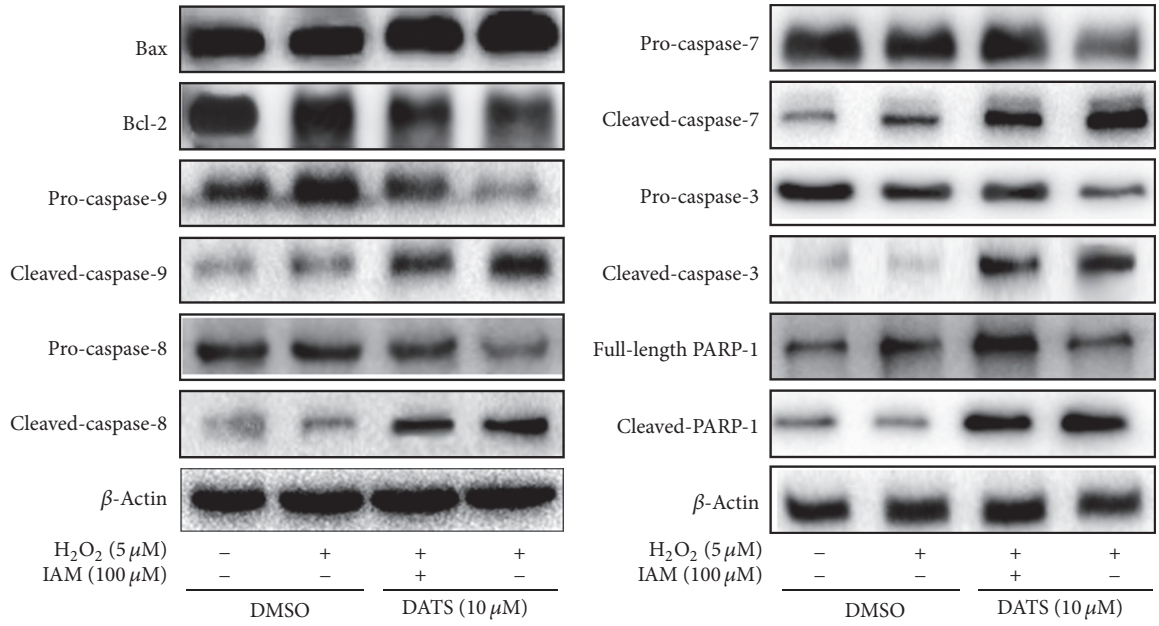


Figure 3: Continued.



(d)

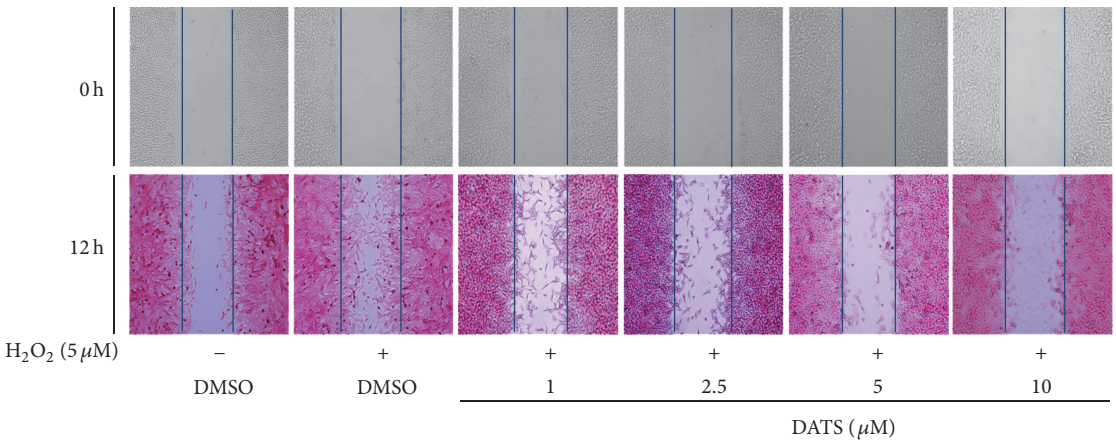


(e)

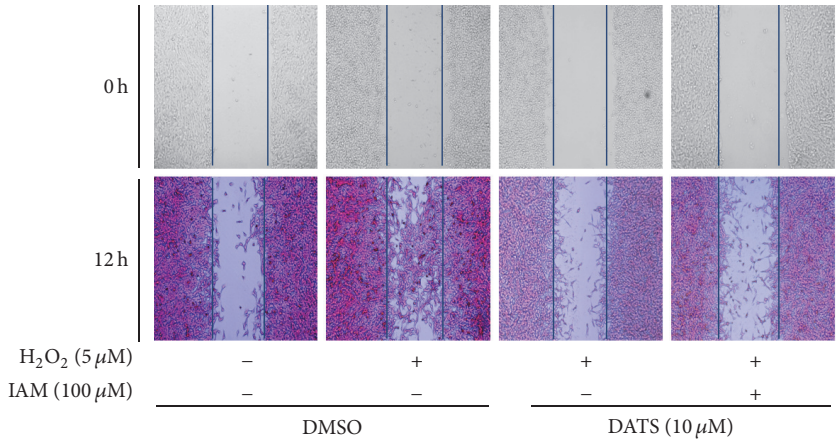
Figure 3: DATS stimulates apoptosis associated with H_2S in HSCs. (a) Hoechst fluorescence staining for morphology of apoptotic HSCs under a fluorescence microscope (200x magnification). (b, c) Flow cytometric analyses of apoptotic rates. Significance: $^{**}P < 0.01$ versus DMSO + H_2O_2 and $^{***}P < 0.001$ versus DMSO + H_2O_2. (d, e) Western blot analyses of apoptosis regulatory proteins.

We identified the H_2S as a molecular link implicated in DATS's antifibrotic activity. Actually, the role of H_2S in hepatology has been studied increasingly during recent years. Malfunction of hepatic H_2S metabolism may be involved in many chronic liver diseases, including hepatic fibrosis and cirrhosis [15]. Exogenous H_2S could reduce hepatotoxicity, liver cirrhosis, and portal hypertension through multiple functions including antioxidation, anti-inflammation, cytoprotection, and antifibrosis [16]. Our current data were consistent with these observations and provided evidence that DATS could be transformed to be H_2S that mediated the potent hepatoprotective and antifibrotic effects in HSCs.

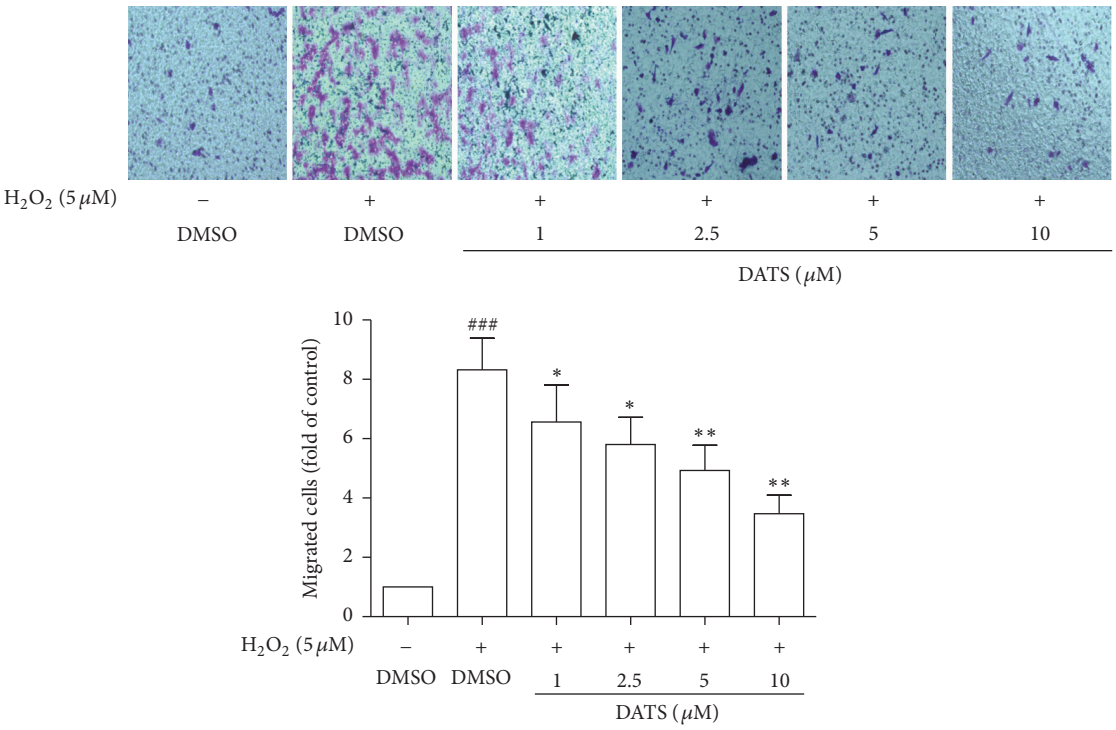
We demonstrated that H_2S was involved in DATS effects on many aspects of HSC activation, including fibrotic marker expression, cell cycle, apoptosis, migration, and the status of oxidative stress. Consistent with our data, recent investigations showed that sodium hydrosulfide, a well-known inorganic H_2S-releasing molecule, suppressed proliferation and induced G1 phase cell cycle arrest in HSCs [17] and attenuated collagen expression in rats with CCl_4-induced hepatic fibrosis [18]. These findings collectively suggested H_2S as a signaling molecule regulating the pathophysiology of HSCs. Actually, H_2S promotes a number of cellular signals that regulate metabolism, proliferation, and apoptosis in various types of cells. For example, exogenous treatment with H_2S led to growth inhibition with cyclin D1 downregulation in rat smooth muscle cells [19] and stimulated apoptosis by downregulation of Bcl-2 and Bcl-xL in portal vein smooth



(a)



(b)



(c)

Figure 4: Continued.

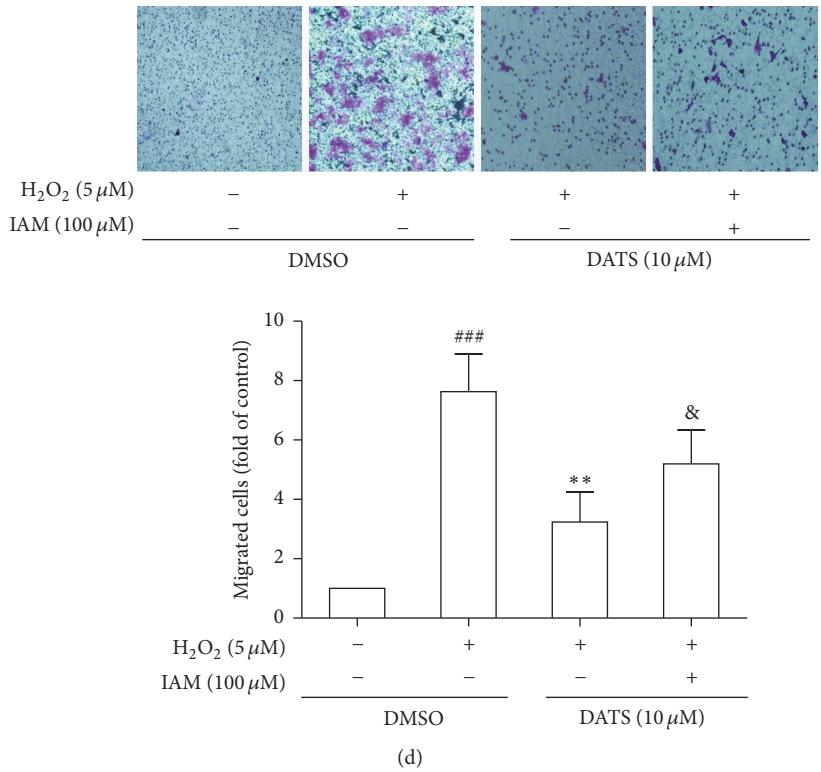


Figure 4: DATS inhibits migration associated with H_2S in HSCs. (a, b) Wound healing assay (50x magnification). (c, d) Boyden chamber assay (50x magnification). Significance: $^{###}P < 0.001$ versus DMSO, $^{*}P < 0.05$ versus DMSO + H_2O_2, $^{**}P < 0.01$ versus DMSO + H_2O_2, and $^{\&}P < 0.05$ versus DATS + H_2O_2.

muscle cells [20]. These observations were recaptured in HSCs by our present study and potentiated the role of H_2S as a gaseous signaling molecule with a DNA damaging function. Interestingly, there was evidence that administration of sodium hydrosulfide significantly inhibited hepatocyte apoptosis in hepatic ischemia reperfusion-induced injury in rats, suggesting a hepatoprotective role for H_2S in vivo [21]. However, it should be noted that this study examined apoptosis molecules in whole liver tissues rather than in single type of cells. This may raise a possibility that the proapoptotic effects of H_2S on HSCs could be masked due to the overwhelming amount of hepatocytes in the liver. It could also be assumed that the different effects of H_2S on apoptosis were cell-specific and disease-specific. Furthermore, we found that DATS inhibited HSC migration associated with H_2S. Consistently, recent studies showed that DATS suppressed migration and invasion in human colon cancer cells [22] and breast cancer cells [23]. Another important issue is that increasing evidence elucidates the fundamental role of HSC in liver immunology, because HSCs represents a versatile source of many soluble immunological active factors and may act as an antigen presenting cell [24]. Understanding the role of HSCs as central regulators of liver immunology may lead to novel therapeutic strategies for chronic liver diseases. Interestingly, H_2S has multiple and complex cellular signaling roles in relation to immunological modulation under certain pathophysiological conditions [25]. However, studies addressing how H_2S regulates HSC immunology during hepatic fibrosis are not seen, which could be an attractive field in hepatology.

We herein evaluated DATS effects in an oxidative stress-induced HSC activation model, because signs of oxidative stress are concomitant or precede HSC activation and collagen deposition [3]. We also suggested a role for H_2S in regulation of oxidative stress in HSCs. H_2S could increase intracellular GSH concentrations and suppress oxidative stress in mitochondria [26]. H_2S could also protect murine liver against ischemia reperfusion injury through upregulation of intracellular antioxidant pathways [27]. The antioxidant effects of H_2S have recently been examined through use of H_2S-releasing derivative ACS67, which was found to inhibit ROS formation and NADH oxidation in endothelial cells [28]. In current study, H_2S also contributed to DATS attenuation of oxidative stress in HSCs. However, whether H_2S exerted its antioxidant effects by virtue of its undoubted reducing activity or by its activation of endogenous defense systems merits further investigation. Furthermore, it is necessary to use an additional HSC line and another blocker of H_2S generation to make the results more credible. There is also a need to carry out in vivo studies using certain H_2S generation blocker to validate the H_2S-dependent mechanism underlying the antifibrotic effects of DATS, which is ongoing in our laboratory.

In summary, DATS inhibited the profibrogenic properties and alleviated oxidative stress in HSCs. These effects were

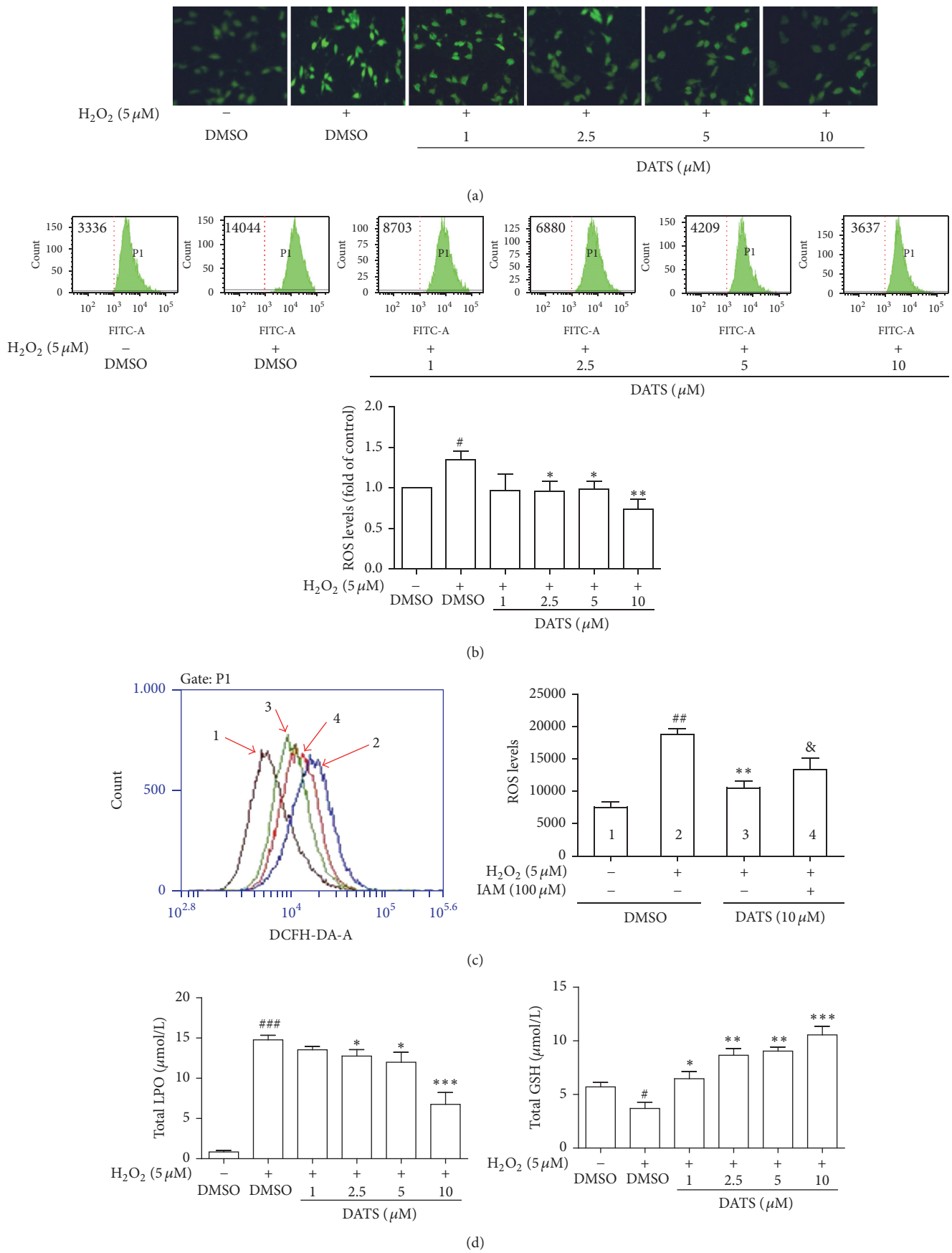
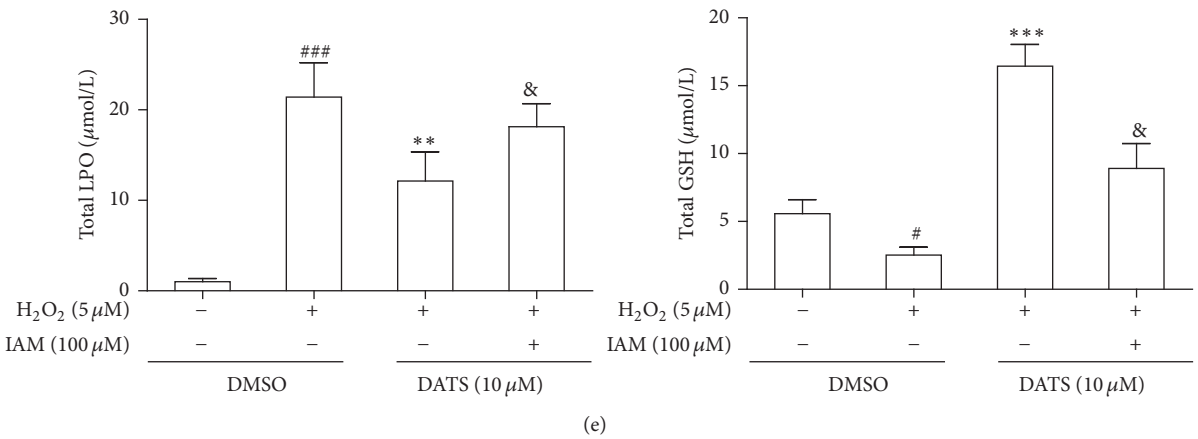


Figure 5: Continued.



(e)

Figure 5: DATS alleviates oxidative stress associated with H_2S in HSCs. (a) Analyses of intracellular ROS using a fluorescence microscope (100x magnification). (b, c) Analyses of intracellular ROS using flow cytometry with quantification. Significance: $^{\#}P < 0.05$ versus DMSO, $^{\#\#}P < 0.01$ versus DMSO, $^{*}P < 0.05$ versus DMSO + H_2O_2, $^{**}P < 0.01$ versus DMSO + H_2O_2, and $^{\&}P < 0.05$ versus DATS + H_2O_2. (d, e) Analyses of intracellular LPO and GSH. Significance: $^{\#}P < 0.05$ versus DMSO, $^{\#\#\#}P < 0.001$ versus DMSO, $^{*}P < 0.05$ versus DMSO + H_2O_2, $^{**}P < 0.01$ versus DMSO + H_2O_2, $^{***}P < 0.001$ versus DMSO + H_2O_2, and $^{\&}P < 0.05$ versus DATS + H_2O_2.

associated with production of H_2S within cells. Our data elucidated, at least partially, the mechanisms underlying DATS's antifibrotic activity and indicated a therapeutic role of targeting H_2S for treatment of liver fibrosis.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by the Natural Science Foundation of Jiangsu Province (BK20140955), the National Natural Science Foundation of China (31571455, 31401210, 31600653, 81600483, and 81270514), the Open Project Program of Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica (JKLPSE201601 and 201502), and the Project of the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) and was also sponsored by the Qing Lan Project of Jiangsu Province.

References

[1] S. L. Friedman, "Evolving challenges in hepatic fibrosis," *Nature Reviews. Gastroenterology & Hepatology*, vol. 7, no. 8, pp. 425–436, 2010.

[2] F. Tacke and R. Weiskirchen, "Update on hepatic stellate cells: pathogenic role in liver fibrosis and novel isolation techniques," *Expert Review of Gastroenterology & Hepatology*, vol. 6, no. 1, pp. 67–80, 2012.

[3] V. Sánchez-Valle, N. C. Chávez-Tapia, M. Uribe, and N. Méndez-Sánchez, "Role of oxidative stress and molecular changes in liver fibrosis: a review," *Current Medicinal Chemistry*, vol. 19, no. 28, pp. 4850–4860, 2012.

[4] F. Zhang, C. Ni, D. Kong et al., "Ligustrazine attenuates oxidative stress-induced activation of hepatic stellate cells by interrupting platelet-derived growth factor-β receptor-mediated ERK and p38 pathways," *Toxicology and Applied Pharmacology*, vol. 265, no. 1, pp. 51–60, 2012.

[5] M. S. Butt, M. T. Sultan, M. S. Butt, and J. Iqbal, "Garlic: nature's protection against physiological threats," *Critical Reviews in Food Science and Nutrition*, vol. 49, no. 6, pp. 538–551, 2009.

[6] T. Ariga and T. Seki, "Antithrombotic and anticancer effects of garlic-derived sulfur compounds: a review," *BioFactors*, vol. 26, no. 2, pp. 93–103, 2006.

[7] X. Zhu, F. Zhang, L. Zhou et al., "Diallyl trisulfide attenuates carbon tetrachloride-caused liver injury and fibrogenesis and reduces hepatic oxidative stress in rats," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 387, no. 5, pp. 445–455, 2014.

[8] S. Fiorucci, E. Distrutti, G. Cirino, and J. L. Wallace, "The emerging roles of hydrogen sulfide in the gastrointestinal tract and liver," *Gastroenterology*, vol. 131, no. 1, pp. 259–271, 2006.

[9] E. J. Norris, C. R. Culberson, S. Narasimhan, and M. G. Clemens, "The liver as a central regulator of hydrogen sulfide," *Shock*, vol. 36, no. 3, pp. 242–250, 2011.

[10] Y. Oya-Ohta, T. Ochi, Y. Komoda, and K. Yamamoto, "The biological activity of hydrogen peroxide. VI. Mechanism of the enhancing effects of l-histidine: the role of the formation of a histidine-peroxide adduct and membrane transport," *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, vol. 326, no. 1, pp. 99–107, 1995.

[11] G. A. Benavides, G. L. Squadrito, R. W. Mills et al., "Hydrogen sulfide mediates the vasoactivity of garlic," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 46, pp. 17977–17982, 2007.

[12] X. Zhang, F. Zhang, D. Kong et al., "Tetramethylpyrazine inhibits angiotensin II-induced activation of hepatic stellate cells associated with interference of platelet-derived growth factor β receptor pathways," *FEBS Journal*, vol. 281, no. 12, pp. 2754–2768, 2014.

[13] G. D'Argenio, D. C. Amoruso, G. Mazzone et al., "Garlic extract prevents CCl_4-induced liver fibrosis in rats: the role of tissue

transglutaminase," *Digestive and Liver Disease*, vol. 42, no. 8, pp. 571–577, 2010.

[14] N. Gedik, L. Kabasakal, Ö. Şehirli et al., "Long-term administration of aqueous garlic extract (AGE) alleviates liver fibrosis and oxidative damage induced by biliary obstruction in rats," *Life Sciences*, vol. 76, no. 22, pp. 2593–2606, 2005.

[15] S. Mani, W. Cao, L. Wu, and R. Wang, "Hydrogen sulfide and the liver," *Nitric Oxide*, vol. 41, pp. 62–71, 2014.

[16] G. Tan, S. Pan, J. Li et al., "Hydrogen sulfide attenuates carbon tetrachloride-induced hepatotoxicity, liver cirrhosis and portal hypertension in rats," *PLoS ONE*, vol. 6, no. 10, Article ID e25943, 2011.

[17] H.-N. Fan, H.-J. Wang, L. Ren et al., "Decreased expression of p38 MAPK mediates protective effects of hydrogen sulfide on hepatic fibrosis," *European Review for Medical and Pharmacological Sciences*, vol. 17, no. 5, pp. 644–652, 2013.

[18] H. N. Fan, H. J. Wang, C. R. Yang-Dan et al., "Protective effects of hydrogen sulfide on oxidative stress and fibrosis in hepatic stellate cells," *Molecular Medicine Reports*, vol. 7, no. 1, pp. 247–253, 2013.

[19] G. Yang, L. Wu, and R. Wang, "Pro-apoptotic effect of endogenous H_2S on human aorta smooth muscle cells," *The FASEB Journal*, vol. 20, no. 3, pp. 553–555, 2006.

[20] C. Wang, J. Han, L. Xiao, C.-E. Jin, D.-J. Li, and Z. Yang, "Role of hydrogen sulfide in portal hypertension and esophagogastric junction vascular disease," *World Journal of Gastroenterology*, vol. 20, no. 4, pp. 1079–1087, 2014.

[21] K. Kang, M. Zhao, H. Jiang, G. Tan, S. Pan, and X. Sun, "Role of hydrogen sulfide in hepatic ischemia-reperfusion-induced injury in rats," *Liver Transplantation*, vol. 15, no. 10, pp. 1306–1314, 2009.

[22] K.-C. Lai, S.-C. Hsu, J.-S. Yang, C.-C. Yu, J.-C. Lein, and J.-G. Chung, "Diallyl trisulfide inhibits migration, invasion and angiogenesis of human colon cancer HT-29 cells and umbilical vein endothelial cells, and suppresses murine xenograft tumour growth," *Journal of Cellular and Molecular Medicine*, vol. 19, no. 2, pp. 474–484, 2015.

[23] K. Chandra-Kuntal, J. Lee, and S. V. Singh, "Critical role for reactive oxygen species in apoptosis induction and cell migration inhibition by diallyl trisulfide, a cancer chemopreventive component of garlic," *Breast Cancer Research and Treatment*, vol. 138, no. 1, pp. 69–79, 2013.

[24] R. Weiskirchen and F. Tacke, "Cellular and molecular functions of hepatic stellate cells in inflammatory responses and liver immunology," *Hepatobiliary Surgery and Nutrition*, vol. 3, no. 6, pp. 344–363, 2014.

[25] M. Whiteman, L. Li, P. Rose, C.-H. Tan, D. B. Parkinson, and P. K. Moore, "The effect of hydrogen sulfide donors on lipopolysaccharide-induced formation of inflammatory mediators in macrophages," *Antioxidants and Redox Signaling*, vol. 12, no. 10, pp. 1147–1154, 2010.

[26] Y. Kimura, Y.-I. Goto, and H. Kimura, "Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria," *Antioxidants & Redox Signaling*, vol. 12, no. 1, pp. 1–13, 2010.

[27] S. Jha, J. W. Calvert, M. R. Duranski, A. Ramachandran, and D. J. Lefer, "Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: role of antioxidant and antiapoptotic signaling," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 295, no. 2, pp. H801–H806, 2008.

[28] N. N. Osborne, D. Ji, A. S. A. Majid, R. J. Fawcett, A. Sparatore, and P. Del Soldato, "ACS67, a hydrogen sulfide-releasing derivative of latanoprost acid, attenuates retinal ischemia and oxidative stress to RGC-5 cells in culture," *Investigative Ophthalmology and Visual Science*, vol. 51, no. 1, pp. 284–294, 2010.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3269405, 11 pages
http://dx.doi.org/10.1155/2016/3269405

Research Article

Proteomics-Based Identification of the Molecular Signatures of Liver Tissues from Aged Rats following Eight Weeks of Medium-Intensity Exercise

Fanghui Li,[1,2] Tao Li,[3] and Yanying Liu[1]

[1] *School of Physical Education and Health, Zhaoqing University, Zhaoqing 526016, China*
[2] *Exercise Health and Technology Centre, Shanghai Jiao Tong University, Shanghai 200240, China*
[3] *Laboratory of Laser Sports Medicine, South China Normal University, Guangzhou 510006, China*

Correspondence should be addressed to Fanghui Li; fanghuili2007@163.com

Received 4 June 2016; Revised 5 September 2016; Accepted 28 November 2016

Academic Editor: Ravirajsinh Jadeja

Physical activity has emerged as a powerful intervention that promotes healthy aging by maintaining the functional capacity of critical organ systems. Here, by combining functional and proteomics analyses, we examined how hepatic phenotypes might respond to exercise treatment in aged rats. 16 male aged (20 months old) SD rats were divided into exercise and parallel control groups at random; the exercise group had 8 weeks of treadmill training with medium intensity. Whole protein samples of the liver were extracted from both groups and separated by two-dimensional gel electrophoresis. Alternatively objective protein spots with >2-fold difference in expression were selected for enzymological extraction and MS/MS identification. Results show increased activity of the manganese superoxide dismutase and elevated glutathione levels in the livers of exercise-treated animals, but malondialdehyde contents obviously decreased in the liver of the exercise group. Proteomics-based identification of differentially expressed proteins provided an integrated view of the metabolic adaptations occurring in the liver proteome during exercise, which significantly altered the expression of several proteins involved in key liver metabolic pathways including mitochondrial sulfur, glycolysis, methionine, and protein metabolism. These findings indicate that exercise may be beneficial to aged rats through modulation of hepatic protein expression profiles.

1. Introduction

Sedentary habits are associated with low levels of physical fitness and a high risk of mortality [1], whereas physical activity has emerged as a powerful intervention that promotes successful aging by maintaining general physical fitness [2]. However, the physiological mechanisms underlying physical activity-induced physical fitness are only partly understood.

Exercise-induced oxygen consumption increases associated with energy metabolism adaptation might be involved in physical fitness [3]. The first direct evidence of this was provided by Davies et al. [4] through the demonstration that high-intensity exercise enhances reactive oxygen species (ROS) production. Although high concentrations of ROS can damage proteins, lipids, and nucleic acids, further evidence suggests that exercise promotes the formation of ROS, which participate in redox regulation. In particular, moderate concentrations of ROS function as regulatory mediators to reestablish "redox homeostasis" in signaling processes [5, 6]. In this context, exercise-induced ROS may be involved in the establishment of physical fitness [7].

Although the potential effects of muscular ROS have been explored in depth, the role and related mechanisms of ROS in the hepatic adaptation induced by exercise have not been extensively studied [8]. Several recent findings have resulted in physical activity being prescribed as part of the overall strategy (or therapy) to reduce the risk of senescence-associated liver metabolic syndrome. The potential mechanisms of this protective role might involve the enhancement of hepatic insulin sensitivity and the activities of antioxidant enzymes, the attenuation of lipid accumulation in the liver, and subsequent prevention of hyperglycemia,

hypercholesterolemia, and liver diseases (such as nonalcoholic steatohepatitis or cirrhosis) [9]. In addition, ROS are direct triggers of liver insulin resistance [10] and inflammation [11]. In contrast, however, evidence from rats indicates that regular exercise can attenuate hepatic oxidative stress [12]. Nonetheless, further research is needed to evaluate the detailed mechanism of ROS in the hepatic adaptation induced by exercise.

Given the multitude of changes that occur in each organ during exercise, genetic and epigenetic studies might be able to elucidate the biology underlying the individual response to exercise [13]. 2D gel tandem mass spectrometry-based proteomic methods are valuable tools for quantifying differences in protein abundance between different physical conditions or treatments [14]. Current proteomic methods allow the identification of several thousand proteins from microgram quantities of proteins in skeletal muscle, cardiomyocytes [15], adipose tissue [16], and liver [7, 17, 18]. In addition, proteomics analysis has previously been performed by several groups to determine how liver proteome profiles differ between exercise-trained and untrained individuals [18], which might provide information that could be of benefit in combating the effects of postmenopause or of nonalcoholic fatty liver disease-related loss of liver function including metabolic syndrome by promoting redox homeostasis. However, insight into the adaptive response of liver tissue proteome signatures during aging under a variety of regular exercise challenges is still required [19]. In the current study, we focused our attention specifically on the putative phenotypic/metabolic adaptations in the liver of aged rats that were induced by eight weeks of medium-intensity exercise.

2. Material and Methods

2.1. Subjects. In total, 16 male adult Sprague-Dawley (SD) rats (*Rattus norvegicus*, Guangdong Medical Laboratory Animal Center (GDMLAC), Guangdong, China) with similar body weights (range of 340–260 g), aged 18 months at the beginning of the training period, were randomly distributed into two groups: treadmill exercise ($n = 8$) (EXE) and sedentary control ($n = 8$) (CON). The animals were fed standard laboratory chow and water ad libitum while housed (3–5 per cage) in a temperature-controlled room (22°C) with 12 h dark/light cycles. All animal protocols were approved by the local Experimental Animal Use Committee.

2.2. Endurance Training. The animal exercise protocol was as follows: the training intensity was progressively increased. For the first 4 weeks, the speed of the treadmill and duration of the training sessions were gradually increased from a speed of 10 m/min for 10 min to a running speed of 15 m/min for 60 min by the end of week 4. For the next 4 weeks, a 5 min warm-up session at a speed of 10 m/min was followed by the 60 min training session at a speed of 15 m/min [20]. This training protocol was developed based on previous experience training in rodents [21], but minor modifications were applied according to another training protocol [22]. The intensity and duration were chosen based on previous reports from the groups of Song and Bejma in which a similar speed of 15 m/min and a similar parameter of 60 min/bout were applied, which resulted in a series of positive changes such as increased activity of antioxidant enzymes in the aging liver [21, 23]. The 8-week exercise program of treadmill running at 0° incline was chosen based on previous reports from the Siu group [22]. Training animals and control animals were sacrificed 6 h after the last training session. The liver tissues were quickly removed and frozen immediately in liquid nitrogen and stored at −80°C until further analysis.

2.3. Assay of Malondialdehyde (MDA) and Glutathione (GSH) Contents as well as Superoxide Dismutase (SOD) Activity in the Livers of Old Rats. For biochemical analyses of in vivo MDA, GSH, and SOD activity, 20 mg of liver tissue was homogenized on ice in phosphate-buffered saline and centrifuged for 10 min at 12,000 ×g at 4°C. The supernatant was collected and analyzed. MDA level, GSH content, and SOD activity were determined using commercial assay kits produced by Nanjing Jiancheng Bioengineering Institute, China. Protein concentrations were determined using a BCA protein assay kit (Nanjing Jiancheng Biochemistry Co.) as described by the manufacturer.

2.4. Proteomics Analysis

2.4.1. Sample Preparation for Two-Dimensional Gel Electrophoresis (2DGE). We selected 8 "EXE" and 8 "CON" rats for proteomics analysis. From these animals, 50 mg of liver was homogenized in 500 μL of sample buffer [(7 M urea, 2 M thiourea, 4% (w/v) dimethyl[3 propyl] azaniumyl propane-1-sulfonate (CHAPS), 1% (v/v) immobilized pH gradient (IPG) buffer pH 3–10 NL) (Amersham Biosciences, Piscataway, NJ, USA), 40 mM dithiothreitol (DTT), and Protease Inhibitor Cocktail Complete (Roche, Mannheim, Germany)] and allowed to stand at room temperature for 1 h, after which the homogenate was then centrifuged at 40,000 ×g for 60 min at 4°C. Subsequently, the supernatant was removed and the protein concentration was measured using the Bradford protein assay. Homogenates were stored at −70°C for later use. 2-DE was performed using a 24 cm (nonlinear, pH 3.0–10.0) IPG gel strip, according to Sun et al. [24]. Spot detection, gel matching, and interclass analysis were performed using PDQuest 8.0 software (Amersham Biosciences, USA) [24].

2.4.2. In-Gel Tryptic Digestion and Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS/MS). In-gel digestion and protein identification were performed as described by Perkins et al. [25]. Candidate spots were excised from the stained gel, destained with 0.1 M ammonium bicarbonate in 50% acetonitrile (Sigma-Aldrich, St. Louis, MO, USA), and dried using a SpeedVac SC110 (Savant Instruments, Holbrook, NY, USA). The gels were then rehydrated and incubated in trypsin solution (Promega, Madison, WI, USA) overnight at 37°C, and the peptide mixtures were then analyzed using MALDI-TOF MS/MS (Applied Biosystems, Foster City, CA, USA) [25].

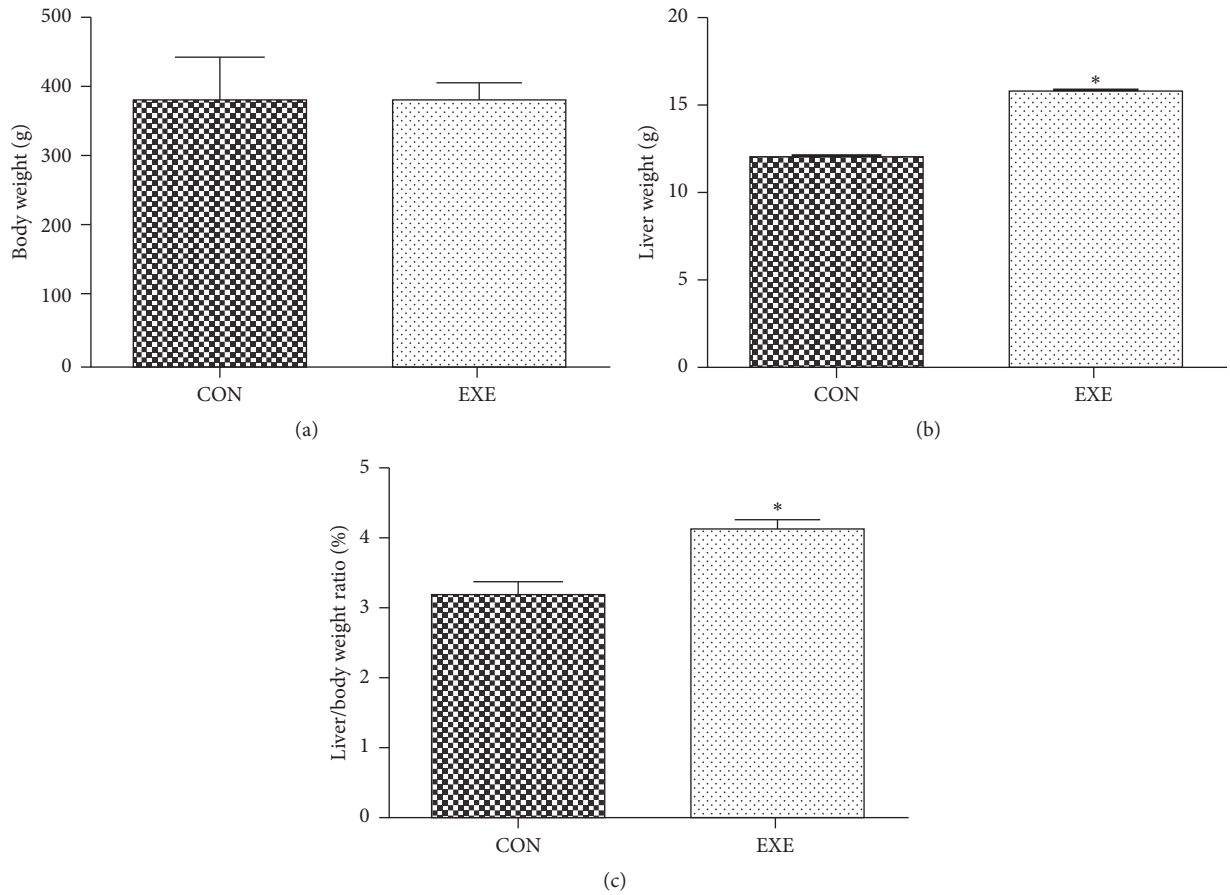


Figure 1: Body weight and ratio of liver weight to whole body weight. Body weight (a), liver weight (b), and liver/body weight (c). Values represent the mean ± SEM; $n = 8$ per group. $^{*}P < 0.05$ versus the control group.

2.4.3. Database Searching. Proteins were identified by peptide mass fingerprinting with the search engine programs MASCOT [25]. The criteria for positive identification of proteins were according to Perkins et al. [25] and were set as follows: (i) at least four matching peptide masses, (ii) 50 ppm or better mass accuracy, and (iii) matching of the molecular weights (Mr) and isoelectric points (pI) of the identified proteins to the estimated values obtained from image analysis.

2.5. Statistical Analysis. Statistical analysis was performed using predictive analytics software statistics 16.0 (SPSS Inc., Chicago, IL, USA). Comparisons across the experimental groups were performed using Student's t-test. For the analyses, the significance level was set at 5% ($P < 0.05$). Data are presented in the figures as the mean ± standard error of the mean (SEM).

3. Results

3.1. Liver Weights and Liver Weight/Body Weight Ratios between Sedentary and Exercised Aged Rats. The ratio of the weight of the liver to the whole animal body weight was determined. As shown in Figure 1, no difference in body weight was observed between exercised (EXE) and sedentary control (CON) groups. However, liver weight and liver weight/body weight ratio were higher for the livers of EXE animals than for those of the CON group ($P < 0.05$).

3.2. Effects of Treadmill Training on the Oxidative Status of the Livers of Aged Rats. Changes in the levels of hepatic MDA and GSH contents as well as in MnSOD activity in response to 8-week moderate-intensity treadmill training were analyzed. As shown in Figure 2, after 8 weeks of treadmill training, MnSOD activity, hepatic GSH level, and GSH/oxidized glutathione (GSSH) ratio in the EXE group were significantly higher in comparison to the CON group ($P < 0.05$, Figure 2), whereas the hepatic MDA and GSSH levels were decreased ($P < 0.01$, Figure 2).

3.3. Proteomics Comparison of Hepatic Expression Profiles between Sedentary and Exercised Aged Rats. The hepatic protein profiles are shown in Figure 3. Following gel image analysis, differentially expressed spots were selected and identified using MALDI-TOF MS when the normalized spot intensities showed differences of at least 2-fold between the groups. The

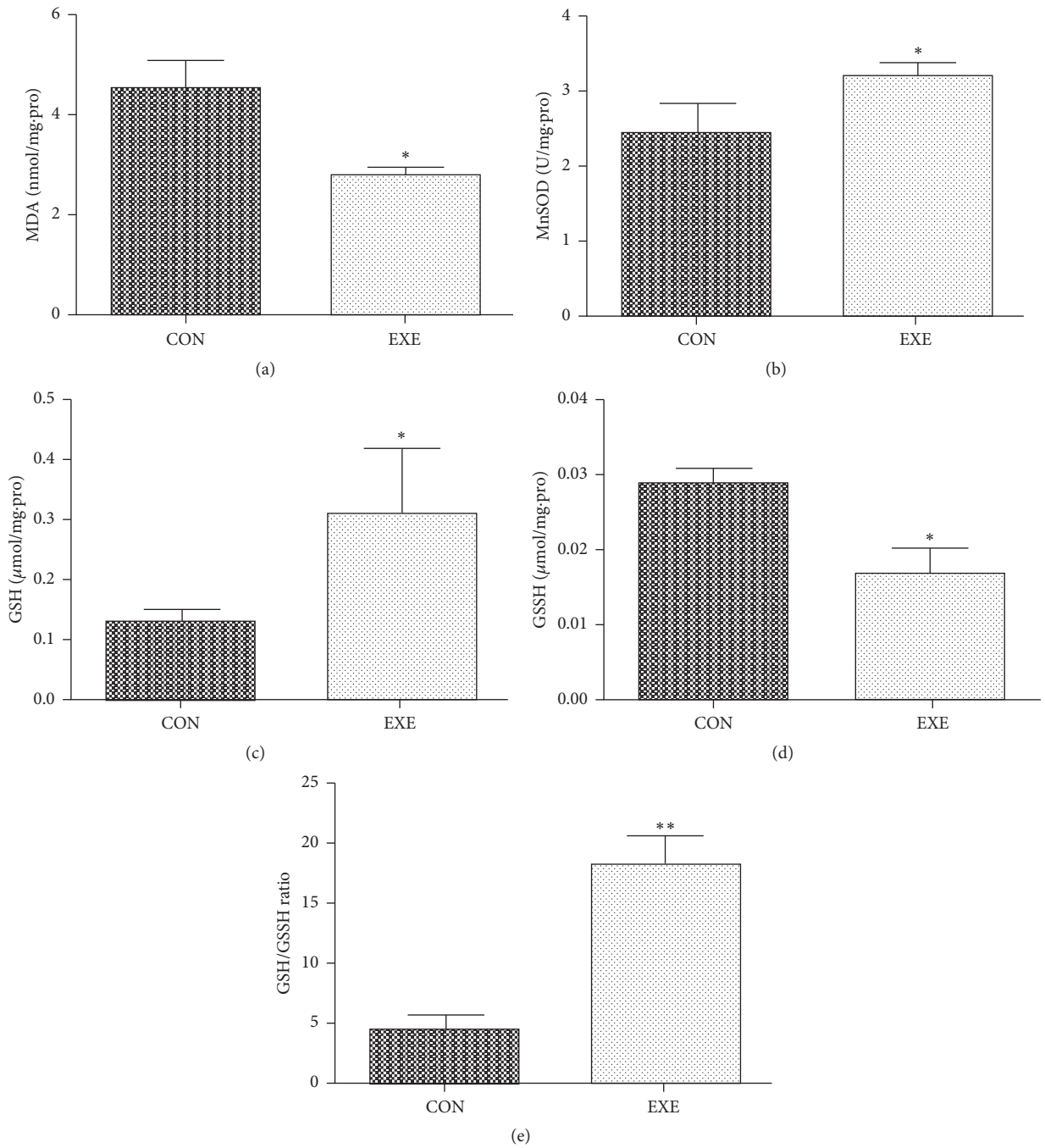


Figure 2: Antioxidant enzyme activity and MDA in the livers of control (CON) and exercised (EXE) groups. MDA (a), MnSOD (b), GSH (c), GSSH (d), and GSH/GSSH ratio (e). Values represent the mean ± SEM; $n = 8$ per group. $^{*}P < 0.05$ versus the control group, $^{**}P < 0.01$ versus the control group.

12 proteins identified as differentially expressed are listed in Table 1. The experimental ratios of the Mr and pIs matched those of the theoretical data, suggesting that the identification of proteins by our proteomic method was reliable.

4. Discussion

The liver is the primary organ required for processing of nutrients, hormones, and drugs; therefore, to evaluate exercise-dependent antiaging effects and adaptive alterations in the proteome of the livers of aged rats after 8 weeks of moderate-intensity training exercise, total liver tissue extracts from sedentary aged rats (22 months, control) and aged rats that were provided exercise training were resolved by 2DGE. 2DGE with medium-range IPGs (pH 3–10) revealed 3839 protein spots in the liver that were matched among all rats. Among these protein spots, 35 were found to be differentially expressed in the liver between sedentary and exercised rats, of

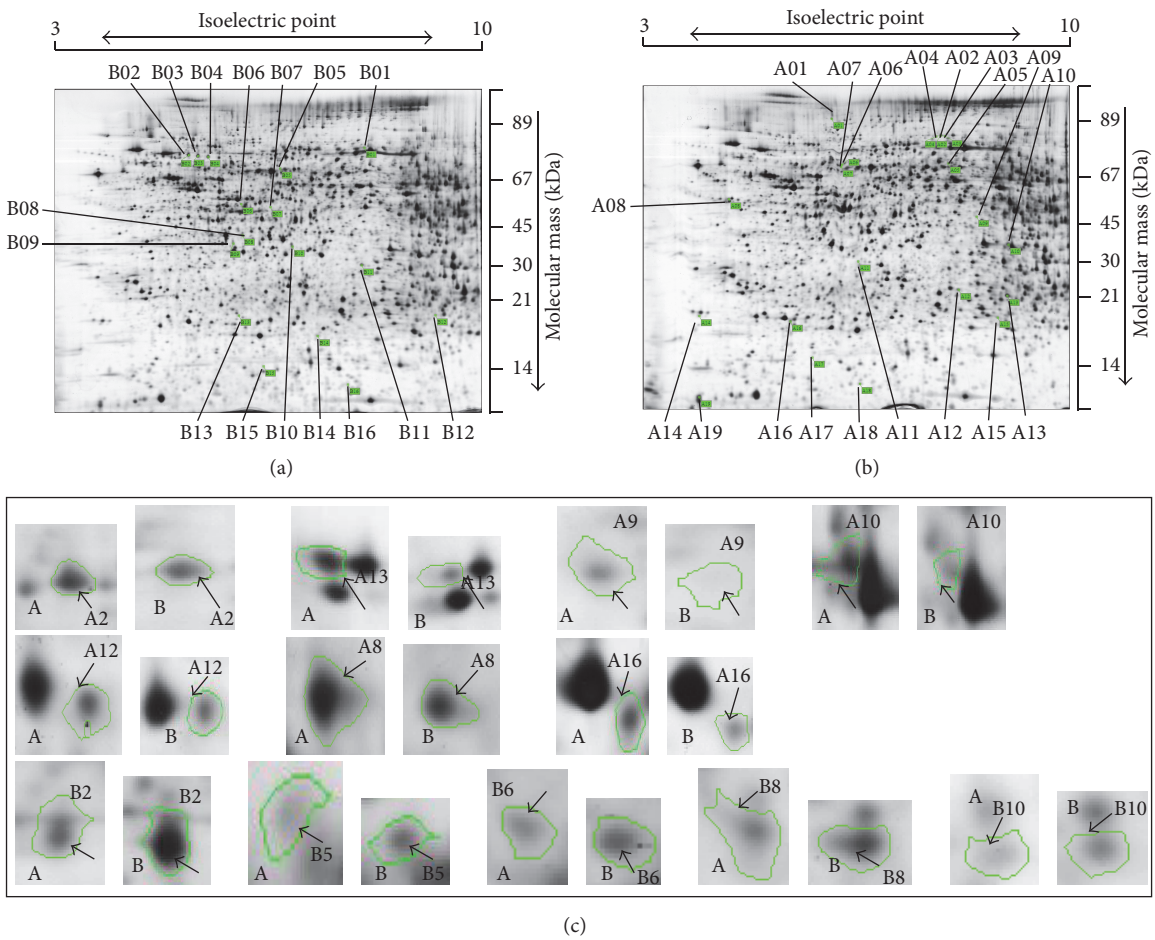


Figure 3: Two-dimensional gel electrophoresis (2DGE) image analysis of the liver of aged rats following exercise. (a) Sedentary control (CON) group. (b) Exercise training (EXE) group. Statistically significant spots are indicated by arrows in each gel; numbers 1–12 indicate the up- or downregulated proteins in the exercise group (c).

which 12 were identified by MALDI-TOF MS/MS (Figure 3). From these proteins, we elucidated that age-related hepatic proteome changes were effectively reversed through exercise, which might be beneficial to combat health conditions related to age-related abnormal liver morphology through regulation of mitochondrial energy metabolism, proteolytic systems, detoxification, and quality control of cellular and cytoskeletal proteins.

Among the identified proteins, FAS activity is believed to be a determinant of the maximal capacity of the liver to synthesize fatty acids by de novo lipogenesis. Advanced age has been associated with upregulation of FAS expression and enzymatic activity, which in turn has been found to be an underlying contributor to many age-related conditions including hepatic steatosis [26] and necroinflammation [27]. Aging is also associated with variable degrees of fibrosis in the liver. Our results showed that moderate-intensity training exercise decreased the abundance of FAS protein, a marker of hepatic fibrosis.

SAFB1 has been shown to be important in numerous cellular processes including cell growth, stress response, and apoptosis [28]. Studies performed by the Gelman group have shown that SAFB1 can repress the transcriptional activity of various other nuclear receptors including estrogen receptor [29], heat shock factor 1 [30], and p53 [31]. Additional studies identified that SAFB1 regulates the expression of xanthine oxidoreductase (XOR) [32], peroxisome proliferator-activator receptor (PPAR) gamma [33], and sterol regulatory element-binding protein- (SREBP-) 1c [33]. SREBP-1c and PPAR gamma are transcription factors that control the expression of hepatic genes involved in fatty acid and triglyceride synthesis during overnutrition to facilitate the conversion of glucose to fatty acids and triglycerides for the storage of the excess energy [34]. Additionally, in this context, it should be noted that SAFB1 was found to be downregulated by exercise, which is probably related to a decrease in lipogenesis as suggested by decreased abundance of the FAS protein. Furthermore, Lee et al. [35] showed that SAFB1 is also involved in the later stages of apoptosis, in which it is possibly associated with endonuclease–mediated DNA cleavage. These data clearly suggest that downregulation of SAFB1 in the liver might play an important role in age-related fatty acid metabolism, apoptosis, and oxidative stress, which are the primary causes of fatty liver disease.

Table 1: Proteins differentially expressed in aged rat livers after exercise training, determined using MALDI-TOF MS/MS.

Spot number	GI number	Sequence Cov. (%)	Measured Mr (kDa)/pI[a]	MOWSE score[b]	Protein identification	Fold change
A2	8394158	7	275101/5.96	197	Fatty acid synthase (FAS)	−2.08
A8	132573	21	51653/4.67	77	Ribonuclease/angiogenin inhibitor 1 (RNASE1)	−2.04
A9	158186651	20	47440/6.16	347	α-Enolase (ENO1)	−2.30
A10	19924087	22	37517/6.67	76	Hydroxyprostaglandin dehydrogenase (11β-HSD1)	−2.64
A12	734703982	10	112398/5.99	52	Scaffold attachment factor B (SAFB)	−3.13
A13	149056650	38	28234/6.78	81	Ethylmalonic encephalopathy protein 1 (ETHE1)	−2.20
A16	54019419	28	25502/4.85	215	Proteasome subunit beta type-6 (PSMB6)	+2.85
B2	80861401	10	48828/6.08	204	T-kininogen	+2.32
B5	730311	18	61650/6.30	149	Glucose phosphomutase (PGM)	+2.60
B6	205057	39	48828/5.83	204	Keratin 8 (K8)	+2.94
B8	77157805	16	44240/5.61	139	S-Adenosylmethionine synthetase isoform type-1 (SAMs)	+2.55
B10	59808182	12	39052/6.10	146	Haptoglobin (HP)	+2.14

[a]Theoretical pI and Mr derived from NCBInr and Swiss Prot/TrEML databases.
[b]Score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event, based on the NCBInr database using the MASCOT searching program as MALDI-TOF MS/MS data (http://www.matrixscience.com/).

In a previous study, perturbations in the energy metabolism of aged livers were indicated by the downregulation of several glycolytic enzymes [36]. Moreover, Rothwell and Sayce found that enhancement of glycolysis via PGM can protect cells from oxidative stress, thus preventing senescence [37]. The classical function of PGM is to link glycolysis and glycogen metabolism by interconverting glucose-1-phosphate and glucose-6-phosphate. PGM has been shown to be expressed ubiquitously; however, high levels of expression are observed in the heart, skeletal muscle, kidney, liver, and lungs [37]. The activity of PGM is under hormonal control and may play a physiological role in glucose metabolism and physical activity [38]. Notably, proteomics analysis has revealed decreased expression of PGM in elderly women [39]. Recent evidence indicates that downregulation of PGM is associated with hepatocyte failure in cirrhotic hepatocytes, especially at the late stages of cirrhosis [40]. In addition, Preisler et al. [41] implied that the liver might be affected by PGM deficiency and that reduced liver glucose levels might contribute to the low blood glucose levels observed during exercise in this condition. In this regard, polymorphism in PGM has been reported to be useful as a genetic biomarker for alcoholic cirrhosis [42], ischemic heart disease [43], and obesity disorders [44, 45]. The results of the current study show that training exercise increased the protein content of PGM in the livers of old rats, which likely contributed to the improved glycolytic metabolism of these animals, despite their advanced cirrhosis.

Notably, α-enolase1 (ENO1), a glycolytic enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, was found to be downregulated in the liver of old rats after moderate-intensity training exercise, as shown in Figure 3. ENO1 is highly expressed in liver tissue and in the cytoplasm of hyperplastic bile ducts [46]. In addition to its key contribution in the regulation of glycolysis and the generation of ATP, ENO1 also plays a crucial role in growth control, hypoxia tolerance, and allergic responses [47]. Notably, a previous study had used an immunoproteomics approach to identify and characterize ENO1 as an autoantigen in liver fibrosis [48]. Although it is unclear whether ENO1 plays an active role during the formation of liver fibrosis, it has also been reported that ENO1 is transcriptionally regulated by the c-Jun NH2-terminal kinase (JNK) signaling pathway, which is frequently activated in liver fibrosis [49]. Furthermore, ENO1 might act as an oxidative stress-related mediator to modulate the tissue and cellular events responsible for the progression of liver fibrosis [50]. Our results show a decreased protein content of ENO1 at 8 weeks after moderate-intensity training exercise in aged rats, in parallel with an increase in MnSOD activity and in the hepatic GSH level and GSH/GSSH ratio (Figure 2), which likely contributes to improved liver fibrosis and liver function. However, whether or not the improvement of liver function mediated by training exercise is associated with modulation of ENO1 in the livers of aged rats requires further study.

A previous study demonstrated that 11β-HSD1 catalyzed the intracellular regeneration of active glucocorticoids, promoting insulin resistance in the liver [51], and elevated mRNA levels for 11β-HSD1 in the liver were associated with aging [52]. Fatty liver occurs in mice that overexpress 11β-HSD1 as a result of increases in gluconeogenesis, fatty acid synthesis, and plasma insulin levels [53]. Thus, the age-dependent increase in the mRNA level of 11β-HSD1 is likely to induce insulin secretion and cause hyperinsulinemia. Recent evidence indicates that exercise protects against the elevations in glucocorticoids associated with the development of insulin

resistance by decreased adrenal sensitivity to the 11β-HSD1 content in the livers of diabetic fatty rats [54]. Consistent with these findings, aged rats that exhibited higher hippocampal corticosterone levels also showed increased 11β-HSD1 protein expression as well as increased levels of the enzyme that catalyzes glucocorticoid formation and greater hippocampal glucocorticoid receptor (GR) activation, whereas exercise as a therapeutic intervention significantly reduced total hippocampal GR and 11β-HSD1 expression in these animals [55]. Similarly, the results of the current study show that 11β-HSD1 protein expression significantly decreased after 8 weeks of aerobic exercise; as discussed above, this decrease in 11β-HSD1 expression is believed to play an important role in mitigating age-related fatty acid metabolism, gluconeogenesis, and hyperinsulinemia, which is a main cause of insulin resistance in the liver.

The liver is an important organ for H_2S production and clearance, and hepatic H_2S metabolism affects multiple metabolic processes. In contrast, malfunction of hepatic H_2S metabolism might be involved in the pathogenesis of many liver diseases such as hepatic fibrosis and cirrhosis [56]. The mitochondrial dioxygenase gene *ETHE1* encodes a mitochondrial enzyme involved in sulfide detoxification, which uses molecular O_2 and water to oxidize the mobile persulfide from quinone oxidoreductase to form sulfite, which in turn is involved in sulfur metabolism and takes part in the pathway in which H_2S is oxidized to thiosulfate in three steps [57]. Although the basal endogenous concentration of H_2S has been found to have a hepatoprotective effect [58], inhibition of this pathway prevents binding of H_2S to quinone oxidoreductase and enables the accumulation of H_2S and thiosulfate, which induces oxidative stress and hepatotoxicity (e.g., by inhibiting the respiratory chain and decreasing cellular NAD^+/NADH) in the liver during aging [59]. Thus, the decreased levels of ETHE1 in the rats fed a high-fat diet might allow the occurrence of higher concentrations of H_2S, which might mitigate some of the risks of high blood pressure and oxidative stress associated with obesity and aging [60]. However, ETHE1 is also an antiapoptotic protein that increases the deacetylase activity of p53 in association with histone deacetylase 1, leading to the suppression of apoptosis [61].

S-Adenosylmethionine (SAMe) is an important methyl donor in various methyltransferase reactions that occur in many lipid metabolic pathways. SAMs catalyzes the synthesis of SAMe from methionine in the presence of ATP, which is the major pathway in mammals for the metabolism of methionine [62]. Previous studies have indicated that a marked loss of SAMs was accompanied by a reduction of the concentration of SAMe in cirrhosis [62] and that treatment with SAMe increased hepatic GSH levels and increased survival in patients with less advanced alcoholic liver cirrhosis [63]. Our data therefore support that SAMs upregulates SAMe via exercise, which in turn increases the levels of hepatic GSH (Figure 2) with a concomitant decline in FAS [64] and in K8 [65] (discussed below), especially in the liver of aged rats.

RNH1 belongs to the leucine-rich repeats (LRR) motif protein superfamily [66] and is a tight-binding cytosolic inhibitor of the RNase A family with both ribonucleolytic and angiogenic activities that are essential for cell survival [67]. In addition, RNH1 is a known regulator of vascularization and a mediator of oxidative stress. Knockdown of RNH1 has been shown to significantly decrease cell survival under stress conditions, accompanied with an increase in cell apoptosis. The reported antioxidant [68] and redox homeostatic [69] effects might also contribute to the regulatory function of RNH1 in cell survival. These results suggest that overexpression of RNH1 has antioxidant effects that protect against hepatic damage and apoptosis induced by H_2O_2 in mice [68]. In this context, it should be noted that RNH1 was observed to be upregulated by exercise in the current study, which is likely related to the increase in antioxidant activity and antiapoptosis effects as suggested by the increase in MnSOD activity and the levels of hepatic GSH (Figure 2).

Tight regulation of proteostasis is essential for maintaining cellular homeostasis in postmitotic tissues [70]. Advanced age is an intrinsic process that results in alterations of tissue protein homoeostasis. These degenerative changes are intimately associated with abnormal protein aggregation and impaired protein degradation pathways. Direct interventions to modulate protein quality control mechanisms might therefore improve health and potentially increase lifespan. Proteasomes can be regarded as the major proteolytic enzymes responsible for the degradation of the majority of cellular proteins [71]. PSMB6 is a member of the 20S proteasomal subunit family, which forms the proteolytic core of the 26S proteasome [71]. Beta subunits are characterized as containing the active sites of proteases, whereas the subunits serve as docking domains for the regulatory particles and form a gate that blocks unregulated access of substrates to the central pore. The age-associated decrease in the activity of the 26S proteasomes, which are responsible for the degradation of ubiquitinated proteins, is in accordance with previous findings that high-molecular-weight ubiquitin-protein conjugates accumulate in various tissues over time [70].

In this context, it is interesting to note that, in addition to the increase in the activity of antioxidant enzymes known to occur in the liver of aged rats as a result of training exercise, the upregulation of proteasomes might also play a significant role in improved metabolism by reducing the amount of damaged proteins in the liver. Radák et al. [72] previously showed that regular moderate exercise initiated at advanced age and continued for two months in BDF1 mice resulted in significant upregulation of the activity of antioxidant enzymes and 26S proteasomes in the brain. Our findings are also in accordance with previous reports that exercise training mitigated to a substantial degree of oxidative stress caused by advanced age, with a concomitant increase in proteasomal peptidase activities in the brain.

K8, a cytoprotective stress protein, belongs to the keratin family of cytoskeletal intermediate filament proteins that play a central role in protecting hepatocytes from apoptosis; accordingly, K8 mutations predispose their carriers to liver cirrhosis [73]. Emerging evidence also indicates that K8 protects cells from nonmechanical injury by cellular survival kinases and cell cycle progression [74]. Subsequent studies

have suggested that keratins modulate apoptosis induced by FAS [73] and tumor necrosis factor [75]. In cirrhotic livers, K8 hyperphosphorylation was previously shown to occur preferentially in hepatic nodule cells adjacent to bridging fibrosis and was associated with increased stress kinase activation, oxidative injury, and susceptibility to undergo apoptosis [65]. Furthermore, according to Kucukoglu et al. [76], a high-fat diet triggers aggregate formation and the development of liver oxidative injury in susceptible individuals through the misfolding and cross-linking of excess K8. The possibility that the improvement in liver fibrosis and fatty liver mediated by exercise is associated with modulation of K8 in the livers of aged rats requires further study.

HP is produced as an acute-phase reactant during liver fibrosis (LF), and it possesses anti-inflammatory and antioxidative properties [77], preventing liver damage. Thus, HP might be a potential molecular target for early LF diagnostics and therapeutic applications [78]. In contrast, lack of HP attenuates the hepatomegaly/steatosis often associated with obesity. When animals were stressed by acute exercise or by a high-fat diet in a previous study, oxidative stress, muscle atrophy, and force drop were exacerbated in HP-knockout mice [79]. Conversely, recent data demonstrated that the increased HP levels in the serum in response to traumatic brain injury were derived from the liver and that IL-6 is an important mediator of this induction [80]. T-kininogen has also been demonstrated to be a cysteine proteinase inhibitor present in rat plasma [81]. In a previous report, preliminary evidence suggested that the degree of reduction in plasma T-kininogen levels was directly related to the severity of impaired liver function [81]. Recent results showed that aged rats present reduced expression of T-kininogen and HP and increased hepatic injury in response to an inflammatory insult [82]. Therefore, the upregulation of HP and T-kininogen following training exercise observed in this study might be the consequence of decreased levels of markers of inflammation and oxidative stress (Figure 2) in the livers of aged rats.

This study has several limitations. First, the changes in protein levels were not confirmed by western blot. Second, comprehensive data were not obtained regarding the phenotype of the aging liver, such as the presence of liver tissue histology, hepatocyte apoptosis, intrahepatic lipid accumulation, or cirrhosis. Third, we only measured levels of primary antioxidative enzymes (MnSOD and GSH), secondary antioxidative enzymes such as glutathione-*S*-transferase (GST) and glucose 6-phosphate dehydrogenase, and molecules such as glutathione.

In summary, we succeeded in objectively identifying 12 proteins that exhibited adaptive changes in their expression associated with extended exercise in the livers of aged rats. Identification and detailed analysis of the impacted proteins indicated that exercise training significantly altered the expression of several proteins involved in key liver metabolic pathways including sulfur, glycolysis, and methionine metabolism as well as general protein metabolism in conjunction with the modulation of overall hepatic protein expression profiles. These proteins are potential disease state- and intervention-specific biomarkers. Further research on these specific proteins is needed to uncover possible pathways involved in the beneficial effects of exercise on metabolic adaptations in the liver of aged rats.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

The current project was supported by the National Natural Science Foundation of China (Grant 31500961) and the Guangdong Scientific Project (Grant 2014A020220015 and Grant 2015A020219015) and partially supported by the Distinguished Young Talents in Higher Education of Guangdong, China (Grant 2014KQNCX225).

References

[1] N. M. Johannsen, D. L. Swift, C. J. Lavie, C. P. Earnest, S. N. Blair, and T. S. Church, "Combined aerobic and resistance training effects on glucose homeostasis, fitness, and other major health indices: a review of current guidelines," *Sports Medicine*, vol. 46, no. 12, pp. 1809–1818, 2016.

[2] P.-S. Lin, C.-C. Hsieh, H.-S. Cheng, T.-J. Tseng, and S.-C. Su, "Association between physical fitness and successful aging in taiwanese older adults," *PLoS ONE*, vol. 11, no. 3, Article ID e0150389, 2016.

[3] Z. Radak, Z. Zhao, E. Koltai, H. Ohno, and M. Atalay, "Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling," *Antioxidants and Redox Signaling*, vol. 18, no. 10, pp. 1208–1246, 2013.

[4] K. J. A. Davies, A. T. Quintanilha, G. A. Brooks, and L. Packer, "Free radicals and tissue damage produced by exercise," *Biochemical and Biophysical Research Communications*, vol. 107, no. 4, pp. 1198–1205, 1982.

[5] L. L. Ji, "Exercise-induced modulation of antioxidant defense," *Annals of the New York Academy of Sciences*, vol. 959, pp. 82–92, 2002.

[6] W. Dröge, "Aging-related changes in the thiol/disulfide redox state: implications for the use of thiol antioxidants," *Experimental Gerontology*, vol. 37, no. 12, pp. 1333–1345, 2002.

[7] X. Hu, Z. Duan, H. Hu et al., "Proteomic profile of carbonylated proteins in rat liver: exercise attenuated oxidative stress may be involved in fatty liver improvement," *Proteomics*, vol. 13, no. 10-11, pp. 1755–1764, 2013.

[8] L. L. Ji, C. Kang, and Y. Zhang, "Exercise-induced hormesis and skeletal muscle health," *Free Radical Biology and Medicine*, vol. 98, pp. 113–122, 2016.

[9] P. Wiklund, X. Zhang, S. Pekkala et al., "Insulin resistance is associated with altered amino acid metabolism and adipose tissue dysfunction in normoglycemic women," *Scientific Reports*, vol. 6, Article ID 24540, 11 pages, 2016.

[10] N. Houstis, E. D. Rosen, and E. S. Lander, "Reactive oxygen species have a causal role in multiple forms of insulin resistance," *Nature*, vol. 440, no. 7086, pp. 944–948, 2006.

[11] S. Goto, Z. Radák, C. Nyakas et al., "Regular exercise: an effective means to reduce oxidative stress in old rats," *Annals of the New York Academy of Sciences*, vol. 1019, pp. 471–474, 2004.

[12] Z. Radak, H. Y. Chung, H. Naito et al., "Age-associated increase in oxidative stress and nuclear factor kappaB activation are attenuated in rat liver by regular exercise," *The FASEB Journal*, vol. 18, no. 6, pp. 749–750, 2004.

[13] R. J. F. Loos, J. M. Hagberg, L. Pérusse et al., "Advances in exercise, fitness, and performance genomics in 2014," *Medicine & Science in Sports and Exercise*, vol. 47, no. 6, pp. 1105–1112, 2015.

[14] H. Yuan, Y. Niu, X. Liu et al., "Proteomic analysis of skeletal muscle in insulin-resistant mice: response to 6-week aerobic exercise," *PLoS ONE*, vol. 8, no. 1, Article ID e53887, 2013.

[15] R. Liu, J. W. Kenney, A. Manousopoulou et al., "Quantitative non-canonical amino acid tagging (QuaNCAT) proteomics identifies distinct patterns of protein synthesis rapidly induced by hypertrophic agents in cardiomyocytes, revealing new aspects of metabolic remodeling," *Molecular & Cellular Proteomics*, vol. 15, no. 10, pp. 3170–3189, 2016.

[16] M. K. Nøhr, T. P. Kroager, K. W. Sanggaard et al., "SILAC-MS based characterization of lps and resveratrol induced changes in adipocyte proteomics—resveratrol as ameliorating factor on LPS induced changes," *PLoS ONE*, vol. 11, no. 7, Article ID e0159747, 2016.

[17] G. Gat-Yablonski, A. Finka, G. Pinto, M. Quadroni, B. Shtaif, and P. Goloubinoff, "Quantitative proteomics of rat livers shows that unrestricted feeding is stressful for proteostasis with implications on life span," *Aging*, vol. 8, no. 8, pp. 1735–1758, 2016.

[18] S. Yoon, J. Lee, and S.-M. Lee, "Combined treatment of isoflavone supplementation and exercise restores the changes in hepatic protein expression in ovariectomized rats—a proteomics approach," *Journal of the International Society of Sports Nutrition*, vol. 11, no. 29, pp. 1–8, 2014.

[19] J. R. Zierath and H. Wallberg-Henriksson, "Looking ahead perspective: where will the future of exercise biology take us?" *Cell Metabolism*, vol. 22, no. 1, pp. 25–30, 2015.

[20] F. Li, H. Yu, L. Xiao, and Y. Liu, "Response of BAX, Bcl-2 proteins, and SIRT1/PGC-1α mRNA expression to 8-week treadmill running in the aging rat skeletal muscle," in *Oxygen Transport to Tissue XXXVIII*, vol. 923 of *Advances in Experimental Medicine and Biology*, pp. 283–289, Springer, 2016.

[21] W. Song, H.-B. Kwak, and J. M. Lawler, "Exercise training attenuates age-induced changes in apoptotic signaling in rat skeletal muscle," *Antioxidants & Redox Signaling*, vol. 8, no. 3-4, pp. 517–528, 2006.

[22] P. M. Siu, R. W. Bryner, J. K. Marty, and S. E. Alway, "Apoptotic adaptations from exercise training in skeletal and cardiac muscles," *FASEB Journal*, vol. 18, no. 10, pp. 1150–1152, 2004.

[23] J. Bejma, R. Ramires, and L. L. Ji, "Free radical generation and oxidative stress with ageing and exercise: differential effects in the myocardium and liver," *Acta Physiologica Scandinavica*, vol. 169, no. 4, pp. 343–351, 2000.

[24] B. Sun, J. H. Wang, Y. Y. Lv, S. S. Zhu, J. Yang, and J. Z. Ma, "Proteomic adaptation to chronic high intensity swimming training in the rat heart," *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, vol. 3, no. 1, pp. 108–117, 2008.

[25] D. N. Perkins, D. J. C. Pappin, D. M. Creasy, and J. S. Cottrell, "Probability-based protein identification by searching sequence databases using mass spectrometry data," *Electrophoresis*, vol. 20, no. 18, pp. 3551–3567, 1999.

[26] A. Kuhla, T. Blei, R. Jaster, and B. Vollmar, "Aging is associated with a shift of fatty metabolism toward lipogenesis," *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, vol. 66, no. 11, pp. 1192–1200, 2011.

[27] L. A. Finlay, A. J. Michels, J. A. Butler et al., "R-α-lipoic acid does not reverse hepatic inflammation of aging, but lowers lipid anabolism, while accentuating circadian rhythm transcript profiles," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 302, no. 5, pp. R587–R597, 2012.

[28] J. P. Garee and S. Oesterreich, "SAFB1's multiple functions in biological control—lots still to be done!," *Journal of Cellular Biochemistry*, vol. 109, no. 2, pp. 312–319, 2010.

[29] S. Oesterreich, Q. P. Zhang, and A. V. Lee, "Inhibition of oestrogen receptor activity by the co-repressor HET/SAF-B is relieved by blockade of histone deacetylase activity," *The European Journal of Cancer*, vol. 36, supplement 4, pp. S43–S44, 2000.

[30] F. Weighardt, F. Cobianchi, L. Cartegni et al., "A novel hnRNP protein (HAP/SAF-B) enters a subset of hnRNP complexes and relocates in nuclear granules in response to heat shock," *Journal of Cell Science*, vol. 112, no. 10, pp. 1465–1476, 1999.

[31] P. Peidis, N. Voukkalis, E. Aggelidou et al., "SAFB1 interacts with and suppresses the transcriptional activity of p53," *FEBS Letters*, vol. 585, no. 1, pp. 78–84, 2011.

[32] J. Lin, P. Xu, P. LaVallee, and J. R. Hoidal, "Identification of proteins binding to E-Box/Ku86 sites and function of the tumor suppressor SAFB1 in transcriptional regulation of the human xanthine oxidoreductase gene," *Journal of Biological Chemistry*, vol. 283, no. 44, pp. 29681–29689, 2008.

[33] M.-B. Debril, L. Dubuquoy, J.-N. Feige et al., "Scaffold attachment factor B1 directly interacts with nuclear receptors in living cells and represses transcriptional activity," *Journal of Molecular Endocrinology*, vol. 35, no. 3, pp. 503–517, 2005.

[34] Y. Omura, Y. Nishio, T. Takemoto et al., "SAFB1, an RBMX-binding protein, is a newly identified regulator of hepatic SREBP-1c gene," *BMB Reports*, vol. 42, no. 4, pp. 232–237, 2009.

[35] Y.-B. Lee, S. Colley, M. Norman, G. Biamonti, and J. B. Uney, "SAFB re-distribution marks steps of the apoptotic process," *Experimental Cell Research*, vol. 313, no. 18, pp. 3914–3923, 2007.

[36] H. Kondoh, M. E. Lleonart, D. Bernard, and J. Gil, "Protection from oxidative stress by enhanced glycolysis; a possible mechanism of cellular immortalization," *Histology and Histopathology*, vol. 22, no. 1, pp. 85–90, 2007.

[37] T. J. Rothwell and M. D. Sayce, "The stability of PGM and AK isoenzymes in human tissues," *Journal of Forensic Sciences*, vol. 19, no. 3, pp. 590–598, 1974.

[38] T. Stojkovic, J. Vissing, F. Petit et al., "Muscle glycogenosis due to phosphoglucomutase 1 deficiency," *New England Journal of Medicine*, vol. 361, no. 4, pp. 425–427, 2009.

[39] M. Gueugneau, C. Coudy-Gandilhon, O. Gourbeyre et al., "Proteomics of muscle chronological ageing in post-menopausal women," *BMC Genomics*, vol. 15, article 1165, 24 pages, 2014.

[40] T. Nishikawa, N. Bellance, A. Damm et al., "A switch in the source of ATP production and a loss in capacity to perform glycolysis are hallmarks of hepatocyte failure in advance liver disease," *Journal of Hepatology*, vol. 60, no. 6, pp. 1203–1211, 2014.

[41] N. Preisler, P. Laforêt, A. Echaniz-Laguna et al., "Fat and carbohydrate metabolism during exercise in phosphoglucomutase type 1 deficiency," *The Journal of Clinical Endocrinology and Metabolism*, vol. 98, no. 7, pp. E1235–E1240, 2013.

[42] V. A. Spitsyn, A. K. Nafikova, N. K. Spitsynaet et al., "Genetic predisposition to development of toxic liver cirrhosis caused by alcohol," *Genetika*, vol. 37, no. 5, pp. 698–707, 2001.

[43] M. Nishinari, N. Aoyama, Z. Ogawa et al., "Phosphoglucomutase activity as a novel biomarker in patients with acute myocardial infarction," *Circulation Journal*, vol. 76, no. 9, pp. 2197–2203, 2012.

[44] F. Gloria-Bottini, A. Magrini, E. Antonacci et al., "Phosphoglucomutase genetic polymorphism and body mass," *American Journal of the Medical Sciences*, vol. 334, no. 6, pp. 421–425, 2007.

[45] S. Krähenbühl, S. Krähenbühl-Glauser, J. Stucki, P. Gehr, and J. Reichen, "Stereological and functional analysis of liver mitochondria from rats with secondary biliary cirrhosis: impaired mitochondrial metabolism and increased mitochondrial content per hepatocyte," *Hepatology*, vol. 15, no. 6, pp. 1167–1172, 1992.

[46] M. Capello, S. Ferri-Borgogno, P. Cappello, and F. Novelli, "α-Enolase: a promising therapeutic and diagnostic tumor target," *The FEBS Journal*, vol. 278, no. 7, pp. 1064–1074, 2011.

[47] V. Pancholi, "Multifunctional α-enolase: its role in diseases," *Cellular and Molecular Life Sciences*, vol. 58, no. 7, pp. 902–920, 2001.

[48] B. Peng, X. Huang, E. S. Nakayasu et al., "Using immunoproteomics to identify alpha-enolase as an autoantigen in liver fibrosis," *Journal of Proteome Research*, vol. 12, no. 4, pp. 1789–1796, 2013.

[49] J. Kluwe, J.-P. Pradere, G.-Y. Gwak et al., "Modulation of hepatic fibrosis by c-Jun-N-terminal kinase inhibition," *Gastroenterology*, vol. 138, no. 1, pp. 347–359, 2010.

[50] G. Poli, "Pathogenesis of liver fibrosis: role of oxidative stress," *Molecular Aspects of Medicine*, vol. 21, no. 3, pp. 49–98, 2000.

[51] D. E. W. Livingstone and B. R. Walker, "Is 11β-hydroxysteroid dehydrogenase type 1 a therapeutic target? Effects of carbenoxolone in lean and obese Zucker rats," *Journal of Pharmacology and Experimental Therapeutics*, vol. 305, no. 1, pp. 167–172, 2003.

[52] T. Honma, N. Shinohara, J. Ito et al., "High-fat diet intake accelerates aging, increases expression of Hsd11b1, and promotes lipid accumulation in liver of SAMP10 mouse," *Biogerontology*, vol. 13, no. 2, pp. 93–103, 2012.

[53] J. M. Paterson, N. M. Morton, C. Fievet et al., "Metabolic syndrome without obesity: hepatic overexpression of 11β-hydroxysteroid dehydrogenase type 1 in transgenic mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 18, pp. 7088–7093, 2004.

[54] J. E. Campbell, M. A. Király, D. J. Atkinson, A. M. D'Souza, M. Vranic, and M. C. Riddell, "Regular exercise prevents the development of hyperglucocorticoidemia via adaptations in the brain and adrenal glands in male Zucker diabetic fatty rats," *American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 299, no. 1, pp. R168–R176, 2010.

[55] R. M. Barrientos, V. M. Thompson, M. M. Kitt et al., "Greater glucocorticoid receptor activation in hippocampus of aged rats sensitizes microglia," *Neurobiology of Aging*, vol. 36, no. 3, pp. 1483–1495, 2015.

[56] S. Mani, W. Cao, L. Wu, and R. Wang, "Hydrogen sulfide and the liver," *Nitric Oxide*, vol. 41, pp. 62–71, 2014.

[57] V. Tiranti, C. Viscomi, T. Hildebrandt et al., "Loss of ETHE1, a mitochondrial dioxygenase, causes fatal sulfide toxicity in ethylmalonic encephalopathy," *Nature Medicine*, vol. 15, no. 2, pp. 200–205, 2009.

[58] D. Mancardi, C. Penna, A. Merlino, P. Del Soldato, D. A. Wink, and P. Pagliaro, "Physiological and pharmacological features of the novel gasotransmitter: hydrogen sulfide," *Biochimica et Biophysica Acta—Bioenergetics*, vol. 1787, no. 7, pp. 864–872, 2009.

[59] B. L. Predmore, M. J. Alendy, K. I. Ahmed, C. Leeuwenburgh, and D. Julian, "The hydrogen sulfide signaling system: changes during aging and the benefits of caloric restriction," *Age*, vol. 32, no. 4, pp. 467–481, 2010.

[60] I. Baiges, J. Palmfeldt, C. Bladé, N. Gregersen, and L. Arola, "Lipogenesis is decreased by grape seed proanthocyanidins according to liver proteomics of rats fed a high fat diet," *Molecular & Cellular Proteomics*, vol. 9, no. 7, pp. 1499–1513, 2010.

[61] H. Higashitsuji, H. Higashitsuji, T. Nagao et al., "A novel protein overexpressed in hepatoma accelerates export of NF-κB from the nucleus and inhibits p53-dependent apoptosis," *Cancer Cell*, vol. 2, no. 4, pp. 335–346, 2002.

[62] C. Cabrero, A. M. Duce, P. Ortiz, S. Alemany, and J. M. Mato, "Specific loss of the high-molecular-weight form of S-adenosyl-L-methionine synthetase in human liver cirrhosis," *Hepatology*, vol. 8, no. 6, pp. 1530–1534, 1988.

[63] J. M. Mato, F. J. Corrales, S. C. Lu, and M. A. Avila, "S-adenosylmethionine: a control switch that regulates liver function," *The FASEB Journal*, vol. 16, no. 1, pp. 15–26, 2002.

[64] C. Dahlhoff, S. Worsch, M. Sailer et al., "Methyl-donor supplementation in obese mice prevents the progression of NAFLD, activates AMPK and decreases acyl-carnitine levels," *Molecular Metabolism*, vol. 3, no. 5, pp. 565–580, 2014.

[65] Q. Zhou, X. Ji, L. Chen, H. B. Greenberg, S. C. Lu, and M. B. Omary, "Keratin mutation primes mouse liver to oxidative injury," *Hepatology*, vol. 41, no. 3, pp. 517–525, 2005.

[66] D. Nadano, T. Yasuda, H. Takeshita, K. Uchide, and K. Kishi, "Purification and characterization of human brain ribonuclease inhibitor," *Archives of Biochemistry and Biophysics*, vol. 312, no. 2, pp. 421–428, 1994.

[67] E. Pizzo, C. Sarcinelli, J. Sheng et al., "Ribonuclease/angiogenin inhibitor 1 regulates stress-induced subcellular localization of angiogenin to control growth and survival," *Journal of Cell Science*, vol. 126, no. 18, pp. 4308–4319, 2013.

[68] X. Y. Cui, P. F. Fu, D. N. Pan, Y. Zhao, J. Zhao, and B. C. Zhao, "The antioxidant effects of ribonuclease inhibitor," *Free Radical Research*, vol. 37, no. 10, pp. 1079–1085, 2003.

[69] D. M. Monti, N. Montesano Gesualdi, J. Matoušek, F. Esposito, and G. D'Alessio, "The cytosolic ribonuclease inhibitor contributes to intracellular redox homeostasis," *FEBS Letters*, vol. 581, no. 5, pp. 930–934, 2007.

[70] S. Goto, R. Takahashi, A. Kumiyama et al., "Implications of protein degradation in aging," *Annals of the New York Academy of Sciences*, vol. 928, pp. 54–64, 2001.

[71] Z. Radák, R. Takahashi, A. Kumiyama et al., "Effect of aging and late onset dietary restriction on antioxidant enzymes and proteasome activities, and protein carbonylation of rat skeletal muscle and tendon," *Experimental gerontology*, vol. 37, no. 12, pp. 1423–1430, 2002.

[72] Z. Radák, T. Kaneko, S. Tahara et al., "Regular exercise improves cognitive function and decreases oxidative damage in rat brain," *Neurochemistry International*, vol. 38, no. 1, pp. 17–23, 2001.

[73] N.-O. Ku, R. M. Soetikno, and M. B. Omary, "Keratin mutation in transgenic mice predisposes to Fas but not TNF-induced apoptosis and massive liver injury," *Hepatology*, vol. 37, no. 5, pp. 1006–1014, 2003.

[74] J. D. Rotty, G. W. Hart, and P. A. Coulombe, "Stressing the role of O-GlcNAc: linking cell survival to keratin modification," *Nature Cell Biology*, vol. 12, no. 9, pp. 847–849, 2010.

[75] C. Caulin, C. F. Ware, T. M. Magin, and R. G. Oshima, "Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis," *The Journal of Cell Biology*, vol. 149, no. 1, pp. 17–22, 2000.

[76] O. Kucukoglu, N. Guldiken, Y. Chen et al., "High-fat diet triggers Mallory-Denk body formation through misfolding and crosslinking of excess keratin 8," *Hepatology*, vol. 60, no. 1, pp. 169–178, 2014.

[77] A. Jelena, M. Mirjana, B. Desanka et al., "Haptoglobin and the inflammatory and oxidative status in experimental diabetic rats: antioxidant role of haptoglobin," *Journal of Physiology and Biochemistry*, vol. 69, no. 1, pp. 45–58, 2013.

[78] A. Zhang, H. Sun, W. Sun, Y. Ye, and X. Wang, "Proteomic identification network analysis of haptoglobin as a key regulator associated with liver fibrosis," *Applied Biochemistry and Biotechnology*, vol. 169, no. 3, pp. 832–846, 2013.

[79] E. Bertaggia, G. Scabia, S. Dalise et al., "Haptoglobin is required to prevent oxidative stress and muscle atrophy," *PLoS ONE*, vol. 9, no. 6, Article ID e100745, 2014.

[80] S. Yang, Y. Ma, Y. Liu, H. Que, C. Zhu, and S. Liu, "Elevated serum haptoglobin after traumatic brain injury is synthesized mainly in liver," *Journal of Neuroscience Research*, vol. 91, no. 2, pp. 230–239, 2013.

[81] C. Henkel, K. Schwamborn, H. W. Zimmermann et al., "From proteomic multimarker profiling to interesting proteins: Thymosin-β_4 and kininogen-1 as new potential biomarkers for inflammatory hepatic lesions," *Journal of Cellular and Molecular Medicine*, vol. 15, no. 10, pp. 2176–2188, 2011.

[82] C. R. Gomez, C. Acuña-Castillo, C. Pérez et al., "Diminished acute phase response and increased hepatic inflammation of aged rats in response to intraperitoneal injection of lipopolysaccharide," *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, vol. 63, no. 12, pp. 1299–1306, 2008.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 3453926, 13 pages
http://dx.doi.org/10.1155/2016/3453926

Review Article

Naturally Occurring Nrf2 Activators: Potential in Treatment of Liver Injury

Ravirajsinh N. Jadeja,[1] Kapil K. Upadhyay,[2] Ranjitsinh V. Devkar,[2] and Sandeep Khurana[1]

[1] *Digestive Health Center, Medical College of Georgia, Augusta, GA 30912, USA*
[2] *Division of Phytotherapeutics and Metabolic Endocrinology, Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat 390002, India*

Correspondence should be addressed to Sandeep Khurana; skhurana@augusta.edu

Received 17 June 2016; Revised 8 November 2016; Accepted 28 November 2016

Academic Editor: Javier Egea

Oxidative stress plays a major role in acute and chronic liver injury. In hepatocytes, oxidative stress frequently triggers antioxidant response by activating nuclear erythroid 2-related factor 2 (Nrf2), a transcription factor, which upregulates various cytoprotective genes. Thus, Nrf2 is considered a potential therapeutic target to halt liver injury. Several studies indicate that activation of Nrf2 signaling pathway ameliorates liver injury. The hepatoprotective potential of naturally occurring compounds has been investigated in various models of liver injuries. In this review, we comprehensively appraise various phytochemicals that have been assessed for their potential to halt acute and chronic liver injury by enhancing the activation of Nrf2 and have the potential for use in humans.

1. Introduction

Liver has an extraordinary capacity to detoxify compounds that have potential to induce liver injury. As a consequence, the liver is also vulnerable to injury. Although liver injury is a major cause of morbidity and mortality, medical therapies to prevent hepatocyte loss or protect hepatocytes are limited. One example is N-acetylcysteine (NAC), which is used for the treatment of acetaminophen- (APAP-) induced liver injury to promote recovery and reduce the need for liver transplantation. Hence, it is imperative to identify better medical therapies that are hepatoprotective and have minimal side effects.

Naturally occurring compounds have long been used as potential hepatoprotective agents. Drafts such as Ayurveda and traditional Japanese, Chinese, and Kampo (traditional Chinese medicine but adapted to the Japanese culture) medicine recommended the use of formulations of specific plants and fruits in the treatment of liver diseases [1–4]. In recent years, technological advances led to the isolation of active phytochemicals, which are now available as potential therapeutic agents [5, 6].

Oxidative stress is a major factor in the mechanism underlying liver diseases. It contributes to the initiation as well as progression of liver injury [7]. Factors such as alcohol, drugs, heavy metals, and high-fat diet are now identified as inducers of hepatic oxidative stress [8]. In liver injury, hepatocytes, the key parenchymal cells, suffer oxidative stress the most. In response to oxidative stress, Kupffer cells produce a variety of cytokines which contribute to hepatocyte apoptosis [9]. Oxidative stress also induces proliferation of stellate cells and collagen synthesis, thus promoting fibrosis and cirrhosis. In response to overwhelming oxidative stress, there is a significant use of antioxidant proteins, along with an increase in lipid peroxidation. However, to maintain redox homeostasis, hepatocytes have a sophisticated antioxidant system comprising antioxidant proteins, enzymes, and transcription factors to combat oxidative stress. Hence, regulation of hepatic oxidative stress can play a critical role in the treatment of various liver diseases.

Nuclear erythroid 2-related factor 2 (Nrf2), a transcription factor of the cap'n'collar basic leucine zipper family [10], is a key regulator of oxidative stress in numerous cell types including hepatocytes [11–16]. Nrf2 is primarily regulated

by Kelch-like ECH-associated protein 1 (Keap1), a substrate adaptor for a cul3-containing E3 ubiquitin ligase [17]. In the absence of oxidative stress, Nrf2 is located in the cytoplasm where it interacts with Keap1 and is rapidly degraded by the ubiquitin-proteasome pathway [18, 19]. However, under oxidative stress, phosphorylation of Nrf2 leads to its dissociation from Keap1 and subsequent translocation to the nucleus [14, 15, 19]. Herein, it binds to antioxidant response element (ARE) sequences and, in partnership with other nuclear proteins, enhances the transcription of ARE-responsive genes such as hemeoxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione-S-transferases (GST), glutamate-cysteine ligase modifier subunit (GCLM), glutathione peroxidase (GPX), and glutamate-cysteine ligase catalytic subunit (GCLC) to mount strong antioxidant and cytoprotective responses [20, 21].

Numerous studies have shown that natural products regulate oxidative stress in the liver by modulating Nrf2-ARE pathway to render hepatoprotective effect. This review discusses the importance of Nrf2-ARE in regulating liver injury and the role of natural product based activators (phytochemicals) of Nrf2-ARE pathway in treating liver injury (Table 1).

2. Nrf2 Signaling in Acetaminophen-Induced Hepatotoxicity

APAP is one of the most widely used over-the-counter analgesics. APAP is safe when taken at therapeutic doses but causes severe liver injury when ingested in higher-than-recommended doses. Acute liver failure due to APAP overdose is associated with high mortality [22]. In the United States, the incidence of APAP overdose is over 100,000 cases each year [23]. When ingested in therapeutic doses, APAP is mainly metabolized by sulfation and glucuronidation, leaving only a small fraction to be metabolized by cytochrome p4502E1 (CYP2E1) [24]. However, upon overdose, glucuronidation and sulfation pathways get saturated leading to APAP's metabolism by CYP2E1, resulting in generation of N-acetyl-p-benzoquinone imine (NAPQI), a toxic intermediate metabolite capable of inducing oxidative stress. NAPQI depletes hepatic glutathione (GSH) content and binds to cellular proteins, with subsequent activation of c-Jun N-terminal kinase (JNK). JNK activation results in overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) resulting in mitochondrial dysfunction and hepatocyte death [25–27]. Within 60 minutes of administration of APAP in mice, there is an increase in Nrf2 nuclear translocation in hepatocytes with concomitant increase in the expression of several Nrf2 target genes [28, 29]. Studies using Nrf2-knockout mice showed that even lower doses of APAP could induce mortality due to suppressed GSH synthesis pathway [30]. Contrary to this, hepatocyte-specific deletion of Keap1 activates Nrf2 and confers resistance against APAP toxicity [31]. Interestingly, in Nrf2-knockout mice, the elimination of APAP metabolites is also decreased as a result of reduced expression of multidrug resistance proteins (MRPs) [32]. And, in Keap1-knockout mice, MRP expression is increased, which enhanced efflux of APAP metabolites [29]. These observations indicate that, in APAP-induced hepatotoxicity, Nrf2 modulates injury not only by regulating antioxidant response but also by modulating APAP elimination.

Protein tyrosine phosphorylation is pivotal in cell survival. In this context, the balance between tyrosine kinases and phosphatases determines cell fate. One of the protein tyrosine phosphatases, protein tyrosine phosphatase 1B (PTP1B), is widely expressed and inactivates many tyrosine kinase family members by dephosphorylation. During APAP toxicity in mice, PTP1B expression is significantly increased. PTP1B deficient mouse hepatocytes are protected against APAP-induced GSH depletion and oxidative stress by prolonged Nrf2 nuclear accumulation [33].

Various classes of phytochemicals have been shown to activate Nrf2 pathway and reduce APAP toxicity (Table 1). Sauchinone, a polyphenol, reduces the impact of APAP overdose by enhancing Nrf2 phosphorylation via protein kinase C-δ (PKCδ) and decreasing interaction amongst Nrf2 and Keap1 [34]. Salvianolic acid B reduces APAP-induced liver injury via phosphoinositide-3-kinase- (PI3K-) and PKC-mediated Nrf2 activation, resulting in enhanced HO-1 and GCLC expressions [35]. Sulforaphane (an isothiocyanate found in cruciferous vegetables, namely, cauliflower, broccoli, kale, cole crops, cabbage, collards, brussels sprouts, and mustard) and oleanolic acid (a triterpenoid found in olives) both reduce APAP-induced oxidative stress and liver injury via Nrf2 and related antioxidant gene activation [36, 37]. A recent study from our laboratory evaluated the hepatoprotective potential of withaferin A, an active ingredient of *Withania somnifera*. We observed that withaferin A treatment 1 h *after* APAP intoxication activates Nrf2 responsive genes such as NQO1 and GCLC to reduce oxidative stress and subsequent liver injury [38]. An in vitro study, using rat hepatocytes, reported that ginsenoside Rg3 (a ginseng saponin) upregulates antioxidant genes (GCLC, GCLM) and basolateral MRPs via Nrf2 activation [39].

Currently, NAC is the only FDA-approved antidote for APAP hepatotoxicity and is most effective within 8–10 h after APAP overdose. Various studies that utilized phytochemicals to reduce APAP toxicity provide a strong proof of concept that naturally existing activators of Nrf2 can be used to reduce APAP-induced hepatotoxicity. However, these studies have to be interpreted carefully. Many studies have used a preventive experimental strategy, where the phytochemical was administered before inducing APAP overdose, or simultaneously, both unlikely clinical scenarios; patients seek care after APAP overdose. Moreover, such strategies suggest that phytochemicals may interfere with APAP metabolism by suppressing CYP2E1 activity and preventing generation of NAPQI, thus making the interpretation of these studies difficult [40, 41]. In our recent work, we employed an experimental strategy that was clinically relevant, that is, administering WA 1 h after APAP overdose, which provides sufficient time for APAP's metabolism into NAPQI and generation of protein adducts to adequately mimic clinical APAP hepatotoxicity in humans [38, 42]. Therefore, additional studies that not only employ clinically relevant strategies but also use a combination of Nrf2 activators and NAC to combat APAP toxicity are warranted.

Table 1: Various Nrf2 activator phytochemicals and their role in liver injury.

Phytochemicals	Effective doses	Experimental procedure (injury model)	Outcomes	References
Acetaminophen toxicity				
Ginsenoside Rg3	3 μg/mL	Rat hepatocytes treated with 200 μM NAPQI	Repletion of GSH content and enhanced expression of Mrp expression	[39]
Oleanolic acid	90 mg/kg	Mice injected with 330 μmol/kg APAP into the right femoral vein	Enhanced antioxidant response to reduce hepatocyte necrosis	[37]
Salvianolic acid B	25 and 50 mg/kg	Mice treated with single dose of 300 mg/kg APAP (i.g.)	Antioxidant response and phase II enzyme induction via activation of PI3K/Akt and PKC signaling to reduce liver injury	[35]
Sauchinone	30 mg/kg	Mice treated with single dose of 500 mg/kg APAP (i.p.)	Induction of antioxidant genes to reduce hepatocyte necrosis	[34]
Oleanolic acid	5 mg/kg	Mice treated with single dose of 300 mg/kg APAP (i.p.)	Reduction of ROS generation, GSH depletion, and lipid peroxidation coupled with upregulation of antioxidant genes	[36]
Withaferin A	40 mg/kg	Mice treated with single dose of 250 mg/kg APAP (i.p.)	Reduced hepatocyte injury by reducing GSH depletion	[38]
Inflammatory injury				
Ellagic acid	5, 10, and 20 mg/kg	Mice treated with single dose of 800 mg/kg Gal + 50 μg/kg LPS (i.p.)	Reduced LPS/GalN-induced NF-κB activation and increased antioxidant genes	[43]
Linalool	10, 20, and 40 mg/kg	Mice treated with single dose of 800 mg/kg Gal + 50 μg/kg LPS (i.p.)	Reduced LPS/GalN-induced NF-κB activation and induction of cytoprotective genes	[44]
Mangiferin	5, 10, and 20 mg/kg	Mice treated with single dose of 800 mg/kg Gal + 50 μg/kg LPS (i.p.)	Reduced liver injury by activating antioxidant pathway and inhibiting NLRP3 inflammasome activation	[45]
Oroxylin A	15, 30, and 60 mg/kg	Mice treated with single dose of 800 mg/kg Gal + 50 μg/kg LPS (i.p.)	Decreased liver injury by activating antioxidant genes and inhibiting TLR4 signaling-mediated inflammation	[46]
Chemical toxicity				
Tungtungmadic acid	5 and 20 μM	Hepa1c1c7 cells treated with 250 μM t-BHP	HO-1 induction via the PI3K/Akt signaling pathway to reduce hepatocyte death	[47]
Antcin C	20 μM in cells, 100 mg/kg in mice	HepG2 cells treated with 10 mM AAPH, mice treated with single dose of 80 mg/kg AAPH (i.p.)	Induction of antioxidant response via increase of JNK1/2 and PI3K/Akt activities	[48]
Butein and phloretin	25 μM in vitro, 30 mg/kg in vivo	Mouse hepatocytes treated with 0.5 mM t-BHP, rats treated with single dose of 1 mL/kg CCl_4 (i.p.)	Upregulation of HO-1 and GCLC expression through ERK2 pathway	[49]
Carthamus red	10 and 20 mg/kg	Mice treated with two doses of 2 mL/kg CCl_4-olive oil mixture (1 : 1)	Upregulation of Nrf2, GSTα, and NQO1 expressions associated with decreased hepatocyte injury and ALT levels	[50]
Curcumin	200 mg/kg	Mice treated with single dose of 20 mg/kg DEN (i.p.)	Nrf2-mediated HO-1 induction and amelioration of hepatocyte injury	[51]
Diallyl disulfide	50 and 100 mg/kg	Rats treated with single dose of 2 mL/kg CCl_4 (i.g.)	Induction of antioxidant and detoxifying enzyme activities and suppressing of inflammatory cytokines production by reducing NF-κB activation	[52, 53]
Ginsenoside Rg1	20 and 40 mg/kg	Rats treated with 2 mL/kg of 50% CCl_4 (s.c.) twice a week for 8 weeks	Reduced liver fibrosis by augmented antioxidant systems	[54]
Glycyrrhetinic acid	25 and 50 mg/kg	Mice treated with 6.4 g/kg CCl_4 (s.c.) for 30 days	Enhanced antioxidant genes expression to reduce hepatocyte injury	[55]
Hesperidin	40 and 80 μM	LO-2 cells treated with 150 μM t-BHP	ERK-mediated nuclear translocation of Nrf2 to induce HO-1 gene expression and antioxidant response	[56]

Table 1: Continued.

Phytochemicals	Effective doses	Experimental procedure (injury model)	Outcomes	References
Isoorientin	5 μg/mL	HepG2 cells treated with 200 μM t-BHP	Upregulation of antioxidant enzyme expression through PI3K/Akt pathway	[57]
Naringenin	50 mg/kg	Rats treated with 2 mL/kg CCl_4-olive oil mixture (1 : 1) on days 2 and 5 (i.p.)	Increase in Nrf2 and HO-1 expression to reduce liver injury	[58]
Oxyresveratrol	10 μM for in vitro study, 10 and 30 mg/kg for in vivo study	200 μM t-BHP treatment to HepG2 cells, ice treated with single dose of 0.5 mL/kg CCl_4 (i.p.)	ERK phosphorylation-mediated induction of antioxidant pathway to protect hepatocytes against oxidative stress, mitochondrial damage, and resultant cell death	[59]
Puerarin	100 μM	500 μM t-BHP treatment to Hepa1c1c7 and HepG2 cells	Augmentation of cellular antioxidant defenses through Nrf2-dependent HO-1 induction via PI3K pathway	[60]
Resveratrol	50 and 75 μM	Primary rat hepatocytes treated with 500 μM t-BHP	Reduced hepatocyte death by improving antioxidant status	[61]
Schisandrin B	15 μM	AML12 cells treated with 20 μM menadione for 1 h	Induction of ERK/Nrf2 signaling to enhance glutathione-mediated antioxidant response to protect hepatocytes against menadione-induced apoptosis	[62]
Metal toxicity				
Curcumin	200 mg/kg	Exposure of mice to $NaAsO_2$ (100 mg/L) in drinking water	Induction of antioxidant genes and enhanced methylation and elimination of arsenic	[63]
Lutein	40 mg/kg	Mice treated with 4 mg/kg As_2O_3 (i.g.)	Reduced liver injury by induction of antioxidant response	[64]
S-Allylcysteine	100 mg/kg	Mice treated with single dose of 17 mg/kg $K_2Cr_2O_7$ (s.c.)	Induction of antioxidant response to reduce liver injury	[65]
Alcohol toxicity				
Lucidone	1, 5, and 10 μg/mL	HepG2 cells treated with 100 mM ethanol	Induction of HO-1 via Nrf2 signaling pathway to enhance antioxidant response	[66]
Quercetin	100 μM	Primary human hepatocytes treated with 100 mM ethanol	ERK- and p38-mediated Nrf2 nuclear translocation and subsequent induction of HO-1 activity	[67, 68]
Quercetin	50 μM	LO-2 cells treated with 100 mM ethanol	Preventing hepatotoxicity by inducing p62 expression and induction of antioxidant response	[69]
Sulforaphane	50 mg/kg	Mice treated with 3 g/kg ethanol (30%) for 5 days (i.g.)	Decreased hepatocyte lipid accumulation and injury without altering CYP2E1 expression	[70]
Nonalcoholic steatohepatitis				
Baicalein	10 mg/kg	Rats fed with MCD diet for 8 weeks	Reduction in inflammation and oxidative hepatocyte injury	[71]
Curcumin	50 mg/kg	Rats fed with HFD for 6 weeks	Reduced hepatocyte lipid accumulation and improved insulin resistance and anti-inflammatory and antioxidant effects	[72]
Gastrodin	10, 20, and 50 mg/kg	HL-7702 cells treated with 0.6 mM of OA for 24 h, mice fed with HFD for 10 weeks	AMPK-mediated induction of Nrf2 pathway to enhance expression of antioxidant enzymes	[73]
Lycopene	15 mg/kg	Mice fed with HFD for the next 6 weeks following a single dose of 30 mg/kg DEN injection	Reduction in hepatocyte injury by induction of antioxidant pathway along with a decrease in CY2E1 expression	[74]
Cholestatic liver injury				
Oleanolic acid	20 mg/kg	Mice treated with 125 mg/kg LCA (i.p.)	Upregulation of Mrp2, Mrp3, and Mrp4 to reduce cholestatic liver injury	[75]
Oleanolic acid	20 mg/kg	Bile duct ligation in mice	Induction of Mrps and FXR antagonism to reduce cholestatic liver injury	[76]

Table 1: Continued.

Phytochemicals	Effective doses	Experimental procedure (injury model)	Outcomes	References
Paeoniflorin	200 mg/kg	Rats treated with 50 mg/kg ANIT for 4 days (i.g.)	Enhanced GSH synthesis by activating Nrf2 through PI3K/Akt-dependent pathway	[8]
Sulforaphane	50 mg/kg	Mice treated with 3 g/kg ethanol (30%) for 5 days (i.g.)	Decreased hepatocyte lipid accumulation and injury without altering CYP2E1 expression	[70]
Sulforaphane	25 mg/kg	Bile duct ligation in mice	Antifibrotic response by inhibition of TGF-β/Smad signaling pathway	[77]

AAPH: 2,2′-azobis(2-amidinopropane) dihydrochloride; MCD: methionine and choline deficient; CCl_4: carbon tetrachloride; DEN: dimethylnitrosamine; HFD: high-fat diet; Gal: galactosamine; LPS: lipopolysaccharide; OA: oleic acid; NAPQI: N-acetylbenzoquinoneimine; i.p.: intraperitoneal; s.c.: subcutaneous; i.g.: intragastric; t-BHP: tert-butyl hydroperoxide; APAP: acetaminophen; LCA: lithocholic acid; ANIT: alpha-naphthylisothiocyanate; Nrf2: nuclear factor (erythroid-derived 2)-like 2; JNK1/2: c-Jun N-terminal kinases 1/2; PI3K/AKT: phosphoinositide 3-kinase/protein kinase B; HO-1: heme oxygenase-1; GCLC: glutamate-cysteine ligase catalytic subunit; ALT: alanine transaminase; GST: glutathione S-transferase; NQO1: NAD(P)H quinone dehydrogenase 1; AMPK: 5′ AMP-activated protein kinase; GSH: glutathione; CYP2E1: cytochrome P450 2E1; NLRP3: NLR family pyrin domain containing 3; Mrp: multidrug resistance-associated protein; TLR4: Toll-like receptor 4; Keap1: Kelch-like ECH-associated protein 1.

3. Acute Inflammatory Liver Injury and Nrf2

Inflammation-mediated repeated liver injury may progress to chronic fibrosis and cirrhosis. Studies in various models of liver injury have shown that Nrf2 plays a role in inflammation-induced liver injury. Lack of an active Nrf2 signaling pathway results in severe inflammation-induced oxidative stress. Concanavalin A (ConA) treatment to mice activates and recruits T-lymphocytes and causes severe inflammation and hepatocyte apoptosis [78, 79]. Following intravenous injection of ConA, Nrf2-KO mice develop increased liver injury compared to WT mice. Hepatocyte-specific conditional Keap1-null mice and WT mice pretreated with three daily doses of CDDO-Im, a Nrf2 activator, had significantly reduced ConA-induced injury compared to Nrf2-KO mice. Similarly, in response to LPS/galactosamine treatment, Nrf2-null mice had increased liver injury compared to WT mice, and, predictably, hepatocyte-specific Keap1-KO mice were protected [80]. These results highlight the importance of Nrf2-mediated regulation of cytokine-dependent hepatocyte apoptosis [81]. In another study, investigators used a short interfering RNA (siRNA) against Keap1 to assess the role of Nrf2 in liver injury; mice were injected with Keap1- or luciferase (control)-siRNA-containing liposomes via the tail vein and, after 48 hours, with ConA. Silencing of hepatic Keap1 attenuated ConA-induced inflammation-associated liver damage [82].

Mangiferin and oroxylin A, the naturally occurring flavonoids, reduce LPS/galactosamine-induced liver injury in mice by reducing oxidative stress and inflammation. Mangiferin enhanced Nrf2/HO-1 signaling and inhibited NLR family, pyrin domain containing 3 (NLRP3) inflammasome [45]. Oroxylin A activated Nrf2 signaling and inhibited Toll-like receptor 4 (TLR4) signaling [46]. Linalool, a major volatile component of essential oils in several aromatic plant species, and ellagic acid (EA), a naturally occurring plant phenol found in certain fruits, nuts, and vegetables, have been shown to possess anti-inflammatory and antioxidant properties. Both of these phytochemicals reduced LPS/galactosamine-induced hepatocyte apoptosis by activating hepatocyte Nrf2-mediated antioxidant response and inflammation by suppressing NF-kB pathway in the liver [43, 44]. These phytochemicals, which reduce both oxidative stress and inflammation, are potential agents for the treatment of fulminant hepatitis in humans.

4. Role of Nrf2 in Chemical-Induced Liver Injury

Carbon tetrachloride (CCl_4) is a potent hepatotoxin known to cause centrilobular hepatic necrosis in rodent models of liver fibrosis. Acute administration of CCl_4 at higher doses causes severe hepatocyte necrosis, while chronic administration at lower doses is used to induce hepatic fibrosis [40]. However, this model is often criticized because it affects the central zone of the hepatic acinus and some nonmetabolized intermediates of CCl_4 can induce lung and kidney injury [83]. In hepatocytes, cytochrome P450 metabolizes CCl_4 to trichloromethyl free radicals ($CCl_3^{\bullet}$ and/or $CCl_3OO^{\bullet}$), which subsequently leads to generation of ROS ($^{\bullet}O_2^{-}$, H_2O_2, and $^{\bullet}OH$) [84]. Chemical hepatotoxins such as bromobenzene, CCl_4, and furosemide trigger hepatic Nrf2 nuclear translocation and Nrf2-regulated gene expression [85]. In Nrf2-deficient mice, repair of liver injury after a single treatment with CCl_4 was severely delayed [86]. Similarly, after 1-bromopropane exposure, compared to WT mice, Nrf2-knockout mice had increased liver injury with reduced antioxidant response [87].

Lee and colleagues studied the efficacy of diallyl disulfide (DADS), a secondary component derived from garlic, in amelioration of CCl_4-induced acute liver injury. Pretreatment with DADS (50 and 100 mg/kg/day) significantly activated Nrf2 expression and antioxidant and phase II enzymes to reduce liver injury [52, 53]. Oxyresveratrol (OXY), an antioxidant present in mulberry fruits and twigs, ameliorated tert-butyl hydroperoxide- (t-BHP-) and CCl_4-induced hepatotoxicity by reducing oxidative stress possibly via extracellular signal-regulated kinase- (ERK-) mediated Nrf2 activation in hepatocytes [59]. Other flavonoids such as carthamus red and naringenin also activate Nrf2 signaling to reduce CCl_4

injury in rats [50, 58]. Glycyrrhetinic acid, a triterpenoid, and ginsenoside Rg1, a phytoestrogen, ameliorated CCl_4-induced liver fibrosis via nuclear Nrf2 translocation and upregulation of antioxidant enzymes to reduce oxidative stress [54, 55].

Several other compounds such as 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), menadione (MEN), and carboxymethyllysine (CML) induce free radical-induced damage, and each of them is a potent inducer of hepatotoxicity. Phytocompounds such as antcin C [48], dimerumic acid [88], and schisandrin B [62] have been shown to ameliorate AAPH-, CML-, and MEN-induced liver injury, respectively. Dimethylnitrosamine (DMN) is a semivolatile organic chemical produced as a byproduct of industrial processes and is also known to cause oxidative liver injury. In DMN-administered rats, oral administration of curcumin enhanced nuclear translocation and ARE binding of Nrf2, producing robust antioxidant response thereby reducing hepatotoxicity [51].

In laboratories, t-BHP is a commonly used inducer of oxidative stress in vitro [53, 89, 90]. In hepatocytes, t-BHP is metabolized by CYP450 into free radical intermediates such as *t*-butoxyl and *t*-methyl radicals [91], which induce lipid peroxidation and glutathione (GSH) depletion resulting in organelle damage and cell death [92]. Hence, t-BHP-induced hepatotoxicity model is widely used for evaluating merits of Nrf2-ARE signaling in hepatocytes. Various plant flavonoids possess excellent antioxidant potential and confer hepatoprotection. Flavonoids such as hesperidin, butein, and phloretin reduce t-BHP toxicity in hepatocytes via ERK phosphorylation [49, 56], whereas another member of the same class, isoorientin, induces Nrf2 activation via PI3K/Akt pathway [57]. Additionally, phenolics such as 3-caffeoyl, 4-dihydrocaffeoyl quinic acid, and resveratrol ameliorate t-BHP-induced hepatocyte oxidative stress via Nrf2 induction [47, 61]. Another phytoestrogen, puerarin, reduces t-BHP-induced oxidative stress in Hepa1c1c7 cells via PI3K/Akt pathway [60]. These findings underscore the importance of phytochemicals-mediated regulation of Nrf2 activity in diverse models of hepatocyte toxicity, but many of these results need to be validated in animal models of liver injury.

5. Metal Toxicity and Nrf2

Heavy metals, such as cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), and mercury (Hg), pose health concerns via exposure through water, food, or environmental waste [93]. In experimental models, these heavy metals cause severe oxidative stress and hepatotoxicity. Since these metals induce ROS, it is conceivable that they might induce Nrf2 activation in hepatocytes. A study using Nrf2-KO, Keap1-KO, and hepatocyte-specific Keap1-knockout mice showed that Cd caused extensive liver damage in Nrf2-null mice, whereas Keap1-KO and Keap1-HKO mice were resistant to injury [94].

Arsenic (a metalloid) is a known pollutant for drinking water and has provoked public health concern worldwide. Arsenic modulates Nrf2 signaling in vivo and in vitro [46, 95, 96]. Lutein (a carotenoid) and curcumin (a diarylheptanoid) have been shown to reduce arsenic toxicity via Nrf2 activation and subsequent antioxidant gene expression [63, 64]. Additionally, in mice, curcumin promoted methylation of arsenic and accelerated its excretion [63].

Several studies demonstrated that Cr(VI) induces hepatotoxicity by increasing ROS generation [97, 98]. In Hepa1c1c7 cells, Cr(VI) induces ROS production and in turn triggers activation of Nrf2 pathway [99]. Garlic and its derivative S-allylcysteine (SAC) reduced Cr(VI)-induced hepatotoxicity [65].

In this industrialization era, metal toxicity is a growing concern, and Nrf2 presents a potential target that can be explored to discover novel hepatoprotective agents. These studies suggest that the use of phytochemicals may be helpful in negating the hepatotoxic effects of heavy metals.

6. Nrf2 and Alcoholic Liver Injury

Chronic alcohol consumption is known to result in liver injury-associated deaths [100]. Alcohol abuse leads to increased production of ROS, depletion of hepatocytes antioxidant levels, and enhanced oxidative stress [101, 102]. However, it also leads to an increase in Nrf2 mRNA and protein in the livers [103]. Gong and Cederbaum speculated that the increase in Nrf2 expression was dependent on ethanol-mediated induction of CYP2E1. Compared to WT mice, ethanol-administered Nrf2-KO mice had depletion of total and mitochondrial GSH in their livers resulting in increased liver failure and mortality, while ethanol-fed Keap1-HKO mice were protected [104]. Keap1 knockdown in mice, which induces Nrf2 activation, also blunted ethanol-mediated increase in serum triglycerides and hepatic free fatty acids [105].

Quercetin (a plant flavonoid), a known antioxidant and free radical scavenger, reduces ethanol toxicity in hepatocytes which is Nrf2-mediated. Quercetin upregulates HO-1 via the MAPK/Nrf2 pathway to confer hepatoprotection [67, 68]. A recent study showed that quercetin interacts with Keap1 and blocks its binding to Nrf2 [69]. Sulforaphane (an isothiocyanate) is a known activator of Nrf2 pathway. Zhou et al. showed that sulforaphane induces Nrf2 activation to reduce lipid accumulation and oxidative stress in hepatocytes, an effect independent of CYP2E1 [70]. Lucidone (a naturally occurring cyclopentenedione in *Lindera* sp.) reduces ethanol-induced oxidative stress in HepG2 cells [66] by inducing Nrf2-mediated antioxidant response via profound upregulation of HO-1.

Despite its known detrimental effects, alcohol remains a major cause of liver injury. These observations from preclinical studies indicate that targeting Nrf2 has merits in treating alcoholic liver injury. These natural Nrf2 activators need to be studied in clinical settings to determine the impact on alcohol abuse-mediated liver injury burden.

7. Nrf2 and Nonalcoholic Steatohepatitis

Nonalcoholic fatty liver disease (NAFLD) is becoming the most common cause of chronic liver disease [106, 107]. In NAFLD, multiple mechanisms operate simultaneously which results in hepatocyte apoptosis, inflammation, and fibrosis,

that is, nonalcoholic steatohepatitis (NASH) [107, 108]. In NASH, excessive lipid accumulation and subsequent generation of ROS from impaired mitochondrial respiratory chain result in GSH depletion [109, 110]. It is interesting to note that two research groups showed in mice that high-fat diet (HFD) can increase or decrease hepatic Nrf2 [111]. However, upon HFD feeding, Nrf2-null mice suffered from severe liver injury compared to wild-type mice [112]. Livers of methionine-choline deficient diet- (MCD-) fed Nrf2-knockout mice had higher levels of oxidative stress, iron accumulation, fibrosis, and inflammation than wild-type mice [113–115]. It is suggested that Nrf2 activation protects against NAFLD and NASH also via controlling inflammation [115]. The overall merits of Nrf2-mediated prevention of oxidative damage and progression of NAFLD imply its potential use in patients suffering from NASH.

Studies have shown the beneficial role of phytochemicals in treating NASH in preclinical and clinical settings [116–118]. Lycopene (a carotenoid derived from tomato) is shown to reduce HFD-induced steatohepatitis in rats [119]. Wang et al. showed that lycopene-induced inhibition of NASH and hepatocarcinogenesis in rats is partially due to induction of Nrf2 and HO-1 genes [74]. Gastrodin (GSTD, a natural compound isolated from *Gastrodia elata* BI, a traditional Chinese herbal medicine) ameliorates oxidative stress and proinflammatory response in cellular and animal models of NAFLD. GSTD-induced AMPK activation phosphorylates Nrf2, which in turn enhances its nuclear translocation and expression of antioxidant genes (HO-1 and SOD1) [73]. In rats, baicalein (a flavonoid) has been shown to ameliorate MCD-diet-induced NASH by activating multiple pathways including Nrf2-ARE pathway [71]. Similar results have been reported in studies with curcumin where Nrf2 activation ameliorated hepatotoxicity and reduced NASH [72]. Scientific data available till date indicate that the role of natural compounds in Nrf2 activation and alleviation of NASH in experimental models warrants additional human studies. Owing to the multifactorial nature of NASH, a combination of Nrf2 activator and lipid-lowering drugs may translate into an effective therapeutic strategy.

8. Nrf2 and Cholestatic Liver Injury

Cholestasis, a reduction in bile flow, results in a dramatic increase in both liver and serum bile acid concentrations leading to acute liver toxicity, proliferation of bile ducts, and eventually cirrhosis [120]. Bile duct ligated- (BDL-) Nrf2-deficient mice showed reduced elimination of bile acids and higher intrahepatic accumulation of toxic bile acids leading to GSH depletion and injury [121, 122]. BDL-Keap1-KO mice had sustained activation of Nrf2 and increased expression of MRP efflux transporters, detoxifying enzymes, and antioxidant genes [121, 123]. Interestingly, following BDL, Nrf2-KO mice developed into a cholestatic phenotype but liver injury was not different from WT mice undergoing BDL, suggesting that Nrf2 plays a role in the regulation of bile acid homeostasis in the liver [121]. Nrf2-KO mice treated with lithocholic acid (LCA, a toxic bile acid) compared to wild-type mice had severe multifocal liver necrosis and increased inflammation and serum ALT levels [124].

Ursodeoxycholic acid (UDCA), a secondary bile acid, is reported to be hepatoprotective [125, 126]. UDCA treatment of WT mice significantly increased nuclear Nrf2 expression and that of hepatic Mrp2, Mrp3, and Mrp4 [127]. In patients with biliary cirrhosis, UDCA treatment enhanced hepatic Nrf2 expression with an upregulated hepatic thioredoxin and thioredoxin reductase 1 expression [128]. These studies indicate a role of Nrf2 and its activation as a viable target in treating cholestatic liver injury.

Oleanolic acid (OA), a natural triterpenoid, has been shown to reduce LCA- and BDL-induced cholestatic injury in mice [75, 76]. Protection of both of these models is thought to be due to Nrf2-mediated upregulation of Mrps. However, high doses of OA induce cholestatic liver injury in mice by downregulation of hepatic transporters and disruption of bile acid uptake and metabolism [129]. Sulforaphane also reduces cholestasis injury in mice by hepatocyte Nrf2 activation [77]. Alpha-naphthylisothiocyanate (ANIT) is used to induce hepatotoxicity in animal models, which mimics drug-induced cholestatic hepatic injury in humans [130]. Chen et al. demonstrated that paeoniflorin (a monoterpene glycoside) ameliorates ANIT-induced cholestasis in rats by activating Nrf2 via PI3K/Akt pathway [8]. Since Nrf2 regulates both antioxidant genes and multidrug resistance-associated proteins, it has great implication in treating cholestasis.

9. In Vitro Nrf2-ARE Pathway Activation by Phytochemicals

Various hepatocyte cell lines and primary hepatocytes have been used to demonstrate mechanisms of phytochemicals-induced Nrf2 activation. A handful of phytochemicals have been reported to activate Nrf2 signaling in hepatocytes and related cell lines even in the absence of exogenous oxidative stress stimuli. For example, compared to vehicle, phytochemicals such as isothiocyanate (6-methylthiohexyl isothiocyanate), capsaicinoids (capsaicin), terpenoids (carnosol and maslinic acid), polyphenols (eckol, quercetin, and phloretamide), chalconoids (xanthohumol), and flavonoids (epicatechin) induce Nrf2 activation in hepatocytes [131–139]. Although these studies comprehensively demonstrate the ability of phytochemicals to induce Nrf2 activation in hepatocytes, one of the merits of these compounds under conditions of oxidative stress, both in vitro and in vivo, is the prevailing lacunae, which leaves many questions unanswered.

10. Cytotoxicity of Phytochemicals

Consumption of herbal mixtures or bioactive molecules can lead to adverse events as well as hepatotoxicity [140]. Phytochemicals found naturally in many plants, fruits, and vegetables are generally believed to be devoid of major side effects [140]. However, safety studies are lacking and many phytochemicals that are marketed as food supplements have already been shown to have adverse effects. Polyphenols and flavonoids, which are popular for their beneficial

effects, have been reported to induce adverse events. For example, consumption of polyphenols inhibits nonheme iron absorption and may lead to iron depletion [141]. At higher doses, flavonoids have been reported to act as mutagens, prooxidants, and inhibitors of key enzymes involved in hormone metabolism [142]. Oleanolic acid, which is a known inducer of Nrf2 pathway, has been shown to cause cholestasis at higher doses [129, 143].

Medical literature is littered with various reports of herb-induced hepatotoxicity [144, 145]. For example, black cohosh used for menopausal symptoms can lead to liver failure. We refer the reader to recent articles [146–149]. Safety studies for most phytochemicals discussed here are lacking. The FDA is not authorized to review dietary supplement products for safety and effectiveness before they are marketed (http://www.fda.gov). Hence, there is a big gap in the knowledge regarding the safety profile of phytochemicals and toxicity studies will be needed before they are considered safe for human usage.

11. Concluding Remarks

Almost all modes of liver injury are associated with increased oxidative stress and an overwhelmed antioxidant defense system. Since Nrf2 activation is associated with the enhancement of endogenous antioxidant system, it can be an ideal therapeutic target for reducing oxidative stress. Nrf2-ARE pathway boosts hepatocyte antioxidant defense system but may not be able to reduce pathogenesis of liver injury. Hence, a synchronal use of natural Nrf2 activators in combination with other pharmacological agents can be a potential multipronged approach to reduce liver injury. The search for treatments for liver injury is ongoing and potential candidates include whole herbal extracts as well as purified phytochemicals. The literature on preclinical use of phytochemicals to treat various modes of liver injury is ever-growing. However, very few phytochemicals have been tested in clinical settings. Some of the key limitations in this regard are (1) poor choice of experimental models and their clinical relevance, (2) lack of a specific identified mechanism of action, and (3) lack of comprehensive time point data for better interpretation of preclinical findings. These limitations have resulted in weakened significance of these compounds as hepatoprotective agents. Therefore, further studies are needed to explore the full potential of these compounds.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

This work was supported by SERB EMR/2015/002001 (Ranjitsinh V. Devkar) and the Medical College of Georgia (Sandeep Khurana).

References

[1] C. Girish and S. C. Pradhan, "Indian herbal medicines in the treatment of liver diseases: problems and promises," *Fundamental and Clinical Pharmacology*, vol. 26, no. 2, pp. 180–189, 2012.

[2] R. K. Dhiman and Y. K. Chawla, "Herbal medicines for liver diseases," *Digestive Diseases and Sciences*, vol. 50, no. 10, pp. 1807–1812, 2005.

[3] A. Kuruvilla, "Herbal formulations as pharmacotherapeutic agents," *Indian Journal of Experimental Biology*, vol. 40, no. 1, pp. 7–11, 2002.

[4] K.-H. Lu, C.-T. Liu, R. Raghu, and L.-Y. Sheen, "Therapeutic potential of chinese herbal medicines in alcoholic liver disease," *Journal of Traditional and Complementary Medicine*, vol. 2, no. 2, pp. 115–122, 2012.

[5] H. Farghali, N. K. Canová, and S. Zakhari, "Hepatoprotective properties of extensively studied medicinal plant active constituents: possible common mechanisms," *Pharmaceutical Biology*, vol. 53, no. 6, pp. 781–791, 2014.

[6] N. Ghosh, R. Ghosh, V. Mandal, and S. C. Mandal, "Recent advances in herbal medicine for treatment of liver diseases," *Pharmaceutical Biology*, vol. 49, no. 9, pp. 970–988, 2011.

[7] H. Cichoz-Lach and A. Michalak, "Oxidative stress as a crucial factor in liver diseases," *World Journal of Gastroenterology*, vol. 20, no. 25, pp. 8082–8091, 2014.

[8] Z. Chen, X. Ma, Y. Zhu et al., "Paeoniflorin ameliorates ANIT-induced cholestasis by activating Nrf2 through an PI3K/Akt-dependent pathway in rats," *Phytotherapy Research*, vol. 29, no. 11, pp. 1768–1775, 2015.

[9] J. B. Hoek and J. G. Pastorino, "Ethanol, oxidative stress, and cytokine-induced liver cell injury," *Alcohol*, vol. 27, no. 1, pp. 63–68, 2002.

[10] P. Moi, K. Chan, I. Asunis, A. Cao, and Y. W. Kan, "Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 21, pp. 9926–9930, 1994.

[11] M. Schäfer and S. Werner, "Nrf2—a regulator of keratinocyte redox signaling," *Free Radical Biology and Medicine*, vol. 88, pp. 243–252, 2015.

[12] I. Buendia, P. Michalska, E. Navarro, I. Gameiro, J. Egea, and R. León, "Nrf2-ARE pathway: an emerging target against oxidative stress and neuroinflammation in neurodegenerative diseases," *Pharmacology and Therapeutics*, vol. 157, pp. 84–104, 2016.

[13] O. Al-Sawaf, T. Clarner, A. Fragoulis et al., "Nrf2 in health and disease: current and future clinical implications," *Clinical Science*, vol. 129, no. 12, pp. 989–999, 2016.

[14] H. Zhang, K. J. Davies, and H. J. Forman, "Oxidative stress response and Nrf2 signaling in aging," *Free Radical Biology and Medicine*, vol. 88, pp. 314–336, 2015.

[15] B. Chen, Y. Lu, Y. Chen, and J. Cheng, "The role of Nrf2 in oxidative stress-induced endothelial injuries," *Journal of Endocrinology*, vol. 225, no. 3, pp. R83–R99, 2015.

[16] A. Abdul-Aziz, D. J. Macewan, K. M. Bowles, and S. A. Rushworth, "Oxidative stress responses and NRF2 in human leukaemia," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 454659, 7 pages, 2015.

[17] M. C. Jaramillo and D. D. Zhang, "The emerging role of the Nrf2-Keap1 signaling pathway in cancer," *Genes & Development*, vol. 27, no. 20, pp. 2179–2191, 2013.

[18] H. K. Bryan, A. Olayanju, C. E. Goldring, and B. K. Park, "The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation," *Biochemical Pharmacology*, vol. 85, no. 6, pp. 705–717, 2013.

[19] J. W. Kaspar, S. K. Niture, and A. K. Jaiswal, "Nrf2:INrf2 (Keap1) signaling in oxidative stress," *Free Radical Biology and Medicine*, vol. 47, no. 9, pp. 1304–1309, 2009.

[20] S. P. Reddy, "The antioxidant response element and oxidative stress modifiers in airway diseases," *Current Molecular Medicine*, vol. 8, no. 5, pp. 376–383, 2008.

[21] C. Espinosa-Diez, V. Miguel, D. Mennerich et al., "Antioxidant responses and cellular adjustments to oxidative stress," *Redox Biology*, vol. 6, pp. 183–197, 2015.

[22] R. J. Fontana, "Acute liver failure including acetaminophen overdose," *Medical Clinics of North America*, vol. 92, no. 4, pp. 761–794, 2008.

[23] W. M. Lee, "Acetaminophen and the U.S. Acute Liver Failure Study Group: lowering the risks of hepatic failure," *Hepatology*, vol. 40, no. 1, pp. 6–9, 2004.

[24] J. R. Mitchell, D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette, and B. B. Brodie, "Acetaminophen induced hepatic necrosis. I. Role of drug metabolism," *Journal of Pharmacology and Experimental Therapeutics*, vol. 187, no. 1, pp. 185–194, 1973.

[25] N. Hanawa, M. Shinohara, B. Saberi, W. A. Gaarde, D. Han, and N. Kaplowitz, "Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury," *Journal of Biological Chemistry*, vol. 283, no. 20, pp. 13565–13577, 2008.

[26] T. R. Knight, Y.-S. Ho, A. Farhood, and H. Jaeschke, "Peroxynitrite is a critical mediator of acetaminophen hepatotoxicity in murine livers: protection by glutathione," *Journal of Pharmacology and Experimental Therapeutics*, vol. 303, no. 2, pp. 468–475, 2002.

[27] C. Saito, J. J. Lemasters, and H. Jaeschke, "c-Jun N-terminal kinase modulates oxidant stress and peroxynitrite formation independent of inducible nitric oxide synthase in acetaminophen hepatotoxicity," *Toxicology and Applied Pharmacology*, vol. 246, no. 1-2, pp. 8–17, 2010.

[28] C. E. P. Goldring, N. R. Kitteringham, R. Elsby et al., "Activation of hepatic Nrf2 in vivo by acetaminophen in CD-1 mice," *Hepatology*, vol. 39, no. 5, pp. 1267–1276, 2004.

[29] L. M. Aleksunes, A. L. Slitt, J. M. Maher et al., "Induction of Mrp3 and Mrp4 transporters during acetaminophen hepatotoxicity is dependent on Nrf2," *Toxicology and Applied Pharmacology*, vol. 226, no. 1, pp. 74–83, 2008.

[30] K. Chan, X.-D. Han, and Y. W. Kan, "An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 8, pp. 4611–4616, 2001.

[31] H. Okawa, H. Motohashi, A. Kobayashi, H. Aburatani, T. W. Kensler, and M. Yamamoto, "Hepatocyte-specific deletion of the *keap1* gene activates Nrf2 and confers potent resistance against acute drug toxicity," *Biochemical and Biophysical Research Communications*, vol. 339, no. 1, pp. 79–88, 2006.

[32] S. A. Reisman, I. L. Csanaky, L. M. Aleksunes, and C. D. Klaassen, "Altered disposition of acetaminophen in Nrf2-null and Keap1-knockdown mice," *Toxicological Sciences*, vol. 109, no. 1, pp. 31–40, 2009.

[33] M. A. Mobasher, A. González-Rodriguez, B. Santamaría et al., "Protein tyrosine phosphatase 1B modulates GSK3b/Nrf2 and IGFIR signaling pathways in acetaminophen-induced hepatotoxicity," *Cell Death and Disease*, vol. 4, no. 5, article e626, 2013.

[34] H. Y. Kay, Y. W. Kim, D. H. Ryu, S. H. Sung, S. J. Hwang, and S. G. Kim, "Nrf2-mediated liver protection by sauchinone, an antioxidant lignan, from acetaminophen toxicity through the PKCδ-GSK3β pathway," *British Journal of Pharmacology*, vol. 163, no. 8, pp. 1653–1665, 2011.

[35] M. Lin, X. Zhai, G. Wang et al., "Salvianolic acid B protects against Acetaminophen hepatotoxicity by inducing Nrf2 and phase II detoxification gene expression via activation of the PI3K and PKC signaling pathways," *Journal of Pharmacological Sciences*, vol. 127, no. 2, pp. 203–210, 2015.

[36] J. Noh, Y. Kim, J. H. Hwang et al., "Sulforaphane protects against acetaminophen-induced hepatotoxicity," *Food and Chemical Toxicology*, vol. 80, pp. 193–200, 2015.

[37] S. A. Reisman, L. M. Aleksunes, and C. D. Klaassen, "Oleanolic acid activates Nrf2 and protects from acetaminophen hepatotoxicity via Nrf2-dependent and Nrf2-independent processes," *Biochemical Pharmacology*, vol. 77, no. 7, pp. 1273–1282, 2009.

[38] R. N. Jadeja, N. H. Urrunaga, S. Dash, S. Khurana, and N. K. Saxena, "Withaferin-a reduces acetaminophen-induced liver injury in mice," *Biochemical Pharmacology*, vol. 97, no. 1, pp. 122–132, 2015.

[39] S. I. Gum and M. K. Cho, "The amelioration of N-acetyl-p-benzoquinone imine toxicity by ginsenoside Rg3: the role of Nrf2-mediated detoxification and Mrp1/Mrp3 transports," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 957947, 11 pages, 2013.

[40] H. Jaeschke, C. D. Williams, M. R. McGill, Y. Xie, and A. Ramachandran, "Models of drug-induced liver injury for evaluation of phytotherapeutics and other natural products," *Food and Chemical Toxicology*, vol. 55, pp. 279–289, 2013.

[41] H. Jaeschke, M. R. McGill, C. D. Williams, and A. Ramachandran, "Current issues with acetaminophen hepatotoxicity—a clinically relevant model to test the efficacy of natural products," *Life Sciences*, vol. 88, no. 17-18, pp. 737–745, 2011.

[42] K. Du, M. R. McGill, Y. Xie, M. L. Bajt, and H. Jaeschke, "Resveratrol prevents protein nitration and release of endonucleases from mitochondria during acetaminophen hepatotoxicity," *Food and Chemical Toxicology*, vol. 81, pp. 62–70, 2015.

[43] L. Gu, W.-S. Deng, Y. Liu et al., "Ellagic acid protects Lipopolysaccharide/D-galactosamine-induced acute hepatic injury in mice," *International immunopharmacology*, vol. 22, no. 2, pp. 341–345, 2014.

[44] J. Li, X. Zhang, and H. Huang, "Protective effect of linalool against lipopolysaccharide/D-galactosamine-induced liver injury in mice," *International Immunopharmacology*, vol. 23, no. 2, pp. 523–529, 2014.

[45] C.-W. Pan, Z.-Z. Pan, J.-J. Hu et al., "Mangiferin alleviates lipopolysaccharide and D-galactosamine-induced acute liver injury by activating the Nrf2 pathway and inhibiting NLRP3 inflammasome activation," *European Journal of Pharmacology*, vol. 770, pp. 85–91, 2016.

[46] H. Huang, X. Zhang, and J. Li, "Protective effect of oroxylin A against lipopolysaccharide and/or D-galactosamine-induced acute liver injury in mice," *Journal of Surgical Research*, vol. 195, no. 2, pp. 522–528, 2015.

[47] Y. P. Hwang, H. J. Yun, H. K. Chun et al., "Protective mechanisms of 3-caffeoyl, 4-dihydrocaffeoyl quinic acid from Salicornia herbacea against tert-butyl hydroperoxide-induced oxidative damage," *Chemico-Biological Interactions*, vol. 181, no. 3, pp. 366–376, 2009.

[48] M. Gokila Vani, K. J. Senthil Kumar, J.-W. Liao et al., "Antcin C from *Antrodia cinnamomea* protects liver cells against free radical-induced oxidative stress and apoptosis in vitro and in vivo through Nrf2-dependent mechanism," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 296082, 17 pages, 2013.

[49] Y.-C. Yang, C.-K. Lii, A.-H. Lin et al., "Induction of glutathione synthesis and heme oxygenase 1 by the flavonoids butein and phloretin is mediated through the ERK/Nrf2 pathway and protects against oxidative stress," *Free Radical Biology and Medicine*, vol. 51, no. 11, pp. 2073–2081, 2011.

[50] S. C. Wu, Y. Yue, H. Tian et al., "Carthamus red from *Carthamus tinctorius* L. exerts antioxidant and hepatoprotective effect against CCl4-induced liver damage in rats via the Nrf2 pathway," *Journal of Ethnopharmacology*, vol. 148, no. 2, pp. 570–578, 2013.

[51] E. O. Farombi, S. Shrotriya, H.-K. Na, S.-H. Kim, and Y.-J. Surh, "Curcumin attenuates dimethylnitrosamine-induced liver injury in rats through Nrf2-mediated induction of heme oxygenase-1," *Food and Chemical Toxicology*, vol. 46, no. 4, pp. 1279–1287, 2008.

[52] I.-C. Lee, S.-H. Kim, H.-S. Baek et al., "The involvement of Nrf2 in the protective effects of diallyl disulfide on carbon tetrachloride-induced hepatic oxidative damage and inflammatory response in rats," *Food and Chemical Toxicology*, vol. 63, pp. 174–185, 2014.

[53] I.-C. Lee, S.-H. Kim, H.-S. Baek et al., "Protective effects of diallyl disulfide on carbon tetrachloride-induced hepatotoxicity through activation of Nrf2," *Environmental Toxicology*, vol. 30, no. 5, pp. 538–548, 2016.

[54] J.-P. Li, Y. Gao, S.-F. Chu et al., "Nrf2 pathway activation contributes to anti-fibrosis effects of ginsenoside Rg1 in a rat model of alcohol- and CCl_4-induced hepatic fibrosis," *Acta Pharmacologica Sinica*, vol. 35, no. 8, pp. 1031–1044, 2014.

[55] S. Chen, L. Zou, L. Li, and T. Wu, "The protective effect of glycyrrhetinic acid on carbon tetrachloride-induced chronic liver fibrosis in mice via upregulation of Nrf2," *PLoS ONE*, vol. 8, no. 1, Article ID e53662, 2013.

[56] M. Chen, H. Gu, Y. Ye et al., "Protective effects of hesperidin against oxidative stress of tert-butyl hydroperoxide in human hepatocytes," *Food and Chemical Toxicology*, vol. 48, no. 10, pp. 2980–2987, 2010.

[57] J. H. Lim, H.-S. Park, J.-K. Choi, I.-S. Lee, and J. C. Hyun, "Isoorientin induces Nrf2 pathway-driven antioxidant response through phosphatidylinositol 3-kinase signaling," *Archives of Pharmacal Research*, vol. 30, no. 12, pp. 1590–1598, 2007.

[58] M. A. Esmaeili and M. Alilou, "Naringenin attenuates CCl_4-induced hepatic inflammation by the activation of an Nrf2-mediated pathway in rats," *Clinical and Experimental Pharmacology and Physiology*, vol. 41, no. 6, pp. 416–422, 2014.

[59] H. Y. Choi, J.-H. Lee, K. H. Jegal, I. J. Cho, Y. W. Kim, and S. C. Kim, "Oxyresveratrol abrogates oxidative stress by activating ERK-Nrf2 pathway in the liver," *Chemico-Biological Interactions*, vol. 245, pp. 110–121, 2016.

[60] Y. P. Hwang and H. G. Jeong, "Mechanism of phytoestrogen puerarin-mediated cytoprotection following oxidative injury: estrogen receptor-dependent up-regulation of PI3K/Akt and HO-1," *Toxicology and Applied Pharmacology*, vol. 233, no. 3, pp. 371–381, 2008.

[61] J. A. Rubiolo, G. Mithieux, and F. V. Vega, "Resveratrol protects primary rat hepatocytes against oxidative stress damage: activation of the Nrf2 transcription factor and augmented activities of antioxidant enzymes," *European Journal of Pharmacology*, vol. 591, no. 1–3, pp. 66–72, 2008.

[62] P. K. Leong, P. Y. Chiu, N. Chen, H. Leung, and K. M. Ko, "Schisandrin B elicits a glutathione antioxidant response and protects against apoptosis via the redox-sensitive ERK/Nrf2 pathway in AML12 hepatocytes," *Free Radical Research*, vol. 45, no. 4, pp. 483–495, 2011.

[63] S. Gao, X. X. Duan, X. Wang et al., "Curcumin attenuates arsenic-induced hepatic injuries and oxidative stress in experimental mice through activation of Nrf2 pathway, promotion of arsenic methylation and urinary excretion," *Food and Chemical Toxicology*, vol. 59, pp. 739–747, 2013.

[64] S. Li, Y. Ding, Q. Niu et al., "Lutein has a protective effect on hepatotoxicity induced by arsenic via Nrf2 signaling," *BioMed Research International*, vol. 2015, Article ID 315205, 2015.

[65] S. Kalayarasan, N. Sriram, A. Sureshkumar, and G. Sudhandiran, "Chromium (VI)-induced oxidative stress and apoptosis is reduced by garlic and its derivative S-allylcysteine through the activation of Nrf2 in the hepatocytes of Wistar rats," *Journal of Applied Toxicology*, vol. 28, no. 7, pp. 908–919, 2008.

[66] K. J. Senthil Kumar, J.-W. Liao, J.-H. Xiao, M. Gokila Vani, and S.-Y. Wang, "Hepatoprotective effect of lucidone against alcohol-induced oxidative stress in human hepatic HepG2 cells through the up-regulation of HO-1/Nrf-2 antioxidant genes," *Toxicology in Vitro*, vol. 26, no. 5, pp. 700–708, 2012.

[67] P. Yao, A. Nussler, L. Liu et al., "Quercetin protects human hepatocytes from ethanol-derived oxidative stress by inducing heme oxygenase-1 via the MAPK/Nrf2 pathways," *Journal of Hepatology*, vol. 47, no. 2, pp. 253–261, 2007.

[68] S. Liu, W. Hou, P. Yao et al., "Heme oxygenase-1 mediates the protective role of quercetin against ethanol-induced rat hepatocytes oxidative damage," *Toxicology in Vitro*, vol. 26, no. 1, pp. 74–80, 2012.

[69] L.-L. Ji, Y.-C. Sheng, Z.-Y. Zheng, L. Shi, and Z.-T. Wang, "The involvement of p62-Keap1-Nrf2 antioxidative signaling pathway and JNK in the protection of natural flavonoid quercetin against hepatotoxicity," *Free Radical Biology and Medicine*, vol. 85, pp. 12–23, 2015.

[70] R. Zhou, J. J. Lin, and D. F. Wu, "Sulforaphane induces Nrf2 and protects against CYP2E1-dependent binge alcohol-induced liver steatosis," *Biochimica et Biophysica Acta (BBA)—General Subjects*, vol. 1840, no. 1, pp. 209–218, 2014.

[71] H.-G. Xin, B.-B. Zhang, Z.-Q. Wu et al., "Treatment with baicalein attenuates methionine-choline deficient diet-induced non-alcoholic steatohepatitis in rats," *European Journal of Pharmacology*, vol. 738, pp. 310–318, 2014.

[72] B. Li, L. Wang, Q. Lu, and W. Da, "Liver injury attenuation by curcumin in a rat NASH model: an Nrf2 activation-mediated effect?" *Irish Journal of Medical Science*, vol. 185, no. 1, pp. 93–100, 2016.

[73] L. L. Qu, B. Yu, Z. Li, W. X. Jiang, J. D. Jiang, and W. J. Kong, "Gastrodin ameliorates oxidative stress and proinflammatory response in nonalcoholic fatty liver disease through the AMPK/Nrf2 pathway," *Phytotherapy Research*, vol. 30, no. 3, pp. 402–411, 2016.

[74] Y. Wang, L. M. Ausman, A. S. Greenberg, R. M. Russell, and X.-D. Wang, "Dietary lycopene and tomato extract supplementations inhibit nonalcoholic steatohepatitis-promoted hepatocarcinogenesis in rats," *International Journal of Cancer*, vol. 126, no. 8, pp. 1788–1796, 2010.

[75] P. Chen, H. Zeng, Y. Wang et al., "Low dose of oleanolic acid protects against lithocholic acid-induced cholestasis in

mice: potential involvement of nuclear factor-E2-related factor 2-mediated upregulation of multidrug resistance-associated proteinss," *Drug Metabolism and Disposition*, vol. 42, no. 5, pp. 844–852, 2014.

[76] P. Chen, J. Li, X. Fan et al., "Oleanolic acid attenuates obstructive cholestasis in bile duct-ligated mice, possibly via activation of NRF2-MRPs and FXR antagonism," *European Journal of Pharmacology*, vol. 765, Article ID 70177, pp. 131–139, 2015.

[77] C. J. Oh, J.-Y. Kim, A.-K. Min et al., "Sulforaphane attenuates hepatic fibrosis via NF-E2-related factor 2-mediated inhibition of transforming growth factor-β/Smad signaling," *Free Radical Biology and Medicine*, vol. 52, no. 3, pp. 671–682, 2012.

[78] F. Gantner, M. Leist, A. W. Lohse, P. G. Germann, and G. Tiegs, "Concanavalin A-induced T-cell-mediated hepatic injury in mice: the role of tumor necrosis factor," *Hepatology*, vol. 21, no. 1, pp. 190–198, 1995.

[79] F. Heymann, K. Hamesch, R. Weiskirchen, and F. Tacke, "The concanavalin a model of acute hepatitis in mice," *Laboratory Animals*, vol. 49, S1, pp. 12–20, 2015.

[80] J. Liu, K. C. Wu, Y.-F. Lu, E. Ekuase, and C. D. Klaassen, "NRF2 protection against liver injury produced by various hepatotoxicants," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 305861, 8 pages, 2013.

[81] W. O. Osburn, M. S. Yates, P. D. Dolan et al., "Genetic or pharmacologic amplification of Nrf2 signaling inhibits acute inflammatory liver injury in mice," *Toxicological Sciences*, vol. 104, no. 1, pp. 218–227, 2008.

[82] A. González-Rodríguez, B. Reibert, T. Amann, R. Constien, C. M. Rondinone, and A. M. Valverde, "In vivo siRNA delivery of Keap1 modulates death and survival signaling pathways and attenuates concanavalin-A-induced acute liver injury in mice," *Disease Models and Mechanisms*, vol. 7, no. 9, pp. 1093–1100, 2014.

[83] M. J. Tuñón, M. Alvarez, J. M. Culebras, and J. González-Gallego, "An overview of animal models for investigating the pathogenesis and therapeutic strategies in acute hepatic failure," *World Journal of Gastroenterology*, vol. 15, no. 25, pp. 3086–3098, 2009.

[84] L. W. D. Weber, M. Boll, and A. Stampfl, "Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model," *Critical Reviews in Toxicology*, vol. 33, no. 2, pp. 105–136, 2003.

[85] L. E. Randle, C. E. P. Goldring, C. A. Benson et al., "Investigation of the effect of a panel of model hepatotoxins on the Nrf2-Keap1 defence response pathway in CD-1 mice," *Toxicology*, vol. 243, no. 3, pp. 249–260, 2008.

[86] W. Xu, C. Hellerbrand, U. A. Köhler et al., "The Nrf2 transcription factor protects from toxin-induced liver injury and fibrosis," *Laboratory Investigation*, vol. 88, no. 10, pp. 1068–1078, 2008.

[87] F. Liu, S. Ichihara, W. M. Valentine et al., "Increased susceptibility of Nrf2-null mice to 1-bromopropane-induced hepatotoxicity," *Toxicological Sciences*, vol. 115, no. 2, pp. 596–606, 2010.

[88] B.-H. Lee, W.-H. Hsu, Y.-W. Hsu, and T.-M. Pan, "Suppression of dimerumic acid on hepatic fibrosis caused from carboxymethyl-lysine (CML) by attenuating oxidative stress depends on Nrf2 activation in hepatic stellate cells (HSCs)," *Food and Chemical Toxicology*, vol. 62, pp. 413–419, 2013.

[89] C.-L. Liu, J.-M. Wang, C.-Y. Chu, M.-T. Cheng, and T.-H. Tseng, "In vivo protective effect of protocatechuic acid on tert-butyl hydroperoxide-induced rat hepatotoxicity," *Food and Chemical Toxicology*, vol. 40, no. 5, pp. 635–641, 2002.

[90] D.-S. Lee, K.-S. Kim, W. Ko et al., "The cytoprotective effect of sulfuretin against tert-Butyl Hydroperoxide-induced hepatotoxicity through Nrf2/ARE and JNK/ERK MAPK-mediated Heme Oxygenase-1 expression," *International Journal of Molecular Sciences*, vol. 15, no. 5, pp. 8863–8877, 2014.

[91] S. Hix, M. B. Kadiiska, R. P. Mason, and O. Augusto, "In vivo metabolism of tert-Butyl hydroperoxide to methyl radicals. EPR spin-trapping and DNA methylation studies," *Chemical Research in Toxicology*, vol. 13, no. 10, pp. 1056–1064, 2000.

[92] J. M. Oh, Y. S. Jung, B. S. Jeon et al., "Evaluation of hepatotoxicity and oxidative stress in rats treated with tert-butyl hydroperoxide," *Food and Chemical Toxicology*, vol. 50, no. 5, pp. 1215–1221, 2012.

[93] L. Jarup, "Hazards of heavy metal contamination," *British Medical Bulletin*, vol. 68, no. 1, pp. 167–182, 2003.

[94] K. C. Wu, J. J. Liu, and C. D. Klaassen, "Nrf2 activation prevents cadmium-induced acute liver injury," *Toxicology and Applied Pharmacology*, vol. 263, no. 1, pp. 14–20, 2012.

[95] D. Liu, X. Duan, D. Dong et al., "Activation of the Nrf2 pathway by inorganic arsenic in human hepatocytes and the role of transcriptional repressor Bach1," *Oxidative Medicine and Cellular Longevity*, vol. 2013, Article ID 984546, 10 pages, 2013.

[96] Y. Abiko, Y. Shinkai, D. Sumi, and Y. Kumagai, "Reduction of arsenic-induced cytotoxicity through Nrf2/HO-1 signaling in HepG2 cells," *Journal of Toxicological Sciences*, vol. 35, no. 3, pp. 419–423, 2010.

[97] X.-F. Wang, M.-L. Xing, Y. Shen, X. Zhu, and L.-H. Xu, "Oral administration of Cr(VI) induced oxidative stress, DNA damage and apoptotic cell death in mice," *Toxicology*, vol. 228, no. 1, pp. 16–23, 2006.

[98] A. K. Patlolla, C. Barnes, C. Yedjou, V. R. Velma, and P. B. Tchounwou, "Oxidative stress, DNA damage, and antioxidant enzyme activity induced by hexavalent chromium in Sprague-Dawley rats," *Environmental Toxicology*, vol. 24, no. 1, pp. 66–73, 2009.

[99] X. Q. He, G. X. Lin, M. G. Chen, J. X. Zhang, and Q. Ma, "Protection against chromium (VI)-induced oxidative stress and apoptosis by Nrf2. Recruiting Nrf2 into the nucleus and disrupting the nuclear Nrf2/Keap1 association," *Toxicological Sciences*, vol. 98, no. 1, pp. 298–309, 2007.

[100] C. Torruellas, S. W. French, and V. Medici, "Diagnosis of alcoholic liver disease," *World Journal of Gastroenterology*, vol. 20, no. 33, pp. 11684–11699, 2014.

[101] D. Wu and A. I. Cederbaum, "Oxidative stress and alcoholic liver disease," *Seminars in Liver Disease*, vol. 29, no. 2, pp. 141–154, 2009.

[102] A. I. Cederbaum, Y. Lu, and D. Wu, "Role of oxidative stress in alcohol-induced liver injury," *Archives of Toxicology*, vol. 83, no. 6, pp. 519–548, 2009.

[103] P. E. Gong and A. I. Cederbaum, "Nrf2 is increased by CYP2E1 in rodent liver and HepG2 cells and protects against oxidative stress caused by CYP2E1," *Hepatology*, vol. 43, no. 1, pp. 144–153, 2006.

[104] J. Lamlé, S. Marhenke, J. Borlak et al., "Nuclear factor-eythroid 2-related factor 2 prevents alcohol-induced fulminant liver injury," *Gastroenterology*, vol. 134, no. 4, pp. 1159–1168, 2008.

[105] K. C. Wu, J. Liu, and C. D. Klaassen, "Role of Nrf2 in preventing ethanol-induced oxidative stress and lipid accumulation," *Toxicology and Applied Pharmacology*, vol. 262, no. 3, pp. 321–329, 2012.

[106] H. Azzam and S. Malnick, "Non-alcoholic fatty liver disease—the heart of the matter," *World Journal of Hepatology*, vol. 7, no. 10, pp. 1369–1376, 2015.

[107] C. D. Byrne and G. Targher, "NAFLD: a multisystem disease," *Journal of Hepatology*, vol. 62, no. 1, pp. S47–S64, 2015.

[108] T. Williams, "Metabolic syndrome: nonalcoholic fatty liver disease," *FP Essentials*, vol. 435, pp. 24–29, 2015.

[109] W. Liu, S. S. Baker, R. D. Baker, and L. Zhu, "Antioxidant mechanisms in nonalcoholic fatty liver disease," *Current Drug Targets*, vol. 16, no. 12, pp. 1301–1314, 2015.

[110] G. Serviddio, F. Bellanti, and G. Vendemiale, "Free radical biology for medicine: learning from nonalcoholic fatty liver disease," *Free Radical Biology and Medicine*, vol. 65, pp. 952–968, 2013.

[111] S. Kim, I. Sohn, J.-I. Ahn, K.-H. Lee, Y. S. Lee, and Y. S. Lee, "Hepatic gene expression profiles in a long-term high-fat diet-induced obesity mouse model," *Gene*, vol. 340, no. 1, pp. 99–109, 2004.

[112] Y. Tanaka, L. M. Aleksunes, R. L. Yeager et al., "NF-E2-related factor 2 inhibits lipid accumulation and oxidative stress in mice fed a high-fat diet," *Journal of Pharmacology and Experimental Therapeutics*, vol. 325, no. 2, pp. 655–664, 2008.

[113] S. Chowdhry, M. H. Nazmy, P. J. Meakin et al., "Loss of Nrf2 markedly exacerbates nonalcoholic steatohepatitis," *Free Radical Biology and Medicine*, vol. 48, no. 2, pp. 357–371, 2010.

[114] K. Okada, E. Warabi, H. Sugimoto et al., "Nrf2 inhibits hepatic iron accumulation and counteracts oxidative stress-induced liver injury in nutritional steatohepatitis," *Journal of Gastroenterology*, vol. 47, no. 8, pp. 924–935, 2012.

[115] H. Sugimoto, K. Okada, J. Shoda et al., "Deletion of nuclear factor-E2-related factor-2 leads to rapid onset and progression of nutritional steatohepatitis in mice," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 298, no. 2, pp. G283–G294, 2010.

[116] R. Jadeja, R. V. Devkar, and S. Nammi, "Herbal medicines for the treatment of nonalcoholic steatohepatitis: Current scenario and future prospects," *Evidence-Based Complementary and Alternative Medicine*, vol. 2014, Article ID 648308, 18 pages, 2014.

[117] J. Xiao, K. F. So, E. C. Liong, and G. L. Tipoe, "Recent advances in the herbal treatment of non-alcoholic fatty liver disease," *Journal of Traditional and Complementary Medicine*, vol. 3, no. 2, pp. 88–94, 2013.

[118] N. Miloševič, M. Milanovič, L. Abenavoli, and N. Milič, "Phytotherapy and nafld-rom goals and challenges to clinical practice," *Reviews on Recent Clinical Trials*, vol. 9, no. 3, pp. 195–203, 2014.

[119] I. H. Bahcecioglu, N. Kuzu, K. Metin et al., "Lycopene prevents development of steatohepatitis in experimental nonalcoholic steatohepatitis model induced by high-fat diet," *Veterinary Medicine International*, vol. 2010, Article ID 262179, 8 pages, 2010.

[120] H. Jaeschke, G. J. Gores, A. I. Cederbaum, J. A. Hinson, D. Pessayre, and J. J. Lemasters, "Mechanisms of hepatotoxicity," *Toxicological Sciences*, vol. 65, no. 2, pp. 166–176, 2002.

[121] J. Weerachayaphorn, A. Mennone, C. J. Soroka et al., "Nuclear factor-E2-related factor 2 is a major determinant of bile acid homeostasis in the liver and intestine," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 302, no. 9, pp. G925–G936, 2012.

[122] L. M. Aleksunes, A. L. Slitt, J. M. Maher et al., "Nuclear factor-E2-related factor 2 expression in liver is critical for induction of NAD(P)H:quinone oxidoreductase 1 during cholestasis," *Cell Stress and Chaperones*, vol. 11, no. 4, pp. 356–363, 2006.

[123] K. Okada, J. Shoda, K. Taguchi et al., "Nrf2 counteracts cholestatic liver injury via stimulation of hepatic defense systems," *Biochemical and Biophysical Research Communications*, vol. 389, no. 3, pp. 431–436, 2009.

[124] K. P. Tan, G. A. Wood, M. Yang, and S. Ito, "Participation of nuclear factor (erythroid 2-related), factor 2 in ameliorating lithocholic acid-induced cholestatic liver injury in mice," *British Journal of Pharmacology*, vol. 161, no. 5, pp. 1111–1121, 2010.

[125] O. Lukivskaya, L. Zavodnik, M. Knas, and V. Buko, "Antioxidant mechanism of hepatoprotection by ursodeoxycholic acid in experimental alcoholic steatohepatitis," *Advances in Medical Sciences*, vol. 51, pp. 54–59, 2006.

[126] K. Ishizaki, T. Imada, and M. Tsurufuji, "Hepatoprotective bile acid 'ursodeoxycholic acid (UDCA)': property and difference as bile acids," *Hepatology Research*, vol. 33, no. 2, pp. 174–177, 2005.

[127] G. Paumgartner and U. Beuers, "Mechanisms of action and therapeutic efficacy of ursodeoxycholic acid in cholestatic liver disease," *Clinics in Liver Disease*, vol. 8, no. 1, pp. 67–81, 2004.

[128] K. Kawata, Y. Kobayashi, K. Souda et al., "Enhanced hepatic Nrf2 activation after ursodeoxycholic acid treatment in patients with primary biliary cirrhosis," *Antioxidants and Redox Signaling*, vol. 13, no. 3, pp. 259–268, 2010.

[129] Y.-F. Lu, X.-L. Wan, Y. Xu, and J. Liu, "Repeated oral administration of oleanolic acid produces cholestatic liver injury in mice," *Molecules*, vol. 18, no. 3, pp. 3060–3071, 2013.

[130] G. L. Plaa and B. G. Priestly, "Intrahepatic cholestasis induced by drugs and chemicals," *Pharmacological Reviews*, vol. 28, no. 3, pp. 207–273, 1976.

[131] Y. Korenori, S. Tanigawa, T. Kumamoto et al., "Modulation of Nrf2/Keap1 system by Wasabi 6-methylthiohexyl isothiocyanate in ARE-mediated NQO1 expression," *Molecular Nutrition and Food Research*, vol. 57, no. 5, pp. 854–864, 2013.

[132] E.-J. Joung, M.-H. Li, H. G. Lee et al., "Capsaicin induces heme oxygenase-1 expression in HepG2 cells via activation of PI3K-Nrf2 signaling: NAD(P)H:quinone oxidoreductase as a potential target," *Antioxidants and Redox Signaling*, vol. 9, no. 12, pp. 2087–2098, 2007.

[133] C.-C. Chen, H.-L. Chen, C.-W. Hsieh, Y.-L. Yang, and B.-S. Wung, "Upregulation of NF-E2-related factor-2-dependent glutathione by carnosol provokes a cytoprotective response and enhances cell survival," *Acta Pharmacologica Sinica*, vol. 32, no. 1, pp. 62–69, 2011.

[134] Y.-J. Jun, M. Lee, T. Shin, N. Yoon, J.-H. Kim, and H.-R. Kim, "Eckol enhances heme oxygenase-1 expression through activation of Nrf2/JNK pathway in HepG2 cells," *Molecules*, vol. 19, no. 10, pp. 15638–15652, 2014.

[135] A. B. Granado-Serrano, M. A. Martín, G. Haegeman, L. Goya, L. Bravo, and S. Ramos, "Epicatechin induces NF-κB, activator protein-1 (AP-1) and nuclear transcription factor erythroid 2p45-related factor-2 (Nrf2) via phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) and extracellular regulated kinase (ERK) signalling in HepG2 cells," *British Journal of Nutrition*, vol. 103, no. 2, pp. 168–179, 2010.

[136] V. Krajka-Kuźniak, J. Paluszczak, L. Celewicz, J. Barciszewski, and W. Baer-Dubowska, "Phloretamide, an apple phenolic compound, activates the Nrf2/ARE pathway in human hepatocytes," *Food and Chemical Toxicology*, vol. 51, no. 1, pp. 202–209, 2013.

[137] S. Tanigawa, M. Fujii, and D.-X. Hou, "Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin," *Free Radical Biology and Medicine*, vol. 42, no. 11, pp. 1690–1703, 2007.

[138] V. Krajka-Kuźniak, J. Paluszczak, and W. Baer-Dubowska, "Xanthohumol induces phase II enzymes via Nrf2 in human hepatocytes in vitro," *Toxicology in Vitro*, vol. 27, no. 1, pp. 149–156, 2013.

[139] W. H. Yap, K. S. Khoo, A. S. H. Ho, and Y. M. Lim, "Maslinic acid induces HO-1 and NOQ1 expression via activation of Nrf2 transcription factor," *Biomedicine and Preventive Nutrition*, vol. 2, no. 1, pp. 51–58, 2012.

[140] S. Chitturi and G. C. Farrell, "Herbal hepatotoxicity: an expanding but poorly defined problem," *Journal of Gastroenterology and Hepatology*, vol. 15, no. 10, pp. 1093–1099, 2000.

[141] L. I. Mennen, R. Walker, C. Bennetau-Pelissero, and A. Scalbert, "Risks and safety of polyphenol consumption," *The American Journal of Clinical Nutrition*, vol. 81, no. 1, supplement, pp. 326S–329S, 2005.

[142] C. F. Skibola and M. T. Smith, "Potential health impacts of excessive flavonoid intake," *Free Radical Biology and Medicine*, vol. 29, no. 3-4, pp. 375–383, 2000.

[143] J. Liu, Y.-F. Lu, Y. Zhang, K. C. Wu, F. Fan, and C. D. Klaassen, "Oleanolic acid alters bile acid metabolism and produces cholestatic liver injury in mice," *Toxicology and Applied Pharmacology*, vol. 272, no. 3, pp. 816–824, 2013.

[144] C. Frenzel and R. Teschke, "Herbal hepatotoxicity: clinical characteristics and listing compilation," *International Journal of Molecular Sciences*, vol. 17, no. 5, p. 588, 2016.

[145] C. Bunchorntavakul and K. R. Reddy, "Review article: herbal and dietary supplement hepatotoxicity," *Alimentary Pharmacology and Therapeutics*, vol. 37, no. 1, pp. 3–17, 2013.

[146] M. Muqeet Adnan, M. Khan, S. Hashmi, M. Hamza, S. Abdul-Mujeeb, and S. Amer, "Black cohosh and liver toxicity: is there a relationship?" *Case Reports in Gastrointestinal Medicine*, vol. 2014, Article ID 860614, 3 pages, 2014.

[147] T. Y. Lim, A. Considine, A. Quaglia, and D. L. Shawcross, "Subacute liver failure secondary to black cohosh leading to liver transplantation," *BMJ Case Reports*, vol. 2013, 2013.

[148] R. Teschke and A. Schwarzenboeck, "Suspected hepatotoxicity by *Cimicifugae racemosae rhizoma* (black cohosh, root): critical analysis and structured causality assessment," *Phytomedicine*, vol. 16, no. 1, pp. 72–84, 2009.

[149] C. R. Lynch, M. E. Folkers, and W. R. Hutson, "Fulminant hepatic failure associated with the use of black cohosh: a case report," *Liver Transplantation*, vol. 12, no. 6, pp. 989–992, 2006.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 7962948, 12 pages
http://dx.doi.org/10.1155/2016/7962948

Research Article

Endogenous n-3 Fatty Acids Alleviate Carbon-Tetrachloride-Induced Acute Liver Injury in *Fat-1* Transgenic Mice

Ruibing Feng,[1] Meng Wang,[1] Chunyan Yan,[2] Peng Li,[1] Meiwan Chen,[1] Chengwei He,[1] and Jian-Bo Wan[1]

[1] *State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau*
[2] *College of Pharmacy, Guangdong Pharmaceutical University, Guangzhou 510006, China*

Correspondence should be addressed to Chengwei He; chengweihe@umac.mo and Jian-Bo Wan; jbwan@umac.mo

Received 6 September 2016; Accepted 11 October 2016

Academic Editor: Ravirajsinh Jadeja

n-3 polyunsaturated fatty acids (PUFAs) are beneficial for numerous models of liver diseases. The probable protective effects of n-3 PUFA against carbon-tetrachloride- (CCl_4-) induced acute liver injury were evaluated in a *fat-1* transgenic mouse that synthesizes endogenous n-3 from n-6 PUFA. *Fat-1* mice and their WT littermates were fed a modified AIN93 diet containing 10% corn oil and were injected intraperitoneally with a single dose of CCl_4 or vehicle. CCl_4 challenge caused severe liver injury in WT mice, as indicated by serum parameters and histopathological changes, which were remarkably ameliorated in *fat-1* mice. Endogenous n-3 PUFA decreased the elevation of oxidative stress induced by CCl_4 challenge, which might be attributed to the activation of Nrf2/keap1 pathway. Additionally, endogenous n-3 PUFA reduces hepatocyte apoptosis via suppressing MAPK pathway. These findings indicate that n-3 PUFA has potent protective effects against acute liver injury induced by CCl_4 in mice, suggesting that n-3 PUFA can be used for the prevention and treatment of liver injury.

1. Introduction

Liver is a vital organ that has extensive synthetic, metabolic, and detoxifying functions [1]. This tissue is also a main target that is subject to acute or chronic injury induced by a variety of drugs or xenobiotics, such as alcohol, heavy metals, and carbon tetrachloride (CCl_4) [2]. CCl_4, an analogue of human hepatotoxin, has been widely used in *in vitro* and *in vivo* models to induce liver injury [3]. CCl_4 is oxidized by cytochrome P4502E1 (CYP2E1) in the liver to generate the highly reactive species, such as trichloromethyl ($^{\bullet}CCl_3$) and peroxy trichloromethyl ($^{\bullet}OOCCl_3$) radicals [4], which can trigger oxidative stress, lipid peroxidation, and hepatocyte apoptosis, leading ultimately to hepatotoxicity [5].

Oxidative stress is mainly responsible for the pathogenesis of CCl_4-induced liver injury, which can disturb the redox homeostasis and elevate the excessive production of reactive oxygen species (ROS) [6]. Antioxidant defense system, including nonenzymatic antioxidants and enzymatic antioxidants, contributes to protecting the liver against oxidative stress in living organisms. The expressions of these antioxidative enzymes are regulated by a redox-sensitive transcription factor, nuclear factor-erythroid 2-related factor-2 (Nrf2), and its downstream proteins, including heme oxygenase-1 (HO-1), glutamate cysteine ligase (GCL), and NAD(P)H:quinone oxidoreductase-1 (NQO1) [7, 8]. Oxidative stress also elevates cytochrome C in the cytoplasm which is released from the mitochondria, which induces the activation of caspase cascades, eventually leading to hepatocyte apoptosis.

Growing evidence indicates that n-3 polyunsaturated fatty acids (PUFAs), mainly α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), exhibit profoundly therapeutic efficacy in several models of liver disease, including nonalcoholic liver disease [9], parenteral nutrition-associated liver disease [10], alcohol-induced liver injury [11], hepatic steatosis [12, 13], and

D-galactosamine/lipopolysaccharide-induced hepatitis [14]. However, the impacts of n-3 PUFA on CCl_4-induced liver injury have not been sufficiently addressed. The *fat-1* transgenic mouse was genetically modified to express a *fat-1* gene that encodes n-3 PUFA desaturase [15, 16]. This enzyme can endogenously convert n-6 PUFA to n-3 PUFA in mammals, leading to higher n-3 PUFA level in tissues from *fat-1* mice, compared to the wild-type (WT) littermates when fed the same diet rich in n-6 PUFA. Thus, *fat-1* mice are a well-established animal model to investigate the role of n-3 PUFA in CCl_4-induced liver injury. Therefore, the aims of current study are to evaluate the probable effects of n-3 PUFA against CCl_4-induced acute liver injury and to elucidate the potential molecular mechanisms underlying this action.

2. Materials and Methods

2.1. Animals and Treatments. *Fat-1* transgenic mice with a genetic background of C57BL/6 were provided by Dr. Jing X. Kang's lab at Massachusetts General Hospital (Boston, MA, USA). Male heterozygous *fat-1* mice were crossed with C57BL/6 female mice to yield heterozygous *fat-1* and WT offspring. The *fat-1* phenotype of each offspring was identified by the analysis of total lipids from mouse tail by using gas chromatography-mass spectrometry (GC-MS). The female *fat-1*-positive and WT littermates were maintained in a specific pathogen-free room at the Experimental Animal Center, Guangdong Pharmaceutical University. Mice were fed an n-6 PUFA-rich but n-3 PUFA-deficient diet (a modified AIN93 containing 10% corn oil), which contains 20% protein, 58% carbohydrate, and 22% fat (TROPHIC Animal Feed High-Tech Co., Ltd., Nantong, China). Mice (10–12 weeks old) were divided into three groups ($n = 10$), that is, WT control, WT/CCl_4, and *fat-1*/CCl_4 groups. The mice in CCl_4 challenge groups were injected intraperitoneally with 10 mL/kg CCl_4 (0.2%, dissolved in olive oil) [17], while WT controls received an equal volume olive oil (i.p.). After 24 h of CCl_4 or vehicle treatment, all mice were anesthetized by an injection (i.p., 100 mg/kg) with sodium pentobarbital. Blood samples and liver tissues were immediately collected. The animal experimental protocols (ICMS-AEC-2015-028) were performed according to the Guide to Animal Use and Care of the University of Macau and were approved by the ethics committee.

2.2. Analysis of Fatty Acid Composition in the Liver. Fatty acid profile was analyzed according to a simplified method by using GC-MS, as described previously [18, 19]. In brief, approximately 10 mg of liver tissue was ground in liquid nitrogen and methylated with 1.5 mL of 14% boron trifluoride-methanol reagent (Sigma-Aldrich) and 1.5 mL of hexane at 100°C for 1 h. After cooling, fatty acid methyl esters (FAME) were extracted in upper hexane layer. GC-MS analysis was conducted on a Thermo Fisher Scientific ISQ Series Single Quadrupole GC-MS system equipped with a TriPlus RSH™ autosampler (Thermo Fisher, Waltham, MA, USA). Separation of FAME was achieved on an Omegawax™ 250 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Supelco, Bellefonte, PA). The optimum oven temperature program was as follows: it was initially set at 180°C for 3 min, ramped to 206°C at 2°C/min, held at 206°C for 25 min, ramped to 240°C at 10°C/min, and held at 240°C for 5 min. Peaks in the chromatogram were identified by comparing their retention times and mass spectrums with GLC-461 reference standard (Nu-Chek Prep, Elysian, MN, USA) containing 32 FAME.

2.3. Measurement of Serum Aminotransferase Levels. Activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were colorimetrically examined by using their commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.4. Determination of Oxidative Stress Parameters in the Liver. Partial liver tissues were weighed and homogenized with cold radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Nanjing, China) to prepare 10% liver homogenate. After centrifugation, the supernatant was subjected to measure the levels of malondialdehyde (MDA), reduced glutathione (GSH), and oxidized glutathione (GSSG) and the activities of catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and superoxide dismutase (SOD) in the liver, by the corresponding kits (Nanjing Jiancheng Bioengineering Institute). Total proteins in the homogenate were quantified using Pierce™ BCA Kit (Thermo Fisher). Values were normalized against hepatic total protein content.

2.5. Histopathological Analysis. Histopathological changes of the liver were observed by hematoxylin and eosin (H&E) staining [20]. The liver tissue from the same lobe in each mouse was fixed in 10% formalin overnight, dehydrated in alcohol with different concentrations, and embedded in paraffin. The liver sections (5 μm thickness) were stained with H&E using a standard protocol. The histopathological changes of each mouse were examined and photographed by an Olympus CX-31 light microscope (Olympus Corp., Tokyo, Japan).

2.6. TUNEL Assay. To evaluate apoptotic cells in the liver tissue, a terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate (dUTP) nick end labeling (TUNEL) assay was conducted by using ApopTag® Plus In Situ Apoptosis Fluorescein Detection Kit (S7111, EMD Millipore Corporation, Billerica, MA, USA). In brief, liver cryostat section (8 μM) was fixed in 1% paraformaldehyde and washed with PBS three times. Then, the section was incubated with green fluorescein labeled dUTP solution at 37°C for 1 h. The section was counterstained with DAPI and examined using an Olympus BX63 fluorescence microscope (Tokyo, Japan).

2.7. Immunofluorescence Assay. Immunofluorescence analysis of hepatic Nrf2 was conducted as described previously [15]. Briefly, the cryostat section of liver tissue (8 μM) was fixed in cooled acetone for 10 min at 4°C and then washed with PBS. After blocking the endogenous peroxidase with 5% goat serum for 20 min, the liver section was incubated with

Table 1: The primer sequences used in quantitative reverse transcription PCR analysis.

Gene	Full name	GenBank accession number	Primer sequences (forward/reverse)
HO-1	Heme oxygenase-1	NM_010442	5′-AAGCCGAGAATGCTGAGTTCA-3′ 5′-GCCGTGTAGATATGGTACAAGGA-3′
GCLC	Glutamate cysteine ligase catalytic subunit	NM_010295	5′-GGGGTGACGAGGTGGAGTA-3′ 5′-GTTGGGGTTTGTCCTCTCCC-3′
GCLM	Glutamate cysteine ligase modifier subunit	NM_008129	5′-AGGAGCTTCGGGACTGTATCC-3′ 5′-GGGACATGGTGCATTCCAAAA-3′
NQO1	Quinone oxidoreductase-1	NM_008706	5′-AGGATGGGAGGTACTCGAATC-3′ 5′-AGGCGTCCTTCCTTATATGCTA-3′
GADPH	Glyceraldehyde-3-phosphate dehydrogenase	NM_008085	5′-TGGATTTGGACGCATTGGTC-3′ 5′-TTTGCACTGGTACGTGTTGAT-3′

1 : 100 rabbit anti-mouse Nrf2 antibody (Santa Cruz Biotechnology, Dallas, USA) at 4°C overnight and then incubated with 1 : 1000 Alexa Fluor® 568-labeled secondary antibody (Life Technologies, Carlsbad, CA, USA) in the dark at room temperature for 1 h. Nuclei were counterstained with DAPI for 10 min. The fluorescence was observed and photographed by an Olympus BX63 fluorescent microscope (Olympus).

2.8. RT-PCR Assay. Total RNA from the same lobe of liver tissue was extracted by a commercial RNAiso Plus kit according to the manufacturer's protocol (Takara, Tokyo, Japan). cDNA was synthesized by reverse transcription and amplified by PCR with the primers shown in Table 1 using PrimeScript RT Reagent kit (Takara). The sequence of primers was designed from the PrimerBank and synthesized by Invitrogen Life Technologies (Shanghai, China). PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

2.9. Western Blotting Assay. Total protein, cytosolic protein (exclusively for Nrf2), and nuclear protein (exclusively for Nrf2) from liver tissues were prepared. Total proteins were isolated from liver tissue by RIPA buffer with 1% phosphatase and protease inhibitors (Beyotime). The extraction and isolation of nuclear and cytosolic proteins were conducted by a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). Protein concentration was quantified by using a Pierce BCA Kit (Thermo Fisher). An aliquot of 20 μg total protein was loaded and separated on 10–15% SDS-PAGE and then, subsequently, electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane. The transferred membrane was incubated with primary antibodies at 4°C overnight (Table 2) and then incubated with the corresponding secondary antibodies (1 : 1000) at room temperature for 1 h. The band was visualized by ECL Detection Reagent (GE Healthcare BioSciences, NJ, USA) in a FluorChem Imaging system (Cell Biosciences, Santa Clara, CA, USA).

2.10. Statistical Analysis. Data are presented as mean ± standard deviation (SD). To test the difference between groups, one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was performed by using GraphPad Prism 6.0 software (San Diego, CA, USA). Statistical significance was accepted at the level of $p < 0.05$.

3. Results

3.1. Fatty Acid Composition in Liver Tissues. To measure the effect of *fat-1* expression on hepatic fatty acid profile, liver tissues from *fat-1* and WT mice were determined by GC-MS. Because *fat-1* gene can encode n-3 PUFA desaturase that allows converting n-6 PUFA to n-3 PUFA in *fat-1* mice, compared with WT/CCl_4 group, liver tissues from *fat-1*/CCl_4 group exhibited higher amounts of n-3 PUFA, including ALA (18:3n-3), EPA (20:5n-3), and DHA (22:6n-3), and lower levels of n-6 PUFA, mainly linoleic acid (LA, 18:2n-6) and arachidonic acid (AA, 20:4n-6), leading to a remarkable increase in total n-3 PUFA and decreases in total n-6 PUFA and n-6/n-3 ratio (Table 3). The level of total saturated fatty acids (SFA) in *fat-1*/CCl_4 mice was significantly higher, and the level of total monounsaturated fatty acids (MUFAs) tended to be lower. These results demonstrated that the expression of *fat-1* gene greatly elevated n-3 PUFA levels in the liver, although both groups were fed the identical diet.

CCl_4 exposure also greatly altered the fatty acid composition in the liver. Compared to WT control, the WT/CCl_4 group showed decreased levels in SFA, mainly 14:0, 16:0, and 18:0, and increased levels in MUFA, mainly 16:1 and 18:1, leading to increased ratios of 16:1/16:0 and 18:1/18:0, the fatty acid desaturation index. These findings also suggest that CCl_4 challenge may increase the activity or expression of stearoyl-CoA desaturase-1 (SCD-1).

3.2. Endogenous n-3 PUFA Ameliorates the Features of Acute CCl_4-Induced Liver Injury. Liver injury was evaluated by serum enzyme activities and hepatic histopathological changes. ALT and AST are released into the blood once the structural integrity of the hepatocyte was damaged; their levels are the most commonly used markers of liver injury [21]. As shown in Figure 1(a), after acute CCl_4 challenge, the serum levels of ALT and AST in WT/CCl_4 group increased 63 and 71 times, respectively, over those in WT group. However, these elevations were significantly blunted in *fat-1*/CCl_4 group. The histological changes in the liver were evaluated by H&E staining (Figure 1(b)). WT group exhibited normal architecture

Table 2: Primary antibodies used in immunoblot analysis.

Primary antibody	Full name	Source	Dilution	Company
Bax	BCL2-associated X protein	Rabbit	1:1000	Cell Signaling Technology
Bcl-2	B-cell lymphoma-2	Rabbit	1:1000	Cell Signaling Technology
Caspase-3	Cysteinyl aspartate specific proteinase-3	Rabbit	1:1000	Cell Signaling Technology
Caspase-9	Cysteinyl aspartate specific proteinase-9	Rabbit	1:1000	Cell Signaling Technology
CYP2E1	Cytochrome P4502E1	Rabbit	1:500	Abcam
Cyto-C	Cytochrome C	Rabbit	1:1000	Cell Signaling Technology
ERK	Extracellular signal-regulated protein kinase	Rabbit	1:1000	Cell Signaling Technology
GADPH	Glyceraldehyde-3-phosphate dehydrogenase	Rabbit	1:1000	Cell Signaling Technology
GCLC	Glutamate cysteine ligase catalytic subunit	Rabbit	1:1000	Abcam
GCLM	Glutamate cysteine ligase modifier subunit	Rabbit	1:500	Santa Cruz Biotechnology
HO-1	Heme oxygenase-1	Rabbit	1:1000	Abcam
JNK	c-Jun N-terminal kinase	Rabbit	1:1000	Cell Signaling Technology
Keap1	Kelch-like ECH-associated protein-1	Rabbit	1:1000	Cell Signaling Technology
Lamin B	Lamin B	Rabbit	1:500	Cell Signaling Technology
Nrf2	Nuclear factor erythroid 2-related factor-2	Rabbit	1:1000	Santa Cruz Biotechnology
NQO1	NAD(P)H:quinone oxidoreductase-1	Rabbit	1:1000	Santa Cruz Biotechnology
p38	p38 mitogen-activated protein kinase	Rabbit	1:1000	Cell Signaling Technology
p62	Nucleoporin p62	Rabbit	1:1000	Cell Signaling Technology
p-ERK	Phosphorylated extracellular signal-regulated protein kinase	Rabbit	1:1000	Cell Signaling Technology
p-JNK	Phosphorylated c-Jun N-terminal kinase	Rabbit	1:1000	Cell Signaling Technology
p-p38	Phosphorylated p38 mitogen-activated protein kinase	Rabbit	1:1000	Cell Signaling Technology

with clear nuclear distribution. CCl_4 induced histological changes including severely disrupted hepatic architecture and extensive hepatocellular necrosis around the blood vessels in WT/CCl_4 group, which was reduced in *fat-1*/CCl_4 group.

3.3. Endogenous n-3 PUFA Reduces CCl_4-Induced Oxidative Stress in the Liver. Oxidative stress is characterized as a redox imbalance between prooxidants and endogenous antioxidants, including nonenzymatic antioxidants (e.g., GSH) and enzymatic antioxidants (e.g., SOD, CAT, and GSH-Px) [22]. MDA is an end product of lipid peroxidation (LPO) and has been widely used as a marker of oxidative stress [22]. As shown in Table 4, CCl_4 exposure induced a remarkable increase of hepatic MDA production by 87.7% (2.52 ± 0.34 versus 4.73 ± 0.52, $p < 0.01$), and a remarkable decrease in hepatic lipid peroxidation was observed in *fat-1*/CCl_4 group. Conversely, CCl_4 challenge depleted endogenous enzymatic and nonenzymatic antioxidants which can protect hepatocytes against oxidative stress, as it is indicated that the activities of SOD, CAT, and GSH-Px and GSH level in WT/CCl_4 group were significantly reduced to 70.5%, 55.7%, 59.0%, and 68.4% of those of WT group, respectively. This depletion of endogenous antioxidants was markedly ameliorated in *fat-1*/CCl_4 group. As a radical scavenger, GSH can be oxidized to GSSG under oxidative stress. GSSG is also reduced back to GSH by glutathione reductase (GR). Hence, GSH/GSSG has been also used as a marker of oxidative stress [23]. CCl_4 exposure significantly increased the GSSG level and decreased GR activity in the liver, leading to a great decrease in GSH/GSSG ratio; these changes were remarkably ameliorated in *fat-1* mice.

3.4. Endogenous n-3 PUFA Upregulates Antioxidant Enzymes via Nuclear Translocation of Nrf2. To understand the underlying molecular mechanisms for the protective effects of endogenous n-3 PUFA against oxidative stress triggered by CCl_4, the activation of Nrf2, a main transcription factor regulating antioxidant responses in the liver, was examined by immunofluorescence assay and immunoblot analysis. As shown in Figure 2(a), the significant nuclear translocation of Nrf2 was detected in *fat-1*/CCl_4 group, compared to WT/CCl_4 group, which was in accordance with the results of immunoblot analysis. Endogenous n-3 PUFA in *fat-1* mice greatly decreased the protein expression of Nrf2 in the cytoplasm but increased Nrf2 expression in the nucleus, without changing the level of total Nrf2 expression in the liver (Figure 2(b)). In addition, the Kelch-like ECH-associated protein-1 (Keap1), a repressor protein, and p62, a substrate adaptor sequestosome-1 protein that competes with Nrf2 for binding to Keap1, were examined in the liver by western blot. As shown in Figure 2(c), the lower protein expression of Keap1 and higher expression of p62 were detected in *fat-1*/CCl_4 mice compared to WT/CCl_4 group.

Nrf2-regulated genes, such as HO-1, GCLC, GCLM, and NQO1, were also examined. As shown in Figure 3, both mRNA and protein levels of HO-1, GCLC, GCLM, and NQO1 in the liver were obviously upregulated in *fat-1*/CCl_4 group compared to WT/CCl_4 group. Interestingly, CCl_4 challenge notably promoted nuclear translocation of Nrf2, elevated Nrf2 expression in the nucleus, and increased the expression of its downstream genes (Figures 2 and 3), which were consistent with the previous studies [3, 4]. A most plausible explanation is the adaptive cytoprotective

Table 3: Fatty acid composition (%) of liver tissues.

Fatty acids		% of total fatty acids		
Common name	Symbol	WT	WT/CCl_4	*Fat-1*/CCl_4
Lauric acid	12:0	0.27 ± 0.22	0.23 ± 0.15	0.26 ± 0.22
Myristic acid	14:0	0.84 ± 0.28	0.60 ± 0.13$^{\#}$	0.76 ± 0.11**
Palmitic acid	16:0	28.5 ± 4.3	24.4 ± 1.6$^{\#}$	26.7 ± 3.8
Palmitoleic acid	16:1	1.16 ± 0.43	1.59 ± 0.42$^{\#}$	1.32 ± 0.38**
Stearic acid	18:0	23.0 ± 7.4	14.5 ± 2.9$^{\#\#}$	19.5 ± 5.4*
Oleic acid	18:1	15.1 ± 5.0	20.8 ± 2.3$^{\#\#}$	17.7 ± 3.1*
Linolenic acid	18:2,6	17.1 ± 5.6	24.6 ± 2.3$^{\#\#}$	21.3 ± 4.2*
γ-Linolenic acid	18:3,6	0.41 ± 0.20	0.35 ± 0.10	0.28 ± 0.16
α-Linolenic acid	18:3,3	0.27 ± 0.05	0.22 ± 0.05	0.36 ± 0.07**
Arachidic acid	20:0	0.45 ± 0.18	0.23 ± 0.08$^{\#\#}$	0.41 ± 0.12**
Eicosenoic acid	20:1	0.26 ± 0.06	0.26 ± 0.03	0.25 ± 0.05
Dihomo-γ-linoleic acid	20:3,6	0.43 ± 0.14	0.60 ± 0.10$^{\#\#}$	0.43 ± 0.08**
Arachidonic acid	20:4,6	9.08 ± 1.48	8.21 ± 1.86	4.73 ± 1.29**
Eicosapentaenoic acid	20:5,3	0.03 ± 0.02	0.04 ± 0.03	0.42 ± 0.16**
Behenic acid	22:0	0.17 ± 0.05	0.15 ± 0.05	0.17 ± 0.01
Erucic acid	22:1	0.20 ± 0.06	0.18 ± 0.11	0.29 ± 0.08*
Docosadienoic acid	22:2,6	0.25 ± 0.10	0.10 ± 0.11$^{\#\#}$	0.02 ± 0.01*
Docosatetraenoic acid	22:4,6	0.28 ± 0.16	0.45 ± 0.12$^{\#\#}$	0.22 ± 0.10**
Docosapentaenoic acid	22:5,3	0.04 ± 0.03	0.07 ± 0.06	0.15 ± 0.21
Lignoceric acid	24:0	0.02 ± 0.01	0.02 ± 0.02	0.01 ± 0.01
Docosahexaenoic acid	22:6,3	1.67 ± 0.39	1.94 ± 0.33	4.16 ± 1.17**
Nervonic acid	24:1	0.09 ± 0.04	0.20 ± 0.09$^{\#\#}$	0.13 ± 0.09
SFAs		53.5 ± 11.9	40.4 ± 4.1$^{\#\#}$	48.1 ± 9.1**
MUFAs		16.9 ± 5.5	23.1 ± 2.7$^{\#\#}$	19.8 ± 3.6*
n-3 PUFAs		2.04 ± 0.38	2.30 ± 0.33	5.1 ± 1.4**
n-6 PUFAs		27.5 ± 6.5	34.3 ± 2.8	27.0 ± 5.6*
Total PUFAs		29.6 ± 6.8	36.6 ± 3.0	32.2 ± 6.8
n-6/n-3 PUFAs		13.4 ± 1.5	15.1 ± 1.7	5.40 ± 0.75**
16:1/16:0		0.04 ± 0.02	0.07 ± 0.02$^{\#\#}$	0.05 ± 0.02
18:1/18:0		0.80 ± 0.52	1.51 ± 0.42$^{\#\#}$	1.01 ± 0.41**

Values are expressed as the means ± SD ($n = 10$); $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus WT group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus WT/CCl_4 group. SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.

reaction of organisms in response to oxidative stimuli. These results demonstrate that the protection of endogenous n-3 PUFA against CCl_4-induced liver damage is correlated with ameliorating oxidative stress in the liver via activating Nrf2 and upregulating its downstream genes.

3.5. Endogenous n-3 PUFA Reduces Hepatocyte Apoptosis via Regulating MAPK Signal Pathway. As cell apoptosis directly reflects the extent of liver injury caused by CCl_4, a TUNEL assay was conducted to estimate the regulation of cell apoptosis by endogenous n-3 PUFA. As shown in Figure 4(a), after 24 h of CCl_4 challenge, the number of TUNEL-positive cells in the liver section was significantly increased over the WT control group. In *fat-1*/CCl_4 group, this increase in apoptotic cells was significantly decreased. In addition, during the CCl_4-induced liver injury, there was a cascade of apoptosis-related molecular events [24, 25]. After CCl_4 challenge, the protein expressions of the proapoptotic proteins, including cytochrome C, caspase-3, caspase-9, and Bax, were obviously increased in liver tissues from WT mice, while the levels of the antiapoptotic factor Bcl-2 was significantly decreased. In agreement with the reduction of TUNEL-positive cells, compared with WT/CCl_4 mice, the *fat-1*/CCl_4 mice showed decreased levels of the aforementioned proapoptotic proteins and increased level of Bax in the liver, as indicated by western blot analyses (Figure 4(b)). These results indicate that endogenous n-3 PUFA effectively prevents CCl_4-induced DNA fragmentation.

To understand the underlying molecular mechanisms for the inhibitory effects of endogenous n-3 PUFA on CCl_4-induced hepatocyte apoptosis, ERK, JNK, and p38, the major components in mitogen-activated protein kinase (MAPK) pathways, which are critical regulators of cell proliferation and death in response to diverse stresses, were examined by immunoblotting. Oxidative stress in the liver activates MAPK after CCl_4 challenge and results in activation of JNK, p38, and

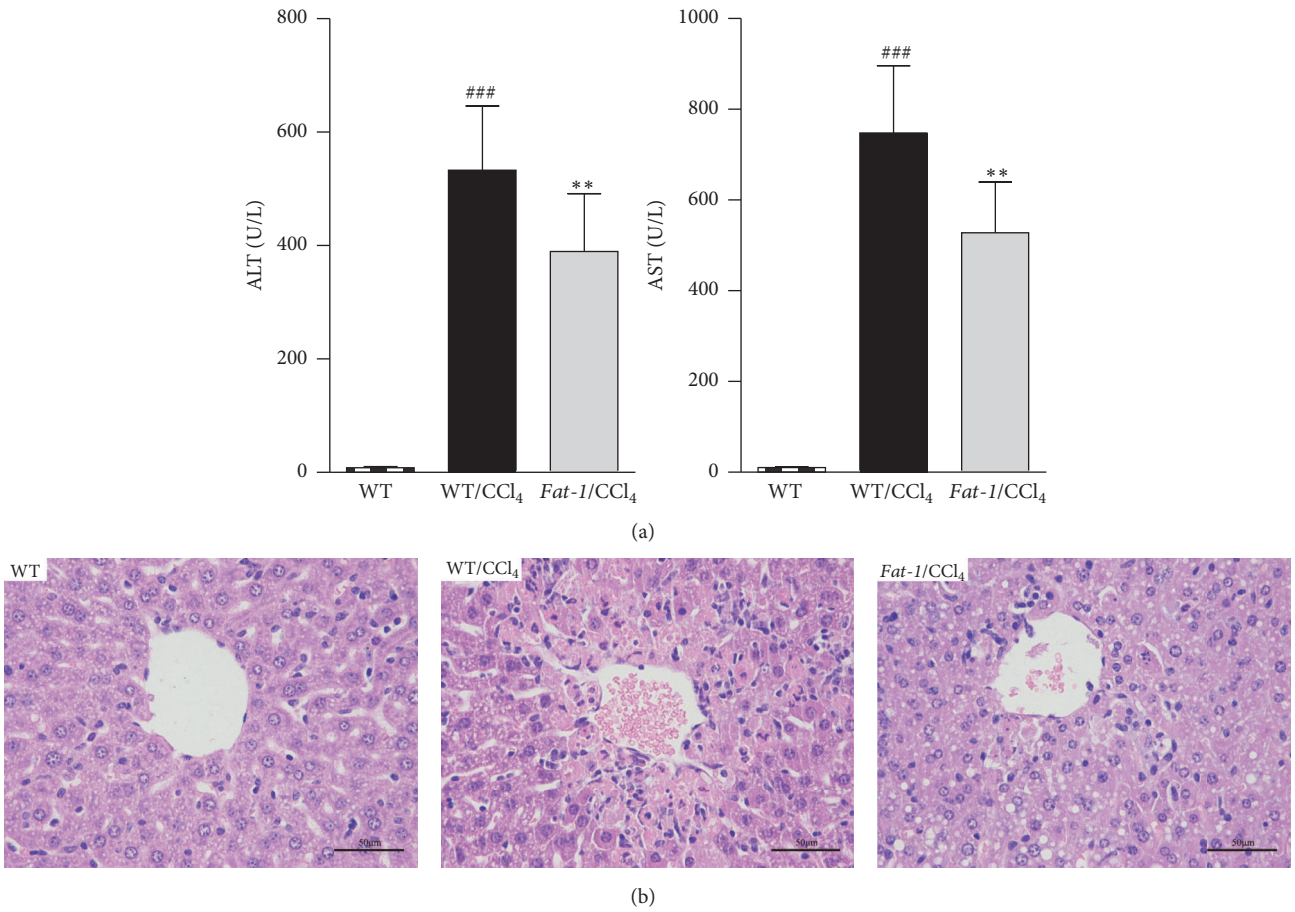


Figure 1: Endogenous n-3 PUFA alleviates CCl_4-induced acute liver injury in *fat-1* mice. (a) Plasma levels of alanine aspartate transaminase (AST) and aminotransferase (ALT). (b) Representative hematoxylin and eosin (H&E) staining of liver tissue sections (magnification: 400x). Values represent the means ± SD ($n = 10$); $^{###}p < 0.001$ versus WT group; $^{**}p < 0.01$ versus WT/CCl_4 group.

Table 4: Effects of endogenous omega-3 fatty acids on oxidative stress parameters in the liver.

Parameters	WT	WT/CCl_4	*Fat-1*/CCl_4
MDA (nmol/mg protein)	2.52 ± 0.34	4.73 ± 0.52$^{##}$	3.99 ± 0.54**
SOD (U/mg protein)	303.8 ± 33.8	214.3 ± 32.8$^{#}$	261.8 ± 44.2**
CAT (U/mg protein)	13.5 ± 1.9	7.52 ± 2.26$^{#}$	9.97 ± 2.10*
GSH-Px (U/mg protein)	975.6 ± 317.2	565.4 ± 199.1$^{##}$	770.1 ± 283.4**
GR (U/g protein)	10.6 ± 1.7	6.22 ± 1.77$^{##}$	7.92 ± 1.72*
GSH (mg/g protein)	11.6 ± 1.8	7.96 ± 0.89$^{##}$	9.27 ± 1.93**
GSSG (mg/g protein)	1.26 ± 0.36	2.29 ± 0.56$^{##}$	1.72 ± 0.40*
GSH/GSSG (fold)	9.72 ± 2.27	3.62 ± 0.89$^{##}$	5.46 ± 0.94**

Data are expressed as mean ± SD ($n = 10$). $^{#}p < 0.05$ and $^{##}p < 0.01$ versus WT group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus WT/CCl_4 group.

ERK [25]. As shown in Figure 4(c), CCl_4 exposure obviously increased the phosphorylated protein levels of JNK, p38, and ERK1/2, without changing their total expressions in liver tissues from WT mice. These increases in phosphorylated kinases were all downregulated by endogenous n-3 PUFA in *fat-1* mice. Thus, protective effects of endogenous n-3 PUFA against CCl_4-induced hepatocyte apoptosis are associated with suppressing MAPK pathways.

4. Discussion

In this study, we used *fat-1* transgenic mice to investigate the role of endogenous n-3 PUFA in CCl_4-caused acute liver damage. We demonstrate that CCl_4 challenge caused severe liver injury in WT mice, as illustrated by markedly elevated serum activities of AST and ALT, oxidative stress, and hepatocyte apoptosis. Those pathological alterations

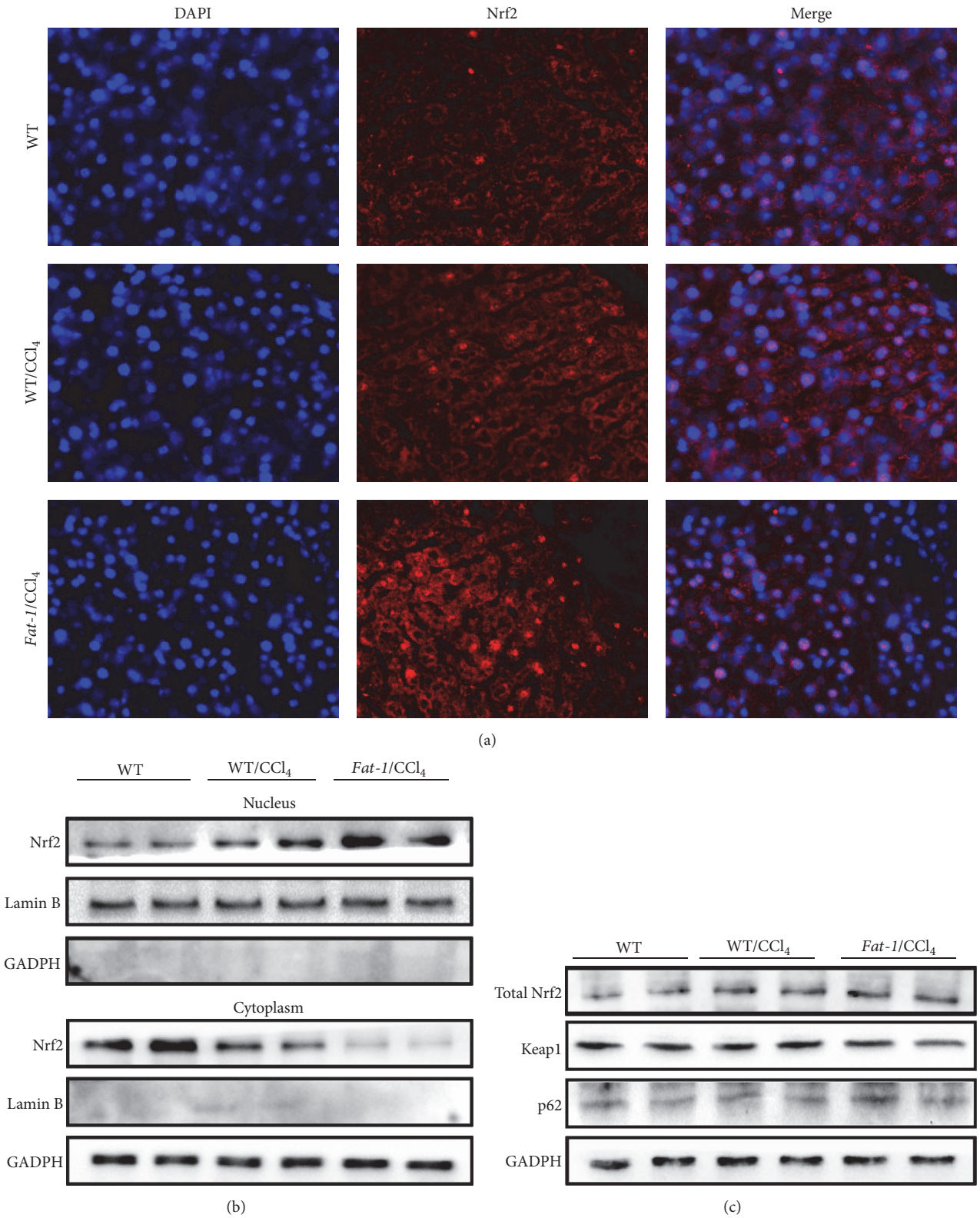


Figure 2: Endogenous n-3 PUFA induces nuclear translocation of Nrf2. (a) Immunofluorescence staining of Nrf2. (b) Western blot analysis of Nrf2 in the nucleus and cytoplasm. (c) Western blot analysis of total Nrf2, keap1, and p62 in liver tissue.

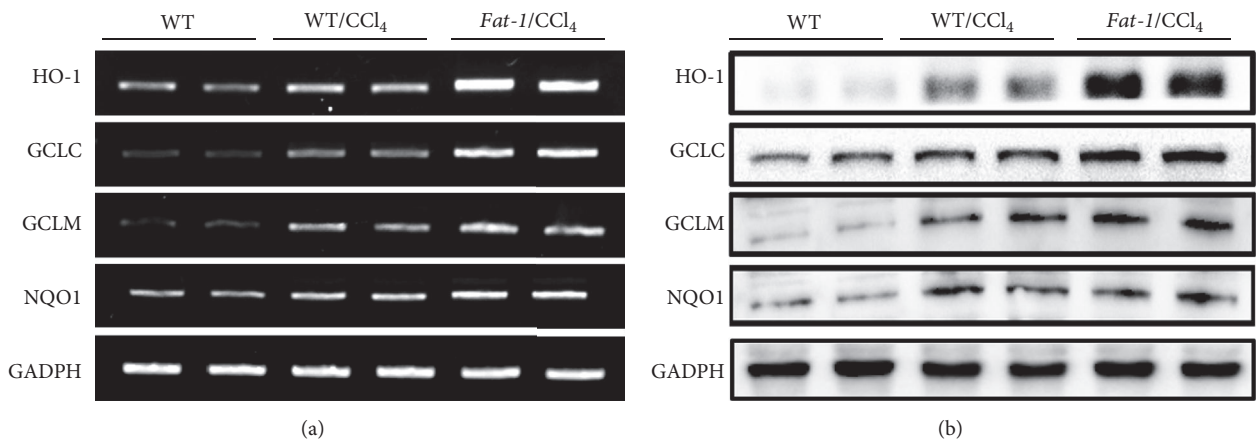


Figure 3: Endogenous n-3 PUFA upregulates mRNA (a) and protein (b) expressions of Nrf2 target genes, including HO-1, GCLC, GCLM, and NQO1.

were remarkably relieved in *fat-1* mice after CCl_4 challenge, which was associated with activating Nrf2 and regulating MAPK signal pathway.

The *fat-1* transgenic mice have been widely used as a novel tool for investigating the benefits of long chain n-3 PUFAs and the mechanisms underlying their actions [26]. *Fat-1* transgenic mice, carrying a *fat-1* gene from *C. elegans*, encode a desaturase that can convert n-3 to n-6 PUFA, resulting in abundant n-3 PUFA, without changing total PUFA in their organs and tissues (Table 3). *Fat-1* mice and WT littermates endogenously generate distinct fatty acid profiles in the liver while feeding them the same diet rich in n-6 PUFA. Thus, several variables arising from different diets, such as flavor, oxidation degree, and unwanted components of fat used, may be well avoided [15]. As expected, in this study, liver tissues from *fat-1*/CCl_4 group exhibited higher amounts of n-3 PUFA, particularly EPA and DHA, and lower level of n-6 PUFA, leading to a remarkable increase in total n-3 PUFA in the liver, compared to WT/CCl_4 group. As a well-characterized animal model, the *fat-1* mice were studied to examine the impacts of endogenous n-3 PUFA on CCl_4-induced acute liver injury.

Oxidative stress is critical during the pathogenesis of CCl_4-induced acute liver injury [27]. CCl_4 challenge produces highly reactive species and increases cellular production of ROS and MDA, leading to oxidative stress in tissues, especially the liver where it is primarily metabolized [6]. Additionally, CCl_4-induced oxidative stress also depletes antioxidant defense system, including endogenous nonenzymatic (e.g., GSH) and enzymatic (e.g., SOD, CAT, GSH-Px, and GR) antioxidants. GSH has been considered to be the first line of defense against free radicals. It was documented that GSH is an important antioxidant in eliminating toxic free radicals and reactive toxic CCl_4 metabolites [28, 29]. The sulfhydryl residues of GSH molecule are easily oxidized to GSSG, which can be reduced back to GSH by GR [30]. Thus, GSH/GSSG ratio serves as a reliable marker to evaluate the redox status and potential of oxidative stress [30]. In our study, CCl_4challenge increased hepatic MDA and GSSG and the ratio of GSH/GSSG and depleted GSH, SOD, CAT, GSH-Px, and GR in livers of WT mice, which was ameliorated in *fat-1* mice after CCl_4 treatment (Table 4). To examine how n-3 PUFA improves the antioxidant defense system, the nuclear translocation of Nrf2 and the expressions of Nrf2 target genes in the liver were evaluated. Nrf2 acts as a transcription factor which plays a key role in regulating the expression of antioxidant proteins in response to oxidative stress [31]. Under physiological condition, Nrf2 is attached to Keap1, a specific repressor, in the cytoplasm, which promotes Nrf2 degradation by the ubiquitin proteasome pathway [32]. In the presence of ROS, Nrf2 degradation ceases, while stabilized Nrf2 translocates into the nucleus, which triggers the expression of a series of antioxidants, including HO-1, GCLC, GCLM, and NQO1, through antioxidant response element (ARE). HO-1 is a strong antioxidant with antiapoptotic and anti-inflammatory effects in the liver. GCLC and GCLM are key rate-limiting enzymes in GSH biosynthesis [33]. p62 is a substrate adaptor sequestosome-1 protein that modulates the Nrf2-Keap1 signaling pathway by competing with Nrf2 for binding to Keap1. In this study, the significant nuclear translocation of Nrf2 was observed in *fat-1*/CCl_4 group, as evidenced by immunofluorescence assay and immunoblot analysis (Figure 2). Additionally, *fat-1*/CCl_4 mice showed lower protein expression of Keap1 and higher expressions of p62, HO-1, GCLC, GCLM, and NQO1 in the liver, when compared to WT/CCl_4 mice (Figure 3). These results suggest that the protective effects of endogenous n-3 PUFA against CCl_4-caused acute liver damage might be attributable to reducing oxidative stress via the activation of Nrf2-keap1 pathway.

The induction of hepatocyte apoptosis has been well studied in acute liver injury induced by CCl_4 exposure [34]. Our data demonstrated that CCl_4 challenge markedly induced hepatocyte apoptosis in WT mice (Figure 4(a)), which was significantly reduced in *fat-1* mice after CCl_4 exposure. The mitochondrial apoptotic pathway was considered to be involved in various types of cellular stress [35]. Oxidative stress causes elevated cytochrome C in the cytoplasm

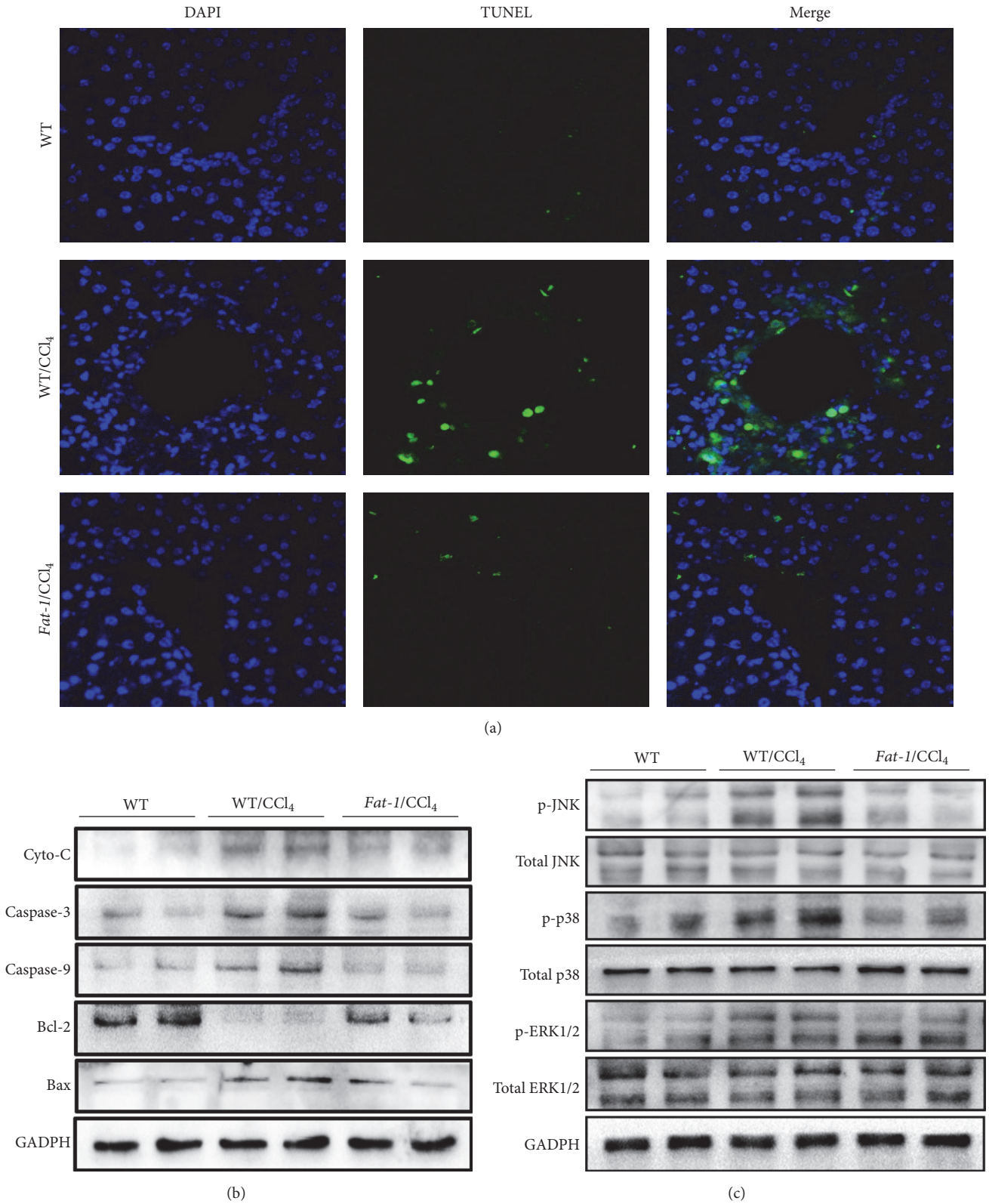


Figure 4: Endogenous n-3 PUFA protects against CCl_4-induced hepatocyte apoptosis in *fat-1* mice via regulating MAPK signaling pathway. (a) Representative images of TUNEL stained liver sections (magnification: 200x): green fluorescence indicates the positive cells, and cellular nucleus is labeled by staining DAPI with blue fluorescence. (b) Western blot analysis of apoptosis-related proteins, including cytochrome C, caspase-3, caspase-9, Bcl-2, and Bax. (c) Western blot analysis of total and phosphorylated protein expression of JNK, p38, and ERK.

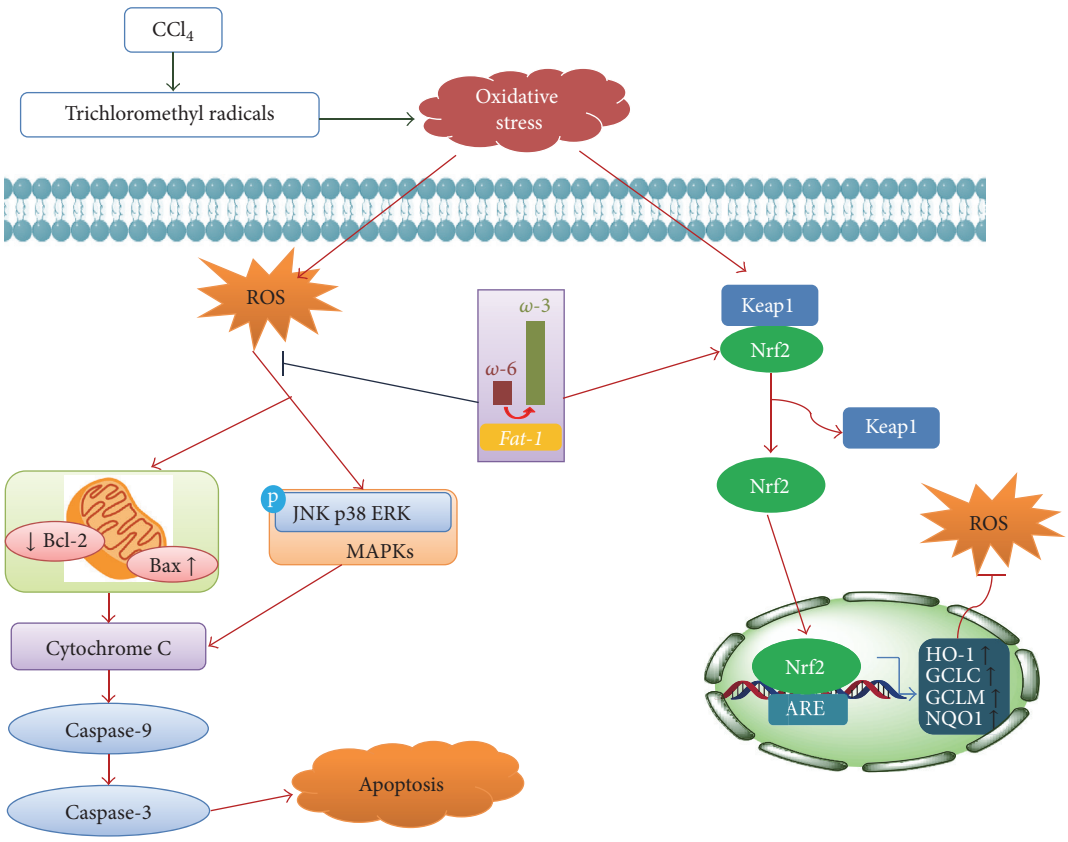


FIGURE 5: A schematic diagram of the potential mechanisms that underlie the protective effects of n-3 PUFA against CCl_4-induced acute liver injury.

which is released from the mitochondria, which consequently induces the activation of caspase cascades, including caspase-3 and caspase-9. The mitochondrial apoptotic pathway is also regulated by several apoptosis-related factors, such as Bax and Bcl-2 [36]. The ratio of proapoptotic protein Bax to antiapoptotic protein Bcl-2 is critical for cell death or survival. Increased Bax/Bcl-2 ratio leads to cytochrome C release, caspase-3 activation, and eventually apoptosis [37]. Our data revealed that endogenous n-3 PUFA significantly inhibited the upregulation of cytochromeC, caspase-3, caspase-9, and Bax and normalized the downregulation of Bcl-2 expression induced by CCl_4 (Figure 4(b)), which was well consistent with TUNEL staining results.

The MAPK family members, including JNK, p38, and JNK, are crucial for the regulation of cell proliferation, differentiation, apoptosis, and cellular responses to oxidative stress [38, 39]. Activated MAPKs can inactivate Bcl-2 by phosphorylation, activate caspase-9, and regulate the release of cytochrome C from the mitochondria [40]. Previous studies revealed that suppressing protein expressions of the phosphorylated MAPK members contributed to the inhibition of CCl_4-induced apoptosis [38, 39]. In this study, the upregulation of phosphorylated JNK, p38, and JNK induced by CCl_4 challenge was attenuated in the livers of *fat-1* mice. These findings suggest that reducing hepatocyte apoptosis via suppressing MAPK pathway might also contribute to the inhibitory function of n-3 PUFA on CCl_4-induced liver injury.

In conclusion, endogenous n-3 PUFA effectively ameliorated CCl_4-induced acute liver injury, and this protective effect might be associated with ameliorating oxidative stress via Nrf2 activation and reducing apoptosis via suppression of MAPK pathway, as illustrated in Figure 5. Our findings suggest that dietary supplement with n-3 PUFA may be beneficial for the prevention of liver injury.

Abbreviations

ALT:	Alanine transaminase
ARE:	Antioxidant response element
AST:	Aspartate transaminase
Bax:	Bcl-2-associated X protein
Bcl-2:	B-cell lymphoma-2
CAT:	Catalase
Caspase-3:	Cysteinyl aspartate specific proteinase-3
Caspase-9:	Cysteinyl aspartate specific proteinase-9
CCl_4:	Carbon tetrachloride
CYP2E1:	Cytochrome P4502E1
Cyto-C:	Cytochrome C
DAPI:	4,6-Diamidino-2-phenylindole
ERK:	Extracellular signal-regulated kinase
FAME:	Fatty acid methyl esters

GADPH: Glyceraldehyde-3-phosphate dehydrogenase
GCLC: Glutamate cysteine ligase catalytic subunit
GCLM: Glutamate cysteine ligase modifier subunit
GSH: Reduced glutathione
GSH-Px: Glutathione peroxidase
GSSG: Oxidized glutathione
H&E: Hematoxylin and eosin
HO-1: Heme oxygenase-1
JNK: c-Jun N-terminal kinase
Keap1: Kelch-like ECH-associated protein-1
MAPK: Mitogen-activated protein kinase
MDA: Malondialdehyde
MUFA(s): Monounsaturated fatty acid(s)
Nrf2: Nuclear factor-erythroid 2-related factor-2
NQO1: NAD(P)H:quinone oxidoreductase-1
PUFA(s): Polyunsaturated fatty acid(s)
p62: Nucleoporin p62
ROS: Reactive oxygen species
SFA(s): Saturated fatty acid(s)
SOD: Superoxide dismutase
TUNEL: Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling
WT: Wild-type.

Competing Interests

The authors declare that there are no competing interests.

Acknowledgments

The present study was supported by grants from the Research Committee of the University of Macau (MYRG111-ICMS13 to Jian-Bo Wan) and from Macao Science and Technology Development Fund (010/2013/A1 to Jian-Bo Wan).

References

[1] L. Qu, H. Xin, G. Zheng, Y. Su, and C. Ling, "Hepatoprotective activity of the total saponins from *Actinidia valvata* dunn root against carbon tetrachloride-induced liver damage in mice," *Evidence-Based Complementary and Alternative Medicine*, vol. 2012, Article ID 216061, 13 pages, 2012.

[2] R. N. Jadeja, N. H. Urrunaga, S. Dash, S. Khurana, and N. K. Saxena, "Withaferin-a reduces acetaminophen-induced liver injury in mice," *Biochemical Pharmacology*, vol. 97, no. 1, pp. 122–132, 2015.

[3] D. Dong, L. Xu, L. Yin, Y. Qi, and J. Peng, "Naringin prevents carbon tetrachloride-induced acute liver injury in mice," *Journal of Functional Foods*, vol. 12, pp. 179–191, 2015.

[4] C. Su, X. Xia, Q. Shi et al., "Neohesperidin dihydrochalcone versus CCl_4-induced hepatic injury through different mechanisms: the implication of free radical scavenging and Nrf2 Activation," *Journal of Agricultural and Food Chemistry*, vol. 63, no. 22, pp. 5468–5475, 2015.

[5] A. F. Ismail, A. A. Salem, and M. M. Eassawy, "Hepatoprotective effect of grape seed oil against carbon tetrachloride induced oxidative stress in liver of γ-irradiated rat," *Journal of Photochemistry and Photobiology B: Biology*, vol. 160, pp. 1–10, 2016.

[6] G. M. Campo, A. Avenoso, S. Campo et al., "Hyaluronic acid and chondroitin-4-sulphate treatment reduces damage in carbon tetrachloride-induced acute rat liver injury," *Life Sciences*, vol. 74, no. 10, pp. 1289–1305, 2004.

[7] B. O. Cho, H. W. Ryu, C. H. Jin et al., "Blackberry extract attenuates oxidative stress through up-regulation of Nrf2-dependent antioxidant enzymes in carbon tetrachloride-treated rats," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 21, pp. 11442–11448, 2011.

[8] Y.-F. Lu, J. Liu, K. C. Wu, Q. Qu, F. Fan, and C. D. Klaassen, "Overexpression of Nrf2 protects against microcystin-induced hepatotoxicity in mice," *PLoS ONE*, vol. 9, no. 3, Article ID e93013, 2014.

[9] E. Scorletti, L. Bhatia, K. G. Mccormick et al., "Effects of purified eicosapentaenoic and docosahexaenoic acids in nonalcoholic fatty liver disease: Results from the WELCOME* study," *Hepatology*, vol. 60, no. 4, pp. 1211–1221, 2014.

[10] E. M. Tillman and R. A. Helms, "Omega-3 long chain polyunsaturated Fatty acids for treatment of parenteral nutrition-associated liver disease: a review of the literature," *The Journal of Pediatric Pharmacology and Therapeutics*, vol. 16, pp. 31–38, 2011.

[11] M. Wang, X. Zhang, C. Yan et al., "Preventive effect of α-linolenic acid-rich flaxseed oil against ethanol-induced liver injury is associated with ameliorating gut-derived endotoxin-mediated inflammation in mice," *Journal of Functional Foods*, vol. 23, pp. 532–541, 2016.

[12] M. Wang, X. Zhang, K. Feng et al., "Dietary α-linolenic acid-rich flaxseed oil prevents against alcoholic hepatic steatosis via ameliorating lipid homeostasis at adipose tissue-liver axis in mice," *Scientific Reports*, vol. 6, p. 26826, 2016.

[13] L. L. Huang, J. B. Wan, B. Wang et al., "Suppression of acute ethanol-induced hepatic steatosis by docosahexaenoic acid is associated with downregulation of stearoyl-CoA desaturase 1 and inflammatory cytokines," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 88, no. 5, pp. 347–353, 2013.

[14] C. Schmöcker, K. H. Weylandt, L. Kahlke et al., "Omega-3 fatty acids alleviate chemically induced acute hepatitis by suppression of cytokines," *Hepatology*, vol. 45, no. 4, pp. 864–869, 2007.

[15] J.-B. Wan, L.-L. Huang, R. Rong, R. Tan, J. Wang, and J. X. Kang, "Endogenously decreasing tissue n-6/n-3 fatty acid ratio reduces atherosclerotic lesions in apolipoprotein E-deficient mice by inhibiting systemic and vascular inflammation," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 12, pp. 2487–2494, 2010.

[16] J. X. Kang, J. Wang, L. Wu, and Z. B. Kang, "Transgenic mice: fat-1 mice convert n-6 to n-3 fatty acids," *Nature*, vol. 427, no. 6974, p. 504, 2004.

[17] H. Xu, T. Guo, Y.-F. Guo et al., "Characterization and protection on acute liver injury of a polysaccharide MP-I from *Mytilus coruscus*," *Glycobiology*, vol. 18, no. 1, pp. 97–103, 2008.

[18] X.-J. Zhang, L.-L. Huang, X.-J. Cai, P. Li, Y.-T. Wang, and J.-B. Wan, "Fatty acid variability in three medicinal herbs of Panax species," *Chemistry Central Journal*, vol. 7, no. 1, article 12, 2013.

[19] X.-J. Zhang, L.-L. Huang, H. Su et al., "Characterizing plasma phospholipid fatty acid profiles of polycystic ovary syndrome patients with and without insulin resistance using GC-MS and chemometrics approach," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 95, pp. 85–92, 2014.

[20] G.-J. Huang, J.-S. Deng, S.-S. Huang, Y.-Y. Shao, C.-C. Chen, and Y.-H. Kuo, "Protective effect of antrosterol from

Antrodia camphorata submerged whole broth against carbon tetrachloride-induced acute liver injury in mice," *Food Chemistry*, vol. 132, no. 2, pp. 709–716, 2012.

[21] R.-B. Ding, K. Tian, L.-L. Huang et al., "Herbal medicines for the prevention of alcoholic liver disease: a review," *Journal of Ethnopharmacology*, vol. 144, no. 3, pp. 457–465, 2012.

[22] R.-B. Ding, K. Tian, Y.-W. Cao et al., "Protective effect of *Panax notoginseng* saponins on acute ethanol-induced liver injury is associated with ameliorating hepatic lipid accumulation and reducing ethanol-mediated oxidative stress," *Journal of Agricultural and Food Chemistry*, vol. 63, no. 9, pp. 2413–2422, 2015.

[23] Y.-T. Tung, J.-H. Wu, C.-C. Huang et al., "Protective effect of Acacia confusa bark extract and its active compound gallic acid against carbon tetrachloride-induced chronic liver injury in rats," *Food and Chemical Toxicology*, vol. 47, no. 6, pp. 1385–1392, 2009.

[24] X.-J. Zhang, C. He, P. Li, H. Su, and J.-B. Wan, "Ginsenoside Rg1, a potential JNK inhibitor, protects against ischemia/reperfusion-induced liver damage," *Journal of Functional Foods*, vol. 15, pp. 580–592, 2015.

[25] H.-Y. Kim, J. Park, K.-H. Lee et al., "Ferulic acid protects against carbon tetrachloride-induced liver injury in mice," *Toxicology*, vol. 282, no. 3, pp. 104–111, 2011.

[26] J. X. Kang, "Fat-1 transgenic mice: a new model for omega-3 research," *Prostaglandins Leukotrienes and Essential Fatty Acids*, vol. 77, no. 5-6, pp. 263–267, 2007.

[27] J.-C. Lin, Y.-J. Peng, S.-Y. Wang et al., "Sympathetic nervous system control of carbon tetrachloride-induced oxidative stress in liver through α-adrenergic signaling," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 3190617, 11 pages, 2016.

[28] G. Ray and S. A. Husain, "Oxidants, antioxidants and carcinogenesis," *Indian Journal of Experimental Biology*, vol. 40, no. 11, pp. 1213–1232, 2002.

[29] M. Boll, L. W. Weber, E. Becker, and A. Stampfl, "Mechanism of carbon tetrachloride-induced hepatotoxicity. Hepatocellular damage by reactive carbon tetrachloride metabolites," *Zeitschrift für Naturforschung C*, vol. 56, pp. 649–659, 2001.

[30] K. Aoyama and T. Nakaki, "Glutathione in cellular redox homeostasis: association with the excitatory amino acid carrier 1 (EAAC1)," *Molecules*, vol. 20, no. 5, pp. 8742–8758, 2015.

[31] Y.-W. Cao, Y. Jiang, D.-Y. Zhang et al., "Protective effects of Penthorum chinense Pursh against chronic ethanol-induced liver injury in mice," *Journal of Ethnopharmacology*, vol. 161, pp. 92–98, 2015.

[32] K. Taguchi, H. Motohashi, and M. Yamamoto, "Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution," *Genes to Cells*, vol. 16, no. 2, pp. 123–140, 2011.

[33] Y. Chen, H. G. Shertzer, S. N. Schneider, D. W. Nebert, and T. P. Dalton, "Glutamate cysteine ligase catalysis: dependence on ATP and modifier subunit for regulation of tissue glutathione levels," *The Journal of Biological Chemistry*, vol. 280, no. 40, pp. 33766–33774, 2005.

[34] E. Karakus, A. Karadeniz, N. Simsek et al., "Protective effect of Panax ginseng against serum biochemical changes and apoptosis in liver of rats treated with carbon tetrachloride (CCl4)," *Journal of Hazardous Materials*, vol. 195, pp. 208–213, 2011.

[35] B.-Y. Yang, X.-Y. Zhang, S.-W. Guan, and Z.-C. Hua, "Protective effect of procyanidin B_2 against CCl_4-induced acute liver injury in mice," *Molecules*, vol. 20, no. 7, pp. 12250–12265, 2015.

[36] K. Kim, N. Jung, K. Lee et al., "Dietary omega-3 polyunsaturated fatty acids attenuate hepatic ischemia/reperfusion injury in rats by modulating toll-like receptor recruitment into lipid rafts," *Clinical Nutrition*, vol. 32, no. 5, pp. 855–862, 2013.

[37] D. M. Finucane, E. Bossy-Wetzel, N. J. Waterhouse, T. G. Cotter, and D. R. Green, "Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL," *The Journal of Biological Chemistry*, vol. 274, no. 4, pp. 2225–2233, 1999.

[38] J.-Q. Ma, J. Ding, L. Zhang, and C.-M. Liu, "Hepatoprotective properties of sesamin against CCl_4 induced oxidative stress-mediated apoptosis in mice via JNK pathway," *Food and Chemical Toxicology*, vol. 64, pp. 41–48, 2014.

[39] D. N. Dhanasekaran and E. P. Reddy, "JNK signaling in apoptosis," *Oncogene*, vol. 27, no. 48, pp. 6245–6251, 2008.

[40] D.-I. Kim, S.-J. Lee, S.-B. Lee, K. Park, W.-J. Kim, and S.-K. Moon, "Requirement for Ras/Raf/ERK pathway in naringin-induced G1 -cell-cycle arrest via p21WAF1 expression," *Carcinogenesis*, vol. 29, no. 9, pp. 1701–1709, 2008.

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 9895176, 13 pages
http://dx.doi.org/10.1155/2016/9895176

Research Article

Liver Cholesterol Overload Aggravates Obstructive Cholestasis by Inducing Oxidative Stress and Premature Death in Mice

Natalia Nuño-Lámbarri,[1,2,3] Mayra Domínguez-Pérez,[1,2] Anna Baulies-Domenech,[4] Maria J. Monte,[5] Jose J. G. Marin,[5] Patricia Rosales-Cruz,[1,2] Verónica Souza,[2,6] Roxana U. Miranda,[2,6] Leticia Bucio,[2,6] Eduardo E. Montalvo-Jave,[7] María Concepción Gutiérrez-Ruiz,[2,6] Carmen García-Ruiz,[4] José C. Fernández-Checa,[4,8] and Luis Enrique Gomez-Quiroz[2,6]

[1] *Postgraduate Program in Experimental Biology, DCBS, Universidad Autónoma Metropolitana Iztapalapa, 09340 Mexico City, DF, Mexico*
[2] *Health Science Department, Universidad Autónoma Metropolitana Iztapalapa, 09340 Mexico City, DF, Mexico*
[3] *Translational Research Unit, Medica Sur Clinic and Foundation, 14050 Mexico City, DF, Mexico*
[4] *Department of Cell Death and Proliferation, Instituto Investigaciones Biomédicas de Barcelona, CSIC, Barcelona and Liver Unit-Hospital Clinic-IDIBAPS and Centro de Investigación Biomédica en Red (CIBERehd), 08036 Barcelona, Spain*
[5] *Laboratory of Experimental Hepatology and Drug Targeting (HEVEFARM), University of Salamanca, Centro de Investigación Biomédica en Red (CIBERehd), Instituto de Investigación Biomédica de Salamanca (IBSAL), 37007 Salamanca, Spain*
[6] *Red "Fisiopatología de Enfermedades Hepáticas" PRODEP-SEP, 09340 Mexico City, DF, Mexico*
[7] *General Surgery Service 304, General Hospital of Mexico, UNAM School of Medicine, 06726 Mexico City, DF, Mexico*
[8] *Research Center for Alcohol Liver and Pancreatic Diseases and Cirrhosis, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA*

Correspondence should be addressed to José C. Fernández-Checa; checa229@yahoo.com and Luis Enrique Gomez-Quiroz; legq@xanum.uam.mx

Received 2 May 2016; Accepted 13 July 2016

Academic Editor: Ravirajsinh Jadeja

Nonalcoholic steatohepatitis is one of the leading causes of liver disease. Dietary factors determine the clinical presentation of steatohepatitis and can influence the progression of related diseases. Cholesterol has emerged as a critical player in the disease and hence consumption of cholesterol-enriched diets can lead to a progressive form of the disease. The aim was to investigate the impact of liver cholesterol overload on the progression of the obstructive cholestasis in mice subjected to bile duct ligation surgery. Mice were fed with a high cholesterol diet for two days and then were subjected to surgery procedure; histological, biochemical, and molecular analyses were conducted to address the effect of cholesterol in liver damage. Mice under the diet were more susceptible to damage. Results show that cholesterol fed mice exhibited increased apoptosis and oxidative stress as well as reduction in cell proliferation. Mortality following surgery was higher in HC fed mice. Liver cholesterol impairs the repair of liver during obstructive cholestasis and aggravates the disease with early fatal consequences; these effects were strongly associated with oxidative stress.

1. Introduction

Obesity is an epidemic problem; it has become a global health concern affecting rich and poor countries [1]. Obesity is the primary cause of nonalcoholic fatty liver disease (NAFLD), the commonest disease that affects liver function. It is highly prevalent and results from excessive fat accumulation, particularly free fatty acids, triglycerides (TG), and

cholesterol [2], inducing a wide range of biochemical and clinical consequences leading to sensitization to damage and running to progressive disease stages such as nonalcoholic steatohepatitis (NASH), and fibrosis [3]. While the two-hit hypothesis posits that fat accumulation is key for progressive NAFLD, some of our studies have suggested that the kind of lipid rather than the amount of fat determines the susceptibility to secondary hits, including inflammatory cytokines such as the TNF-α family [4].

Although cholesterol is a critical component of membrane bilayers, its accumulation disrupts membrane fluidity or dynamics and promotes cellular dysfunction that could lead to disease progression [5]. The liver plays key role in the maintenance of cholesterol homeostasis, whose levels are determined by *de novo* synthesis and supply from the diet by serum lipoproteins [6]. Increased cholesterol deposition in the liver, in particular its accumulation in mitochondria, has emerged as one of the main toxic lipids in NAFLD due to cholesterol dependent mitochondrial dysfunction and sensitization to oxidative stress and inflammatory cytokines secondary to mitochondrial GSH depletion [4, 7–9]. The sensitization to the damage mediated by cholesterol overload can occur independently of nutritional oversupply as ob/ob mice and Niemann-Pick type C1- (NPC1-) lacking mice exhibit increased mitochondrial cholesterol accumulation and sensitization to inflammatory cytokines-mediated oxidative stress and cell death [4]. In line with these findings, there has been evidence that cholesterol intake increases the risk and severity of NAFLD and that patients with NASH have a higher expression of StARD1, a cholesterol transporting protein that regulates mitochondrial cholesterol homeostasis [8, 10, 11].

Despite this knowledge, the role of hepatic cholesterol accumulation in cholestatic liver disease has not been thoroughly examined. Hepatic accumulation of bile acids due to impairment in bile flow is central to the pathogenesis of cholestasis liver disease and leads to hepatic injury and, in severe cases, organ failure [12]. Bile acids' accumulation exerts noxious cellular events, ranging from oxidative stress and inflammation to apoptosis and necrosis [13, 14], leading to acute liver toxicity, proliferation of bile ducts, and fibrosis that eventually progresses to cirrhosis and liver failure. Moreover, it has been reported that patients with NAFLD exhibit alterations in bile acids homeostasis [15], suggesting that hepatic steatosis may determine the clinical presentation of cholestatic liver disease.

In the present work we addressed the impact of liver cholesterol overload in the BDL model of obstructive cholestasis. Our data show that nutritional hepatic cholesterol accumulation sensitizes to BDL-mediated liver injury and death.

2. Material and Methods

2.1. Animal Models. C57BL/6 male mice (8–10 weeks old) were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and were maintained in pathogen-free conditions with controlled temperature and humidity on a 12 h light-dark cycle in the animal care facility at the Universidad Autónoma Metropolitana and the Medical School of Universitat de Barcelona. The experimental protocols used were approved and performed in accordance with the Animal Care Committee of the University of Barcelona.

2.2. Experimental Design. Forty C57BL/6 mice were separated into two diet groups; the first one was fed with a high cholesterol (HC) (2% cholesterol, 0.5% sodium cholate) diet for two days as previously reported [4, 16]; the second group was fed with control standard Chow diet for the same period of time. Animals received water *ad libitum*. Afterwards, each group was subject to bile duct ligation (BDL) surgery or control Sham procedure. Five mice per time were sacrificed at days 1, 2, and 3, after surgery as depicted in Supplementary Figure 1(A) of the Supplementary Material available online at http://dx.doi.org/10.1155/2016/9895176. Five Sham animals were sacrificed only at day three. Liver tissue and serum were recovered for analysis. Six extra animals were used to figure out the effect of sodium cholate alone; we used a diet supplemented with 0.5% of the salt for 2 and 30 days (three mice per time) and liver gross inspection and AST and ALT serum activities were also determined (Supplementary Figures 1(B) and 1(C)).

2.3. Bile Duct Ligation Surgery. Surgery was performed under aseptic conditions. Anesthesia was induced with 2% isoflurane (Abbott Laboratories #B506) and 2-liter/min oxygen flow for a mouse of 25 g body weight. After median laparotomy, the common bile duct was ligated close to the liver *hilus*, below the bifurcation with 4-0 silk suture (Ethicon), to impede bile flow. The gallbladder was left intact. The abdominal muscle was sutured with a 5-0 surgical silk suture and the wound was closed with surgical staples. Control mice underwent a Sham surgery without BDL. All animals were allowed to wake up on a heating pad, and analgesia was given to all animals.

2.4. Determination of Liver Function Tests. Blood samples were obtained from the portal vein under isoflurane anesthesia before sacrifice. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were determined by the automated method using Reflovet Plus (Roche, Mannheim, Germany).

2.5. Histology and Immunohistochemical Studies. Liver tissue was fixed in 10% formalin/phosphate-buffered saline, dehydrated in alcohols, incubated in xylene, and embedded in paraffin. Paraffin molds containing liver sections were cut into 5 μm sections and mounted on HistoGrip-coated slides. H&E was performed following standard procedures. Apoptosis was addressed by TUNEL detection kit (Trevigen Inc.) according to the manufacturer's protocol. Oil Red O (ORO) solution was used to detect neutral lipids in tissue.

For the immunohistochemistry of Ki67, paraffin sections were antigen unmasked with citrate buffer. Endogenous peroxidases were blocked with 3% H_2O_2; afterward slides were incubated with primary antibody overnight in a wet chamber at 4°C. After rinsing with PBS 1x, the slides were

incubated with a biotinylated antibody for 45 min in a wet chamber and developed with the ABC kit with peroxidase substrate (DAB) and peroxidase buffer. After rinsing with tap water, slides were counterstained with hematoxylin. For filipin staining, frozen sections were fixed for 1 h at room temperature with formalin 10%, washed with PBS, and incubated with 0.2 mg/mL filipin over night at 4°C protected from light. After 3 final washes in PBS, tissues were mounted and confocal images (Carl Zeiss, LSM 780 multiphoton, Jena, Germany) were collected using UV excitation.

2.6. Cholesterol Determination. Ten mg of liver tissue or 200 μL of serum was saponified with alcoholic KOH in a 60°C heating block for 15 min. After the mixture had cooled, 2 mL of hexane and 600 μL of distilled water were added and shaken to ensure complete mixing. Appropriate aliquots of the hexane layer were evaporated under nitrogen and used for cholesterol measurement with *O*-phthalaldehyde reagent (Sigma-Aldrich) dissolved in acetic acid; after that sulfuric acid was added and then read at 550 nm in the spectrophotometer.

2.7. Triglycerides Determination. Triglyceride (TG) content was determined using the triglycerides determination kit (Sigma-Aldrich, San Louis, MO) following the manufacture's instructions.

2.8. Western Blot. Western blotting was performed following the protocol previously reported by Clavijo-Cornejo et al. [17]. Briefly, total proteins were isolated from liver homogenate with T-PER (Pierce) extraction reagents, supplemented with protease and phosphatase cocktail inhibitors (Roche Inc.). One hundred μg of total protein was separated on precast 4–20% gels (Invitrogen), transferred to polyvinylidene difluoride membranes (PVDF, Invitrogen), and probed with different antibodies as shown in Supplementary Figure 2. Membranes were incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody depending on the origin of the primary antibody. Immunoreactive bands were identified with ECL-Plus Western blotting detection reagents (GE Healthcare). Equal loading was demonstrated by probing the same membranes with actin antibody (Sigma-Aldrich).

2.9. Caspase 3 Activity. Tissue was lysed in reaction buffer and incubated for 30 min on ice. For each reaction, caspase 3 synthetic fluorogenic tetrapeptide substrate (Ac-DEVD-AMC) was added (BD Pharmingen) and then the tissue lysate; after that samples were incubated for one hour. The amount of AMC released was measured by fluorometry at an excitation wavelength of 380 nm and an emission wavelength of 420 nm.

2.10. In Situ ROS Determination. Animals were sacrificed in parallel exclusively for *in situ* ROS determination. Fresh tissue was rapidly sectioned, frozen in liquid nitrogen, and embedded in optimum cutting temperature compound (OCT, Sakura Finetek, Torrance, CA); subsequently, 8 μm frozen sections were obtained in a cryostat (Leica CM-3050S, Heerbrugg, Switzerland) at −20°C and the slides were immediately incubated for 15 min, in the dark, at room temperature with either DCFH (5 μM), a cell-permeable nonfluorescent probe that is deesterified intracellularly and converted to the highly fluorescent 2′,7′-dichlorofluorescein upon oxidation by ROS, particularly peroxides (H_2O_2), or with dihydroethidium(DHE, 50 μM) for determination of superoxide anion radical (O_2^-) detecting ethidium fluorescence. Samples were covered and observed using a confocal microscope at excitation and emission wavelengths of 480 and 520 nm, respectively, for DCFH and 485 and 570 nm, respectively, for DHE-derived ethidium fluorescence, as previously we reported [18].

2.11. Glutathione (GSH) Measurements. GSH levels in tissue homogenates were analyzed by HPLC as previously we reported [18].

2.12. Bilirubins Determination. Bilirubins content was assayed by using Bilirubin Jendrassik-Grof FS kit (DiaSys Inc.) following the manufacturer's instructions.

2.13. Statistical Analysis. Data are presented as mean ± SEM for at least four different animals, and each experiment was carried out in triplicate. Comparisons between groups were performed using the ANOVA test followed by a *post hoc* Bonferroni test.

Additional materials and methods can be found in Supplementary Material.

3. Results

3.1. A High Cholesterol Diet Induces Liver Steatosis. To address the role of cholesterol hepatic overloading in BDL-mediated liver injury, we fed mice with a diet enriched in cholesterol (HC), as previously described [4, 16]. As seen, HC fed mice exhibited the characteristic pale color of the steatotic liver compared to mice fed with the control Chow diet (Figure 1(a)). In addition, filipin and ORO staining in liver tissue revealed a high content of both free cholesterol and neutral lipids (Figure 1(b)), findings that were biochemically confirmed in tissue homogenates (Figure 1(c)). Moreover, in line with findings in intact liver, isolated hepatocytes from HC fed mice revealed increased filipin and ORO staining (Supplementary Figure 3), confirming the accumulation of cholesterol and neutral lipids in parenchymal cells. This steatotic phenotype was associated with liver injury, as revealed by serum AST and ALT activities (Figure 1(d)).

3.2. Cholesterol Overload Sensitizes to BDL-Induced Liver Damage and Aggravates Obstructive Jaundice. We next examined the impact of HC feeding in BDL-induced liver injury and survival. Mice fed with the HC diet and subjected to BDL surgery died between 60 and 115 h after procedure, compared to mice fed with control Chow diet (Figure 2(a)); none of the HC fed mice survived beyond 5 days after BDL. Although BDL is a model for acute obstructive cholestasis, control mice survived up to 6 weeks after surgery, in agreement with previous results [19, 20].

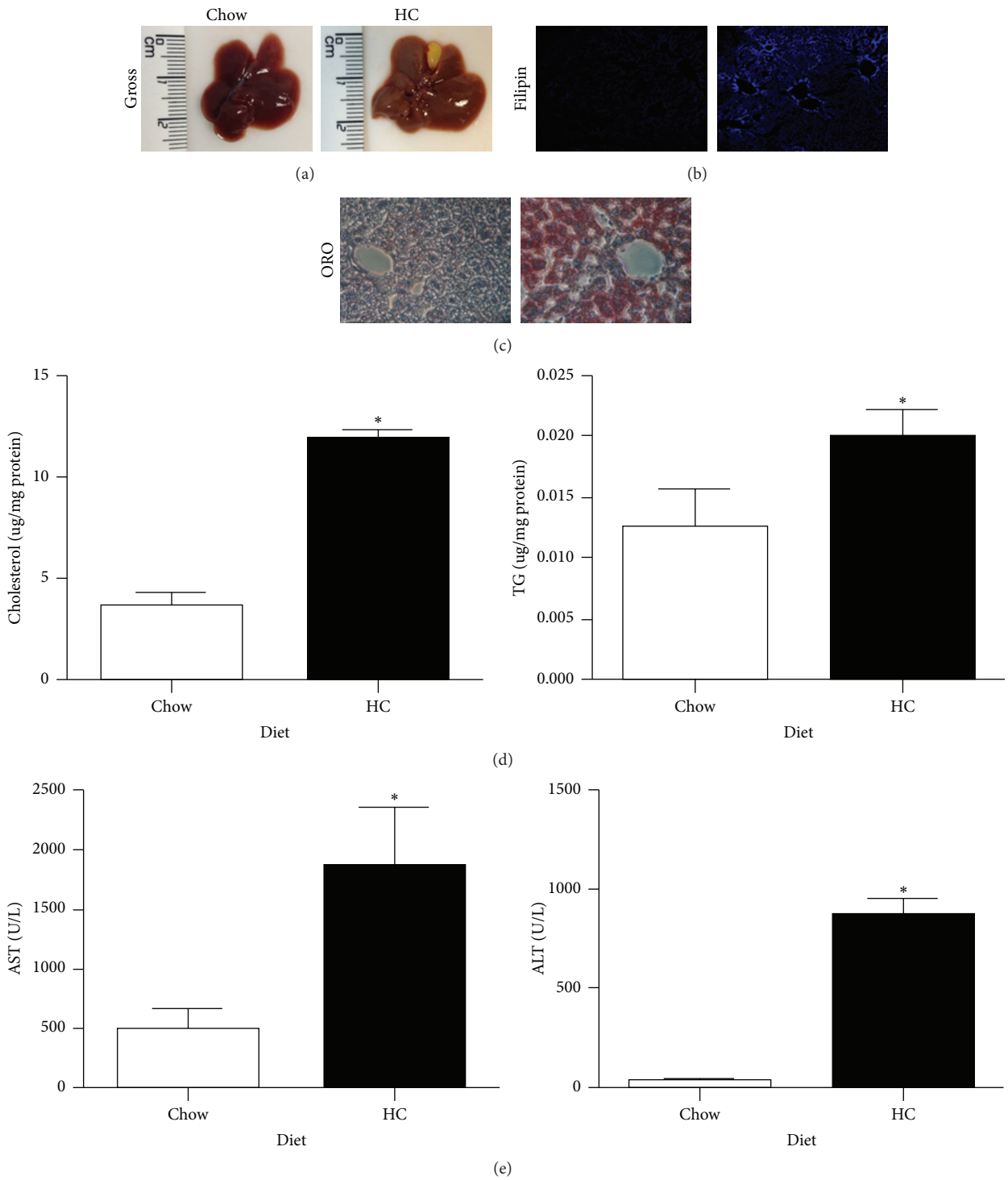


Figure 1: A high cholesterol diet induces liver steatosis. (a) Macroscopic liver inspection of standard control Chow diet (Chow) and high cholesterol (HC) diet. (b) Free cholesterol determination by filipin. (c) Neutral lipid determined by Oil Red O staining (ORO) in Chow and HC liver sections. (d) Biochemistry determination in liver tissue of total cholesterol and triglycerides (TG) content. (e) Liver function tests: aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Each column represents mean ± SEM of three independent experiments. Differences were considered significant at $^{*}p \leq 0.01$ versus Chow. Images are representative of at least three independent experiments. Original magnification: 200x.

As a high percentage of HC fed mice died within 3 days after surgery, we decided to characterize liver function at 1, 2, and 3 days after BDL to assess the mechanisms contributing to the early death of HC fed mice.

Liver gross inspections revealed no significant differences between groups; mice presented bile duct stasis and greenish gallbladder in HC group, in comparison with Chow animals, which exhibited a darker gallbladder (Figure 2(b)). Cholesterol content, assayed by filipin staining, increased in HC animals. At day 3 cholesterol content was less diffused in the tissue, displaying spots with high fluorescence, while Chow fed animals liver cholesterol content was uniform and unchanged following BDL.

Serum cholesterol and TG content were also elevated in HC fed mice and remained increased after BDL. Liver injury and function tests exhibited a significant increase in HC fed mice since day one after BDL in AST and ALT and at two days in ALP. In all cases, values raised until day three in HC fed mice. In contrast, Chow fed animals showed a slight increase at day two in AST, and no significant changes were observed up to the third day.

Figure 2(c) depicts the main biochemical tests to address liver function and damage. Total and free bilirubins were also increased in HC fed animals since the first day of surgery, and a similar effect was observed in serum total bile acids (TBA). Interestingly, liver TBA were significantly augmented in Sham animals due to HC feeding with no changes in the course of the study, while Chow fed animals exhibited significantly increased liver TBA at day two, peaking at the third day.

No significant changes were found in mice fed with a diet supplemented with 0.5% sodium cholate alone, as control diet, and subjected to BDL comparing with Chow diet fed animals.

In order to figure out the mechanism that could explain the premature death in HC fed animals, we focused on studying the phenotype and molecular changes at day three.

Histological analysis of livers from HC fed mice (Figure 3) revealed lymphocytes (black arrow head) infiltration and prominent bridging hepatic necrosis seen in HC fed animals comparing with Chow fed animals, which presented this characteristic to a lesser extent. Chow fed animals presented evidence of cell proliferation (white arrow head), suggesting an ongoing repair process.

3.3. HC Feeding Decreases Cell Proliferation following BDL. In order to address whether cholesterol loading interfered with cell proliferation during BDL, as suggested by H&E staining, we performed Ki67 staining in samples from both groups of mice at the third day after BDL. Figure 4(b) shows more positive Ki67 cells (black arrows) in liver samples from Chow fed mice than liver from HC fed mice (Figure 4(d)). Results were in agreement with the expression of the main cell cycle proteins cyclins D1 and A and cdk2 and cdk4, showing a sustained expression in a time-dependent manner in Chow fed animals, particularly in cdk2 and cdk4, which decreased in HC mice, particularly at day three. Cell cycle inhibitory proteins, such as p27 and p21, presented different comportment; p27 increased in time-dependent manner, but p21 decreased (Figure 4(e)).

3.4. BDL in HC Fed Mice Induces Apoptosis and Oxidative Stress. It is well known that bile salts' accumulation in the liver induces apoptosis in hepatocytes [21, 22]. Figure 5 shows that HC fed mice subjected to BDL surgery presented more apoptotic cells, assayed by TUNEL (Figure 5(d)), than Chow fed animals (Figure 5(b)). The quantification of apoptotic cells revealed a significant increment in positive cells in HC fed animals (Figure 5(e)). These data were corroborated by measurement of caspase 3 activity (Figure 5(f)).

Oxidative stress is one of the major inducers of hepatic apoptosis [23]. Therefore, we measured the *in situ* content of ROS by DCFH and DHE, as previously reported [18]. As seen, DCFH and DHE staining increased in livers sections from HC fed animals at day 3 after BDL, compared to Chow fed animals (Figure 6(a)); these results were in agreement with a decrease in total GSH levels (Figure 6(b)). GSH levels decreased as a result of HC feeding and continued to decline after BDL.

Importantly, Chow fed animals exhibited decreased GSH content at day one of treatment, which subsequently augmented at day two reaching basal GSH levels at the third day. The analysis of the main antioxidant enzymes (Figure 6(c)) revealed an induction of γ-GCS and GPX4 in Chow fed animals at days two and three, an effect that was not observed in HC animals; even more, GPX4 decreased in HC fed animals in time-dependent manner. GPX1/2 were slightly induced in HC fed mice. GST exhibited a time-dependent increment in Chow fed animals, with opposite effect in HC fed mice. Other antioxidant enzymes such as SOD1, SOD2, and NQO1 showed an increment in Chow fed mice but not so in HC fed mice; even more, SOD2 showed a decrease in the time of the study in HC fed animals.

4. Discussion

Bile duct obstruction-mediated liver injury is a key feature of cholestasis, which underlies several chronic liver diseases, such as gallstone impaction, biliary atresia, and tumor compression [24]. The accumulation of toxic bile acids during bile duct obstruction contributes to the hepatocellular injury. The mechanism of damage is complex and involves oxidative stress generation and inflammation [14, 24, 25].

It is well known that bile acids arise from enzymatic oxidation of cholesterol and that hypercholesterolemia leading to hepatic cholesterol accumulation is one of the key hallmarks in NAFLD and NASH [8, 10]. Consumption of cholesterol-enriched diets could aggravate NAFLD, particularly in susceptible individuals related to congenital risks or deficient dietary habits [26, 27]. For instance, in Hispanics, the consumption of high cholesterol diets is very common, which may account for the high prevalence of NAFLD in this population [28].

In the present work we used a nutritional model of hepatic cholesterol overload to examine the impact of cholesterol

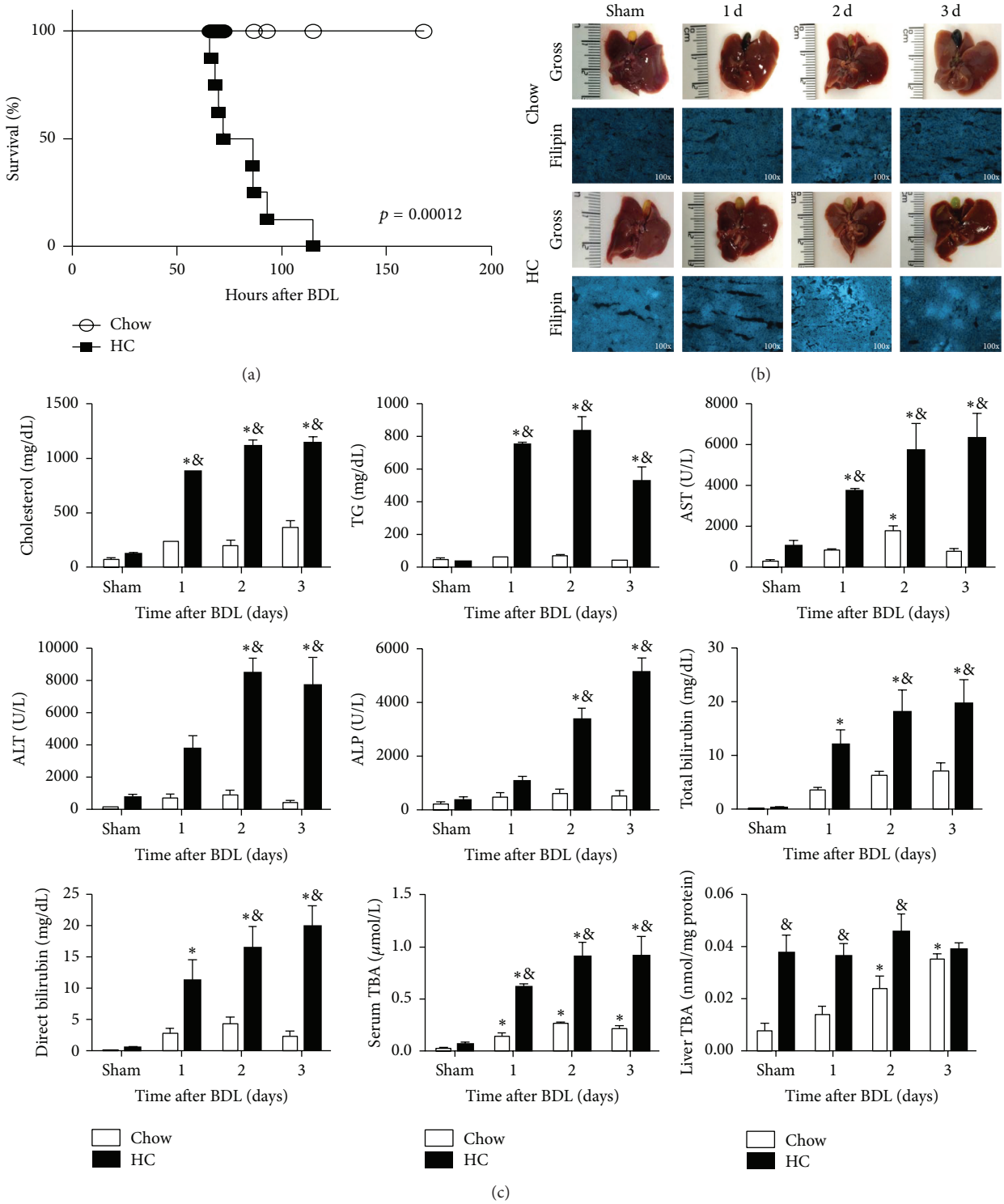


Figure 2: Cholesterol overload sensitizes to BDL-induced liver damage and aggravates obstructive jaundice. (a) Survival plot after BDL; at least nine animals were considered for the survival experiment. (b) Liver gross inspection and free cholesterol determination by filipin. Images are representative of at least three independent experiments. Original magnification for filliping staining: 100x. (c) Biochemical serum cholesterol and triglycerides (TG) determination. Liver function test: aspartate aminotransferase (AST); alanine aminotransferase (ALT); alkaline phosphatase (ALP); total bilirubin; direct bilirubin; serum total bile acids (TBA); and liver tissue TBA. Each column represents mean ± SEM of three independent experiments. Differences were considered significant at $^{*}p \leq 0.01$ versus Sham Chow and $^{\&}p < 0.01$ versus Chow fed mice at the same time.

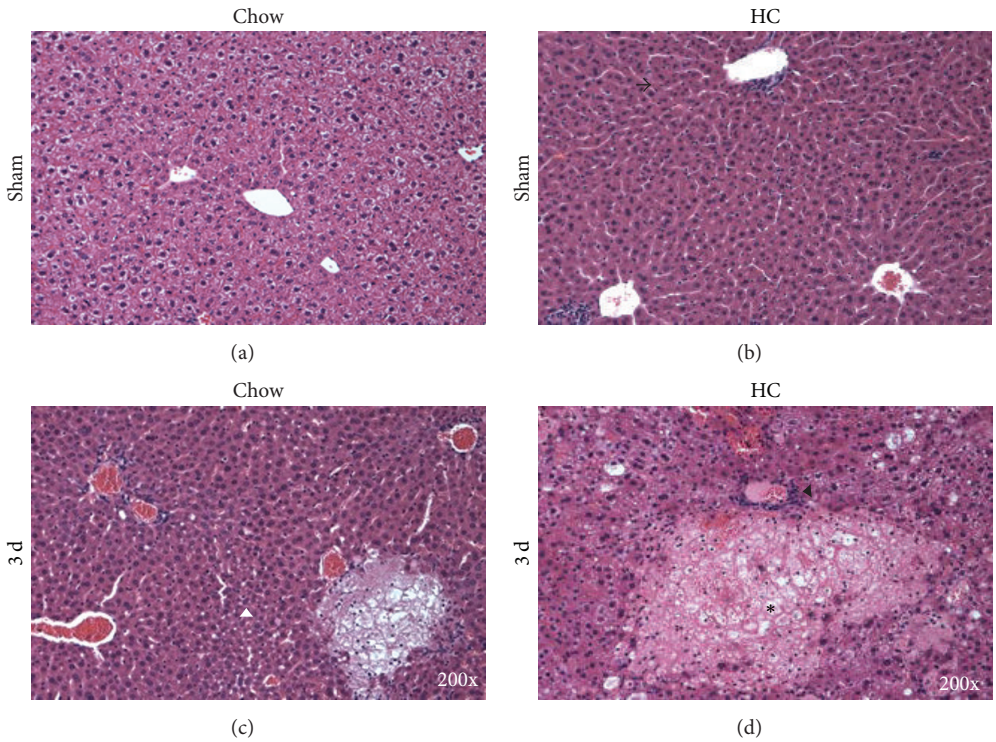


Figure 3: Cholesterol overload exacerbates liver tissue damage. Histology analysis by H&E staining. Liver tissue was obtained from animals fed with standard Chow control diet (Chow) or high cholesterol (HC) diet for 2 days and subjected to BDL. Bridging hepatic necrosis areas (asterisks), polymorphonuclear leukocytes and lymphocytes infiltration (black arrow head), inflammatory cells infiltration (arrow), and cell proliferation (white arrow head). Representative images from at least four different animals. Original magnification: 200x.

in obstructive cholestasis-mediated liver injury. Mice fed a HC-enriched diet for two days showed a steatotic phenotype with particular lipid overload in hepatocytes, comprising primarily increased cholesterol accumulation (2-3-fold), and exhibited liver damage, as revealed by serum AST and ALT values and histology examination. Although HC diet was supplemented with 2% cholesterol, HC fed mice exhibited a mild increase in TG content in hepatocytes. Whether TG accumulation reflects increased FFA esterification into TG or impairment in mitochondrial β-oxidation due to cholesterol trafficking to mitochondria, as we reported previously [4], remains to be further established.

In line with the emerging role of cholesterol in NAFLD progression to NASH in mice and humans and the role of cholesterol in liver fibrogenesis, we show the sensitization of mice fed HC diet to BCL-mediated liver injury and premature death. Moreover, cholesterol overload exacerbates BDL-induced deterioration of liver function, including increased total and free bilirubin and TBA in serum since day one after BDL, but not in Sham animals, indicating that this response is a consequence of bile duct obstruction. Interestingly, hepatic TBA content increased as a result of HC feeding, with no significant further increment following BDL as opposed to chow fed mice, which exhibit a time-dependent increase in liver TBA, reaching similar values observed in HC fed animals at day three. The toxic effect of bile acids is well documented [21, 29, 30] and accumulation in the liver is one of the main factors associated with cholestasis.

Besides accumulation of toxic bile acids, our data suggest that cholesterol overload impairs cell proliferation and tissue repair as revealed by Ki67 immunostaining. This response is accompanied by decreased p21 expression levels in cholesterol loaded livers and a compensatory increased expression of cdk2. We observed that HC fed mice exhibit an increased content of total bile acids, which are associated with cell death. These findings suggest that cholesterol antagonizes the reported protective effect mediated by FXR activation. In support of this link, Cheng and coworkers recently reported that FXR transgenic mice exhibited increased sensitivity to a high cholesterol diet-induced hepatotoxicity [31], indicating that FXR is unable to protect from cholesterol-mediated liver injury.

On the other hand, it is well known that oxidative stress mediates the deleterious effects of toxic bile acids and cholesterol [9, 21, 25, 32]. Our findings are in agreement with these observations, as HC fed mice exhibit increased DCFH and DHE staining following BDL compared to Chow fed mice. In line with previous findings linking cholesterol accumulation in the liver with mitochondria-mediated ROS generation and impairment in antioxidant defense, we observed the downregulation of GPX4 and SOD2, two of the main mitochondrial protective enzymes [17, 33] in HC fed mice after BDL. Moreover, GST and γ-GCS display a similar behavior, strongly supporting the fact that the disruption in GSH homeostasis is an important contributor to cholesterol-mediated hepatocellular damage.

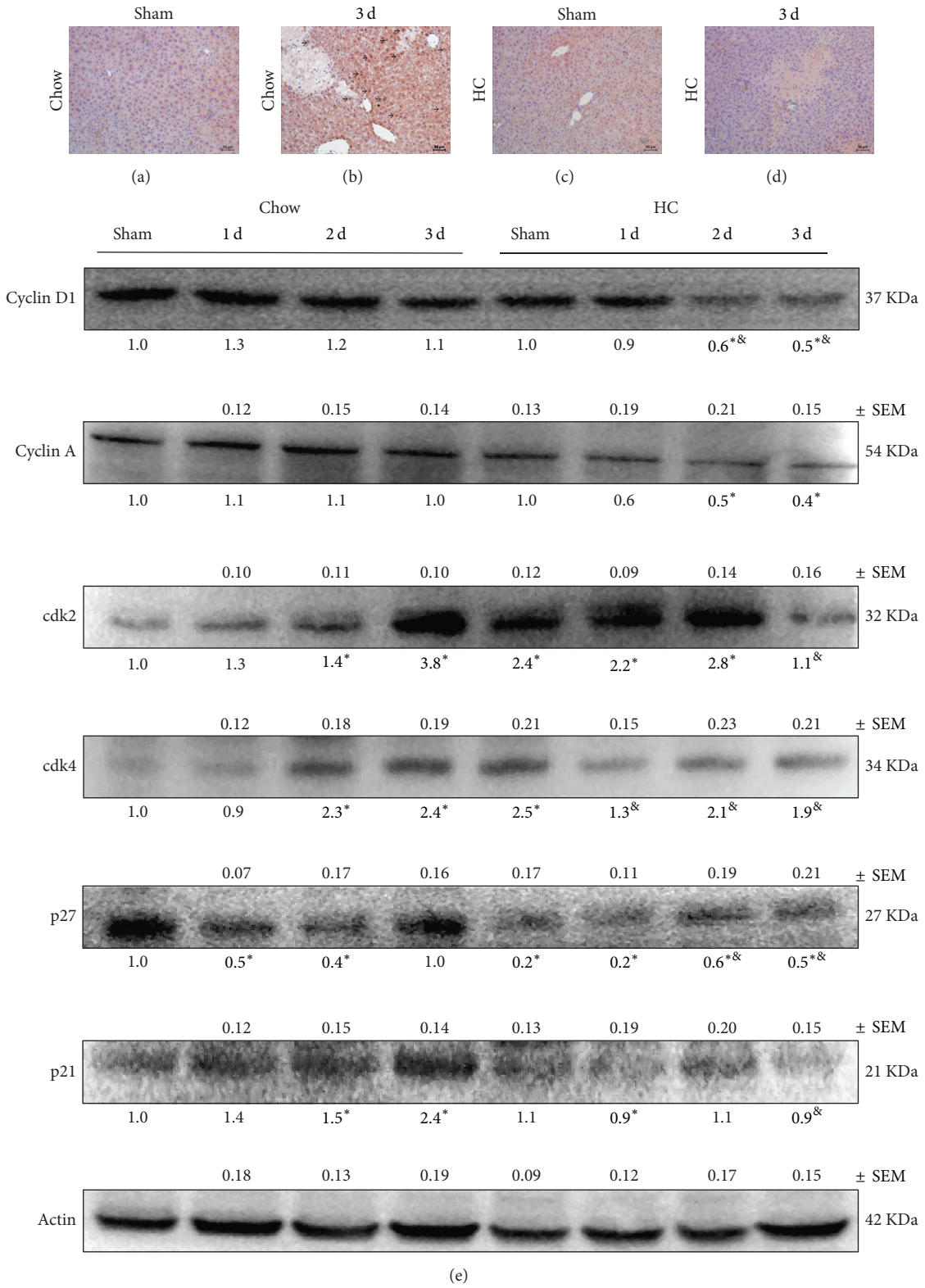


Figure 4: Cholesterol excess impairs cell proliferation. Tissue was obtained from Sham and three days after BDL animals, under (a-b) standard Chow control diet (Chow) or (c-d) high cholesterol (HC) diet, and Ki67 immunohistochemistry was assayed; arrows show positive cells (magnification at 100x). (e) Western blot and densitometric analysis of main cell cycle proteins. Actin was used as loading control; $^{*}p < 0.05$ versus Sham Chow fed mice, $^{\&}p < 0.05$ versus Sham HC fed mice. Images are representative from at least four independent mice.

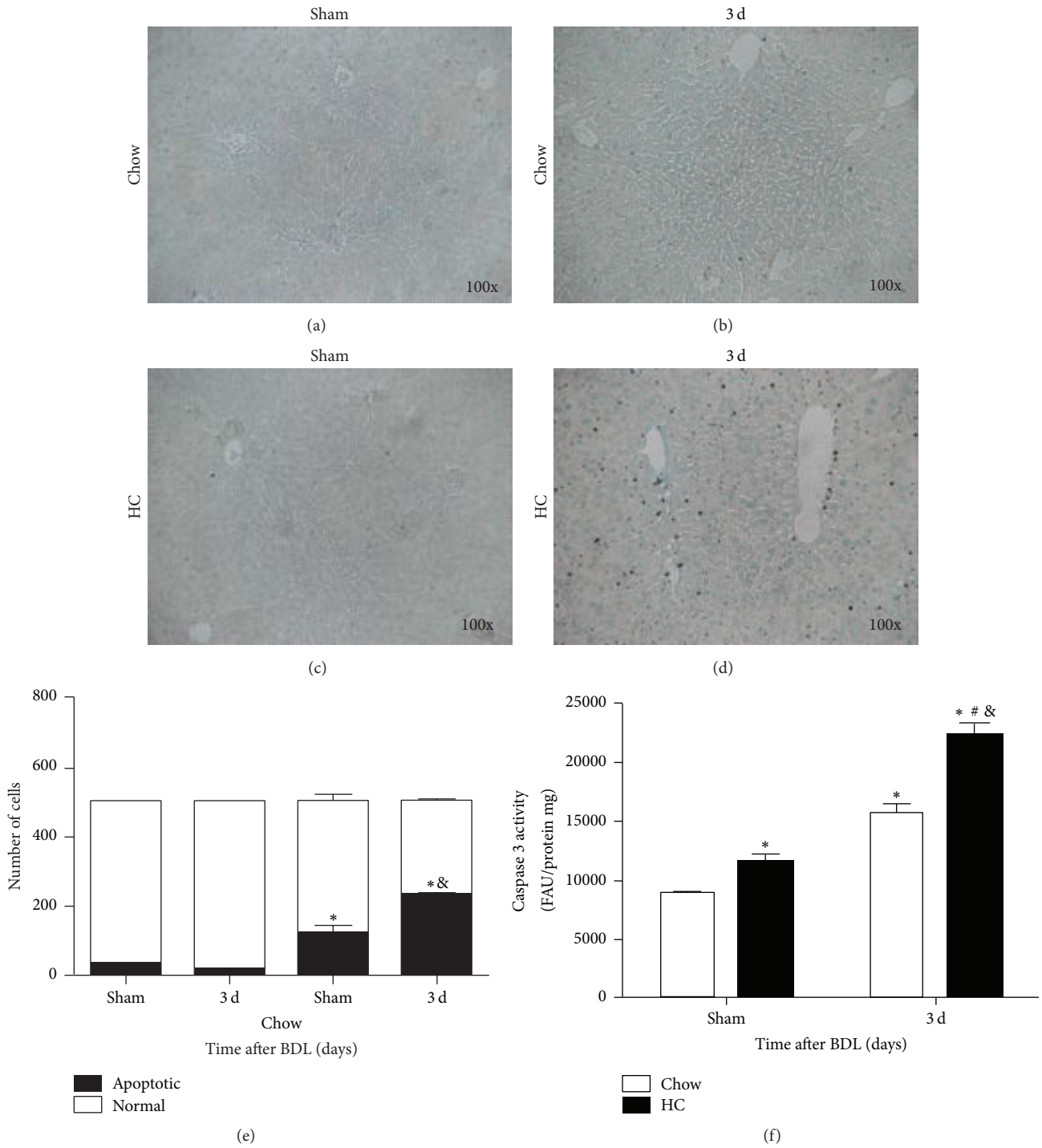
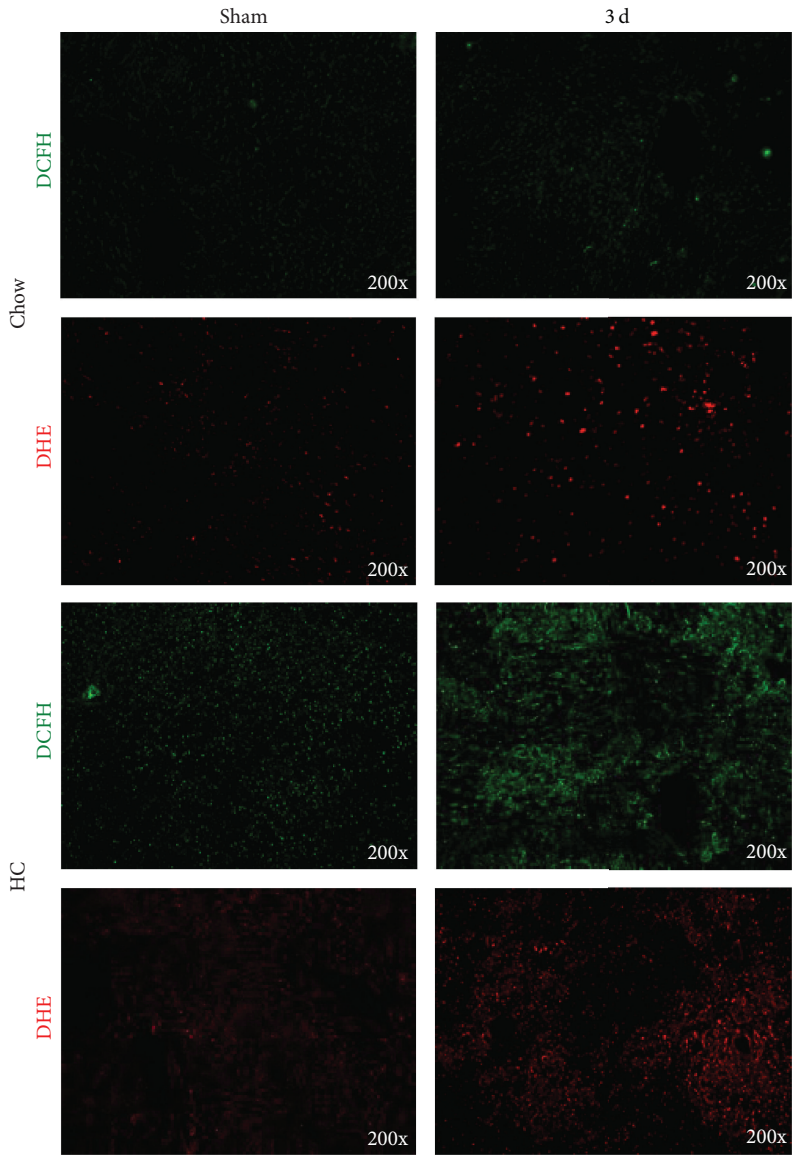


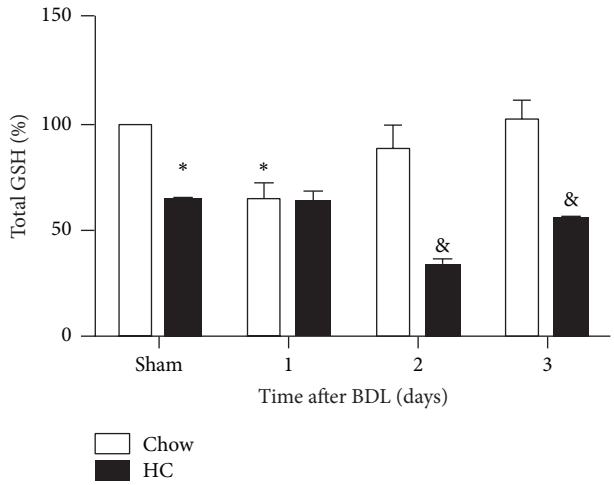
Figure 5: BDL in HC fed mice increases apoptosis. Tissue was obtained from Sham and three days after BDL animals, under (a-b) standard Chow control diet (Chow) or (c-d) high cholesterol (HC) diet, and apoptosis was assayed by (e) TUNEL immunohistochemistry; (e) quantification of positive TUNEL cells or by (f) caspase 3 activity. Images are representative from at least four independent mice. Original magnification: 100x. $^{*}p < 0.01$ versus Chow fed mice, $^{\#}p < 0.01$ versus 3 days Chow fed mice, and $^{\&}p < 0.01$ versus HC fed mice.

It is clear that cholesterol overload in the liver aggravates the organ injury decompensating GSH system, which could be related to a rapid cell death, particularly by apoptosis, potentiated by the overproduction of bile salts that eventually conducts to organ failure and animal death. Animals subjected to sodium cholate alone diet do not exhibit any significant change during the five days of the study.

It is clear that liver with cholesterol overload is sensitized to damage aggravating the diseases; this could be particularly considered in patients with genetic susceptibility to

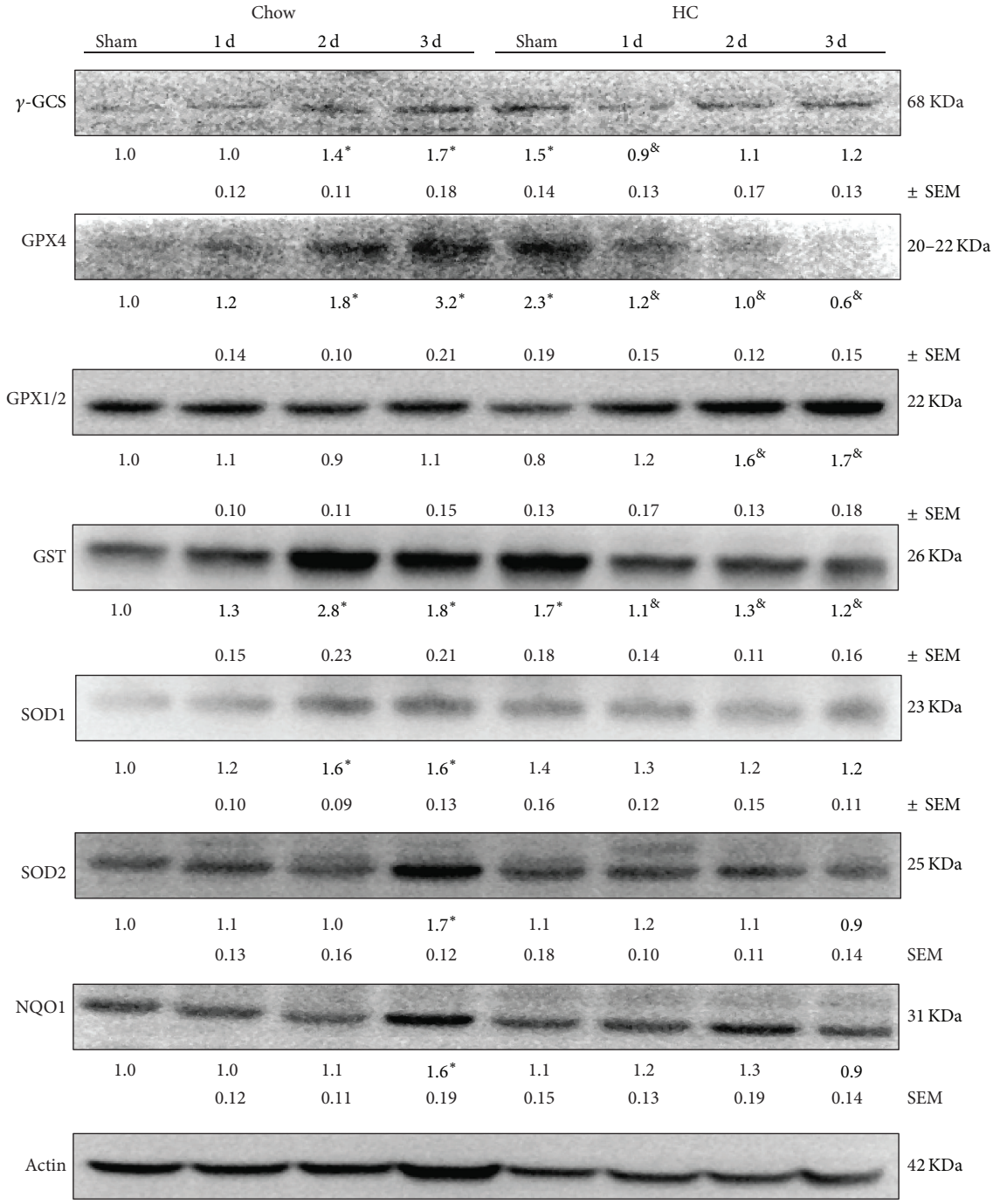


(a)



(b)

Figure 6: Continued.



(c)

Figure 6: HC diet increases *oxidative stress in mice with BDL*. (a) ROS content determined in fresh liver sections. Peroxides content was assayed by DCFH (5 μM) for 30 min, and superoxide content was determined by DHE (50 μM) for 30 min. (b) GSH content determined by HPLC. (c) Western blot and densitometric analysis of main oxidative stress-related proteins. Actin was used as loading control. Each column represents mean ± SEM of three independent experiments. Differences were considered significant at $^{*}p \leq 0.01$ versus Sham Chow fed animals; $^{\&}p \leq 0.01$ versus Sham Chow fed animals at the same time. Images are representative of at least three independent experiments. Original magnification: 200x.

cholesterol overload, such as early infantile forms of Niemann-Pick C disease, where canonical liver insults must be under surveillance.

Overall, our findings may be of clinical relevance suggesting that patients with cholesterol liver overload can be more at risk of developing clinical complications associated with cholestasis. In this context the use of statins may be of potential benefit by targeting the mevalonate pathway and perhaps other noncholesterol related mechanisms involved in cholestasis-mediated liver injury.

Disclosure

José C. Fernández-Checa and Luis Enrique Gomez-Quiroz shared senior authorship.

Competing Interests

The authors declare no competing financial interests.

Acknowledgments

This work was supported by grants of the CONACYT 252942, 236558, and 166042, CONACYT Fronteras de la Ciencia 1320, Asociación Mexicana de Hepatología, and SEP-PRODEP-913026-1461211, Universidad Autónoma Metropolitana Iztapalapa, by grants SAF-2011-23031 and SAF-2012-34831 from Plan Nacional de I+D, Spain, Fundació la Marató de TV3, La Mutua Madrileña, PI11/0325 (META) Grant from the Instituto Salud Carlos III, by the support of CIBEREHD, and by the Center Grant P50-AA-11999 from Research Center for Liver and Pancreatic Diseases funded by NIAAA/NIH.

References

[1] M. M. Finucane, G. A. Stevens, M. J. Cowan et al., "National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9·1 million participants," *The Lancet*, vol. 377, no. 9765, pp. 557–567, 2011.

[2] R. Lomonaco, N. E. Sunny, F. Bril, and K. Cusi, "Nonalcoholic fatty liver disease: current issues and novel treatment approaches," *Drugs*, vol. 73, no. 1, pp. 1–14, 2013.

[3] K. Tomita, T. Teratani, T. Suzuki et al., "Free cholesterol accumulation in hepatic stellate cells: mechanism of liver fibrosis aggravation in nonalcoholic steatohepatitis in mice," *Hepatology*, vol. 59, no. 1, pp. 154–169, 2014.

[4] M. Marí, F. Caballero, A. Colell et al., "Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis," *Cell Metabolism*, vol. 4, no. 3, pp. 185–198, 2006.

[5] C.-J. Lin, C.-K. Lai, M.-C. Kao et al., "Impact of cholesterol on disease progression," *BioMedicine (Netherlands)*, vol. 5, no. 2, pp. 1–7, 2015.

[6] G. Arguello, E. Balboa, M. Arrese, and S. Zanlungo, "Recent insights on the role of cholesterol in non-alcoholic fatty liver disease," *Biochimica et Biophysica Acta*, vol. 1852, no. 9, pp. 1765–1778, 2015.

[7] V. Ribas, C. García-Ruiz, and J. C. Fernández-Checa, "Glutathione and mitochondria," *Frontiers in Pharmacology*, vol. 5, article 151, 2014.

[8] F. Caballero, A. Fernández, A. M. De Lacy, J. C. Fernández-Checa, J. Caballería, and C. García-Ruiz, "Enhanced free cholesterol, SREBP-2 and StAR expression in human NASH," *Journal of Hepatology*, vol. 50, no. 4, pp. 789–796, 2009.

[9] M. C. Gutierrez Ruiz, M. Dominguez Perez, S. Rodriguez Gonzalez, N. Nuno Lambarri, C. Licona Retama, and L. E. Gomez-Quiroz, "High cholesterol diet modifies the repairing effect of the hepatocyte growth factor," *Gaceta Medica de Mexico*, vol. 148, pp. 236–242, 2012.

[10] G. Musso, R. Gambino, and M. Cassader, "Cholesterol metabolism and the pathogenesis of non-alcoholic steatohepatitis," *Progress in Lipid Research*, vol. 52, no. 1, pp. 175–191, 2013.

[11] G. Musso, R. Gambino, F. De Michieli et al., "Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis," *Hepatology*, vol. 37, no. 4, pp. 909–916, 2003.

[12] Y. Chen, J. Kong, and S. Wu, "Cholesterol gallstone disease: focusing on the role of gallbladder," *Laboratory Investigation*, vol. 95, no. 2, pp. 124–131, 2015.

[13] K. Allen, H. Jaeschke, and B. L. Copple, "Bile acids induce inflammatory genes in hepatocytes: a novel mechanism of inflammation during obstructive cholestasis," *American Journal of Pathology*, vol. 178, no. 1, pp. 175–186, 2011.

[14] M. G. Roma and E. J. Sanchez Pozzi, "Oxidative stress: a radical way to stop making bile," *Annals of Hepatology*, vol. 7, no. 1, pp. 16–33, 2008.

[15] B. C. Ferslew, G. Xie, C. K. Johnston et al., "Altered bile acid metabolome in patients with nonalcoholic steatohepatitis," *Digestive Diseases and Sciences*, vol. 60, no. 11, pp. 3318–3328, 2015.

[16] M. Domínguez-Pérez, N. Nuño-Lámbarri, D. Clavijo-Cornejo et al., "Hepatocyte growth factor reduces free cholesterol-mediated lipotoxicity in primary hepatocytes by countering oxidative stress," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 7960386, 8 pages, 2016.

[17] D. Clavijo-Cornejo, M. Gutiérrez-Carrera, M. Palestino-Domínguez et al., "Acetaldehyde targets superoxide dismutase 2 in liver cancer cells inducing transient enzyme impairment and a rapid transcriptional recovery," *Food and Chemical Toxicology*, vol. 69, pp. 102–108, 2014.

[18] C. Enriquez-Cortina, M. Almonte-Becerril, D. Clavijo-Cornejo et al., "Hepatocyte growth factor protects against isoniazid/rifampicin-induced oxidative liver damage," *Toxicological Sciences*, vol. 135, no. 1, pp. 26–36, 2013.

[19] A. M. Geerts, E. Vanheule, M. Praet, H. Van Vlierberghe, M. De Vos, and I. Colle, "Comparison of three research models of portal hypertension in mice: macroscopic, histological and portal pressure evaluation," *International Journal of Experimental Pathology*, vol. 89, no. 4, pp. 251–263, 2008.

[20] J. Kountouras, B. H. Billing, and P. J. Scheuer, "Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat," *British Journal of Experimental Pathology*, vol. 65, no. 3, pp. 305–311, 1984.

[21] R. Reinehr, S. Becker, V. Keitel, A. Eberle, S. Grether-Beck, and D. Häussinger, "Bile salt-induced apoptosis involves NADPH oxidase isoform activation," *Gastroenterology*, vol. 129, no. 6, pp. 2009–2031, 2005.

[22] C. R. Webster, A. N. Johnston, and M. S. Anwer, "Protein kinase Cδ protects against bile acid apoptosis by suppressing proapoptotic JNK and BIM pathways in human and rat hepatocytes," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 307, no. 12, pp. G1207–G1215, 2014.

[23] A. Valdés-Arzate, A. Luna, L. Bucio et al., "Hepatocyte growth factor protects hepatocytes against oxidative injury induced by ethanol metabolism," *Free Radical Biology & Medicine*, vol. 47, no. 4, pp. 424–430, 2009.

[24] A. F. Hofmann, "Cholestatic liver disease: pathophysiology and therapeutic options," *Liver*, vol. 22, supplement 2, pp. 14–19, 2002.

[25] B. L. Copple, H. Jaeschke, and C. D. Klaassen, "Oxidative stress and the pathogenesis of cholestasis," *Seminars in Liver Disease*, vol. 30, no. 2, pp. 195–204, 2010.

[26] K. Yasutake, M. Kohjima, K. Kotoh, M. Nakashima, M. Nakamuta, and M. Enjoji, "Dietary habits and behaviors associated with nonalcoholic fatty liver disease," *World Journal of Gastroenterology*, vol. 20, no. 7, pp. 1756–1767, 2014.

[27] P. Puri, R. A. Baillie, M. M. Wiest et al., "A lipidomic analysis of nonalcoholic fatty liver disease," *Hepatology*, vol. 46, no. 4, pp. 1081–1090, 2007.

[28] E. R. Kallwitz, M. L. Daviglus, M. A. Allison et al., "Prevalence of suspected nonalcoholic fatty liver disease in hispanic/latino individuals differs by heritage," *Clinical Gastroenterology and Hepatology*, vol. 13, no. 3, pp. 569–576, 2015.

[29] B. L. Woolbright, K. Dorko, D. J. Antoine et al., "Bile acid-induced necrosis in primary human hepatocytes and in patients with obstructive cholestasis," *Toxicology and Applied Pharmacology*, vol. 283, no. 3, pp. 168–177, 2015.

[30] S. Chatterjee, I. T. G. W. Bijsmans, S. W. C. van Mil, P. Augustijns, and P. Annaert, "Toxicity and intracellular accumulation of bile acids in sandwich-cultured rat hepatocytes: role of glycine conjugates," *Toxicology in Vitro*, vol. 28, no. 2, pp. 218–230, 2014.

[31] Q. Cheng, Y. Inaba, P. Lu et al., "Chronic activation of FXR in transgenic mice caused perinatal toxicity and sensitized mice to cholesterol toxicity," *Molecular Endocrinology*, vol. 29, no. 4, pp. 571–582, 2015.

[32] A. Bashiri, G. Tavallaee, L. Li, and D. S. Ng, "Emerging role of cellular cholesterol in the pathogenesis of nonalcoholic fatty liver disease," *Current Opinion in Lipidology*, vol. 24, no. 3, pp. 275–276, 2013.

[33] P. Cole-Ezea, D. Swan, D. Shanley, and J. Hesketh, "Glutathione peroxidase 4 has a major role in protecting mitochondria from oxidative damage and maintaining oxidative phosphorylation complexes in gut epithelial cells," *Free Radical Biology & Medicine*, vol. 53, no. 3, pp. 488–497, 2012.