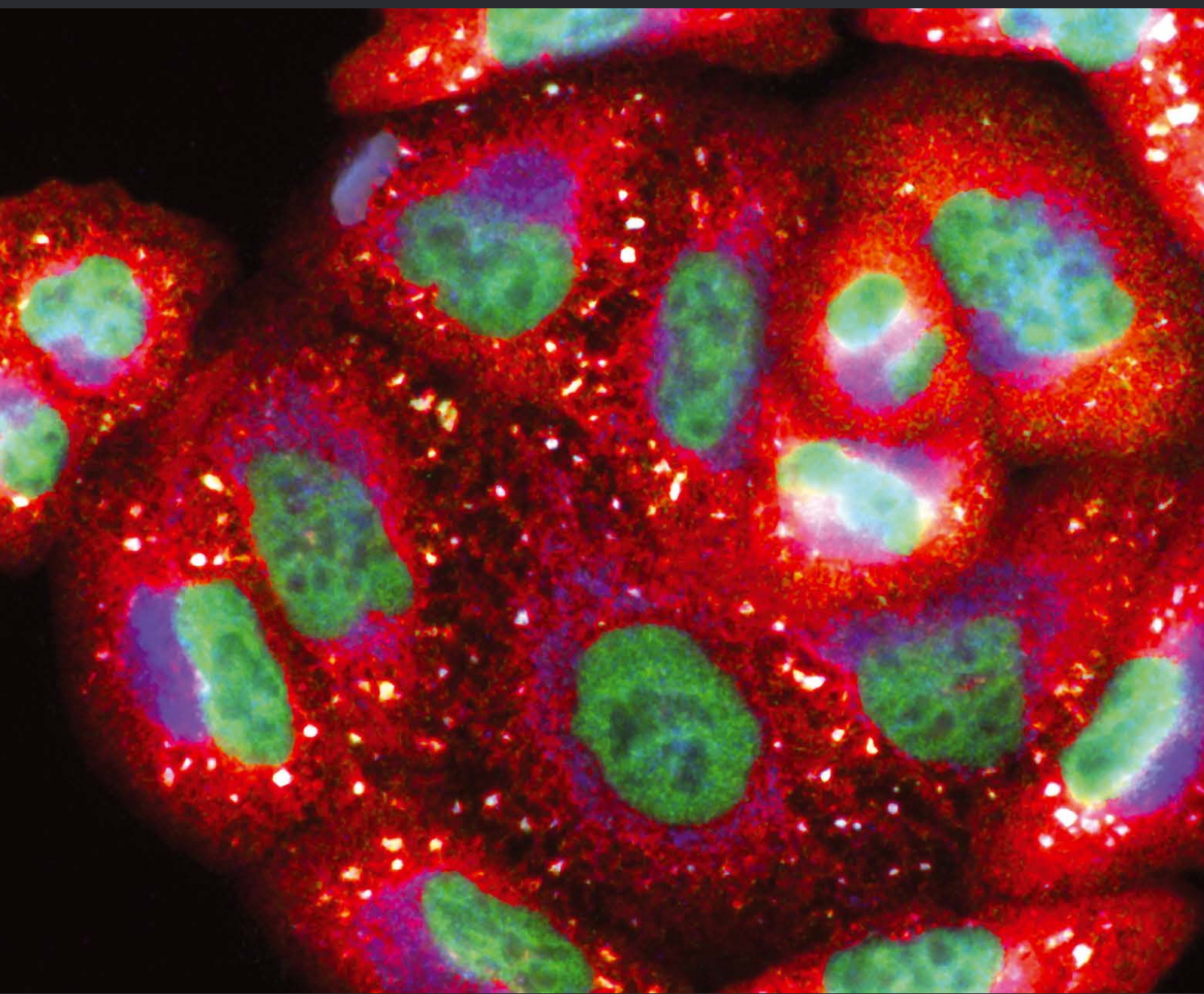
Oxidative Medicine and Cellular Longevity

Oxidative Stress and Inflammation in Hepatic Diseases: Current and Future Therapy

Guest Editors: Karina Reyes-Gordillo, Ruchi Shah, and Pablo Muriel



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Editorial

Oxidative Stress and Inflammation in Hepatic Diseases: Current and Future Therapy

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Liver disease is a highly prevalent disease that is one of the leading causes of death worldwide. The continuous exposure of the liver to some factors such as viruses, alcohol, fat, and biotransformed metabolites can cause hepatic injury, which can lead to inflammation and liver degeneration. When the injury is sustained for long time, it can cause chronic liver diseases (CLDs), which include a spectrum of disease states ranging from simple steatosis and steatohepatitis (steatosis with inflammation and hepatocyte injury and death) to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Multiple evidences indicate that oxidative stress and inflammation are the most important pathogenic events in liver diseases regardless of etiology. Oxidative stress and inflammation are not always harmful; they help phagocytes to kill microorganisms and modulate signaling events through redox regulation. However, unregulated and prolonged imbalance in the liver between the production of free radicals and/or reactive oxygen species (ROS) and their elimination by protective mechanisms (antioxidants) leads to damage of important biomolecules and cells, with potential impact on the whole organism causing many chronic diseases. During liver damage, ROS can induce the generation of proinflammatory genes. A critical component of inflammation is the infiltration of inflammatory cells, like neutrophils, monocytes, and lymphocytes, to the site of stimulus. At the site of inflammation, the activated inflammatory cells release chemical mediators (eicosanoids, cytokines, chemokines, nitric oxide, etc.) that induce tissue damage and augmented oxidative stress and reactive species (superoxide, hydrogen peroxide, hydroxyl radical, etc.). Thus, overexpression of the proinflammatory genes provokes an intracellular signaling cascade that produces more ROS, resulting in a vicious cycle, where increased oxidative stress and inflammatory lesion promote the pathogenesis of liver diseases. A better understanding of the basic pathophysiology underlying the development of steatosis, steatohepatitis, fibrosis, cirrhosis, and HCC is needed, so that better treatments can evolve for liver diseases. Thus, this special issue is dedicated to study the implications of the central roles that oxidative stress and inflammation play in CLDs, as well as the associated current and future therapies.

Antioxidant and anti-inflammatory therapy has been considered to have the possibility of beneficial effects in the management of liver diseases. In this regard, the group of S. Li et al. from China (in "Insights into the Role and Interdependence of Oxidative Stress and Inflammation in Liver Diseases") summarize the following: (i) the crucial roles of oxidative stress and inflammation in the development of liver damage and (ii) the relationship and interdependence of these processes and also describe (iii) the different herbal medicines or derived compounds targeting oxidative stress and inflammation in various liver diseases. Also from China, the group of Z. Wang et al. (in "Oxidative Stress and Liver Cancer: Etiology and Therapeutic Targets")

provided a review about the development of liver cancer from the perspective of cellular and molecular mechanisms and reported the therapeutic targets of hepatocarcinoma, suggesting that antioxidants are urgently needed to prevent carcinogenesis in the liver. On the other hand, U. S. U. Kumar et al. from Malaysia (in "Redox Control of Antioxidant and Antihepatotoxic Activities of *Cassia surattensis* Seed Extract against Paracetamol Intoxication in Mice: In Vitro and In Vivo Studies of Herbal Green Antioxidant") reported the protective effect of *Cassia surattensis* seed extract against paracetamol-induced liver toxicity in mice and described the antagonist effects of antioxidants during mild colitis. Moreover, the group of R. Chaphalkar et al. from India (in "Antioxidants of *Phyllanthus emblica* L. Bark Extract Provide Hepatoprotection against Ethanol-Induced Hepatic Damage: A Comparison with Silymarin") observed that PEE possesses potent antioxidant activity against free radicals and provides significant protection against alcohol-induced liver damage, thus supporting the therapeutic claims made in *Ayurveda* about *Phyllanthus emblica* for treatment of hepatic disorders. In recent times, a new puzzle in medical science has appeared: antioxidants may exert either beneficial or harmful effects depending on the cellular requirement for ROS at a particular situation. In this regard, the group of F. A. Moura et al. from Brazil (in "Colonic and Hepatic Modulation by Lipoic Acid and/or N-Acetylcysteine Supplementation in Mild Ulcerative Colitis Induced by Dextran Sodium Sulfate in Rats") observed that *N*-acetylcysteine is a promising antioxidant toward alleviating ulcerative colitis and hepatotoxicity, but the combination of lipoic acid and *N*-acetylcysteine in contrast causes hepatic injury and colonic inflammation.

Antioxidants and anti-inflammatories also play a crucial role in metabolic liver diseases. From Brazil, A. Paiva et al. (in "Apolipoprotein CIII Overexpression Induced Hypertriglyceridemia Increases Nonalcoholic Fatty Liver Disease in Association with Inflammation and Cell Death") demonstrated that persistent hypertriglyceridemia might be more relevant to liver inflammation than intracellular lipid accumulation and that overexpression of apo-CIII increases severity of diet-induced fatty liver disease. This study will be useful to develop new targets to treat metabolic liver diseases. In addition, P. K. Leong and K. M. Ko from China (in "Schisandrin B: A Double-Edged Sword in Nonalcoholic Fatty Liver Disease") suggest that Schisandrin B, a traditional Chinese herb, may offer potential as a therapeutic agent for NAFLD, due to its antihyperlipidemic, antioxidant, anti-ER stress, anti-inflammatory, and anticarcinogenic activities in cultured hepatocytes in vitro and in rodent livers in vivo.

The reduction of oxidative stress is suggested to be one of the main mechanisms to explain the benefits of subnormothermic perfusion against ischemic liver damage. In this regard, T. Carbonell et al. from Spain (in "Subnormothermic Perfusion in the Isolated Rat Liver Preserves the Antioxidant Glutathione and Enhances the Function of the Ubiquitin Proteasome System") found that subnormothermic perfusion in the liver can induce oxidative stress concomitantly with antioxidant glutathione preservation, triggering antioxidant mechanisms, protecting against ischemic, hypoxic, and toxic damage. In addition, the group of Y. Zhang et al. from China (in "Hyperglycemia Aggravates Hepatic Ischemia Reperfusion Injury by Inducing Chronic Oxidative Stress and Inflammation") suggested that chronic oxidative stress, inflammation, and potential malfunction of antioxidative system are the reasons why hyperglycemia aggravates hepatic ischemia reperfusion injury. Biomarkers are necessary for the evaluation of the severity of oxidative stress in I/R injury; thus, the group of H. Li et al. form China (in "Renalase as a Novel Biomarker for Evaluating the Severity of Hepatic Ischemia-Reperfusion Injury") demonstrated that renalase, a ubiquitous flavin adenine dinucleotide-containing amino oxidase, is a sensitive ROS-responsive gene in hepatocytes which can serve as an efficient and sensitive biomarker for the early warning or evaluation of the severity of hepatic I/R injury.

Liver diseases remain a significant and major health problem around the world. Current therapies in chronic liver diseases are limited and liver transplantation is the only available treatment for end-stage liver disease. This special issue believes to provide novel, effective, and safe approaches to create future antioxidant and anti-inflammatory therapies for patients with CLDs.

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Research Article

Antioxidants of *Phyllanthus emblica* L. Bark Extract Provide Hepatoprotection against Ethanol-Induced Hepatic Damage: A Comparison with Silymarin

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Phyllanthus emblica L. (amla) has been used in Ayurveda as a potent rasayan for treatment of hepatic disorders. Most of the pharmacological studies, however, are largely focused on PE fruit, while the rest of the parts of PE, particularly, bark, remain underinvestigated. Therefore, we aimed to investigate the protective effect of the hydroalcoholic extract of *Phyllanthus emblica* bark (PEE) in ethanol-induced hepatotoxicity model in rats. Total phenolic, flavonoid, and tannin content and in vitro antioxidant activities were determined by using H_2O_2 scavenging and ABTS decolorization assays. Our results showed that PEE was rich in total phenols (99.523 ± 1.91 mg GAE/g), total flavonoids (389.33 ± 1.25 mg quercetin hydrate/g), and total tannins (310 ± 0.21 mg catechin/g), which clearly support its strong antioxidant potential. HPTLC-based quantitative analysis revealed the presence of the potent antioxidants gallic acid (25.05 mg/g) and ellagic acid (13.31 mg/g). Moreover, one-month PEE treatment (500 and 1000 mg/kg, p.o.) followed by 30-day 70% ethanol (10 mL/kg) administration showed hepatoprotection as evidenced by significant restoration of ALT ($p < 0.01$), AST ($p < 0.001$), ALP ($p < 0.05$), and TP ($p < 0.001$) and further confirmed by liver histopathology. PEE-mediated hepatoprotection could be due to its free radical scavenging and antioxidant activity that may be ascribed to its antioxidant components, namely, ellagic acid and gallic acid. Thus, the results of the present study support the therapeutic claims made in Ayurveda about *Phyllanthus emblica*.

1. Introduction

Alcohol consumption is very common in most cultures which could be one of the reasons for alcohol abuse. Additionally, alcoholic liver disease (ALD) represents a spectrum of clinical illness and morphological changes that range from fatty liver to hepatic inflammation to progressive fibrosis and ultimately cirrhosis. Well accepted mechanisms for ethanol-induced liver injury are fat accumulation in hepatocytes, CYP2E1 induction, and oxidative stress-mediated hepatocyte damage [1]. CYP2E1, the main variant of cytochrome P450 enzymes and also a component of the microsomal ethanol oxidizing system (MEOS), plays an important role in ethanol metabolism. Following ethanol consumption, CYP2E1 activity can increase up to fourfold [2]. Alcohol dehydrogenase- and MEOS-mediated ethanol metabolism leads to production of acetaldehyde, which generates ROS. The resultant ROS production causes oxidative stress, endoplasmic reticulum stress, and steatosis. Another source of ROS production is through activation of NADPH oxidase in hepatocytes. ROS-mediated oxidative stress is considered as the most important causative factor in the pathology of ALD. The ROS contribute to liver

damage through a variety of mechanisms including inactivation of antioxidant enzymes, depletion of reduced glutathione, alteration of the breakdown of fat molecules, and lipid peroxidation. Collectively all these alterations lead to inflammation and apoptosis in hepatocytes [3]. On the other hand, the cellular antioxidant milieu gets depleted; particularly, chronic consumption of ethanol leads to reduced glutathione depletion, which makes hepatocytes more sensitive to ROS-mediated oxidative stress [4].

Herbal extracts exhibit protective mechanism against oxidative stress by enhancing antioxidant enzyme activities and averting GSH depletion [5]. *Phyllanthus emblica* L. (PE) is a tropical and subtropical tree that belongs to the family Euphorbiaceae and is distributed throughout the Deccan, coastal districts, Kashmir, and deciduous forests of Madhya Pradesh in India. PE is also widely distributed in most tropical and subtropical countries including China and Indonesia and in the Malay Peninsula. PE is native to tropical southeastern Asia, particularly, in central and southern India, Nepal, Pakistan, Bangladesh, Bhutan, Sri Lanka, Myanmar, and the Mascarene Islands [6]. PE is a medium to large deciduous plant 7 to 19 meters in height growing wild or cultivated throughout tropical India with a grey bark and reddish wood. The bark of PE appears to be shiny greyish brown or greyish green and thick up to 12 mm. According to the two main classic texts on Ayurveda, Charak Samhita, and Sushruta Samhita, amla is regarded as "the best among rejuvenating herbs and sour fruits." Various beneficial effects of *emblica* have been described in "Materia Medica," a classical Indian text on the Ayurvedic system of medicine [7]. PE is a major ingredient in many Ayurvedic preparations including Triphala and Chyawanprash, a general tonic for people of all ages for overall mental and physical well-being. Traditionally PE has been used in Ayurveda for the treatment of diarrhoea and fever, as a diuretic, in inflammation, skin sores, and wounds, and as a potent rasayan in hepatic disorders [8, 9]. The fruit of the plant has been prescribed for different pharmacological activities like antioxidant [10], antitumor [11], gastroprotective [12], antitussive [13], hepatoprotective [14], and antidiabetic [15].

Entire parts of PE, including fruit, flower, seed, leaf, root, and bark, have been widely used in various folk systems, such as traditional system of Indian medicine (Ayurveda), traditional Chinese medicine, Tibetan medicine, and Arab medicine (Unani). The minorities living in the southwest of China use the root for the treatment of eczema and fruits for the treatment of jaundice and diarrhoea, whereas, in the Nepal, it is used as an astringent and hemostatic [16–18]. Bark of PE possesses strong antioxidant and radical scavenging activities demonstrating that it can be correlated to the presence of polyphenols [19]. Being a rich source of tannin (21–33%), the bark of PE is one of the main raw materials used for tannin extract production in China. Most of the pharmacological studies, however, are largely focused on PE fruit, while the rest of the parts of PE, particularly, bark, remain underinvestigated. Currently only wound healing activity of the bark of PE has been reported [20].

As PE contains antioxidant constituents like total phenols, flavonoids, and tannins in abundance and ROS-mediated oxidative stress is considered as major mechanism responsible for alcohol-induced liver damage, we intended to evaluate the hepatoprotective activity of hydroalcoholic bark extract of *P. emblica* (PEE) in rats intoxicated with ethanol.

2. Materials and Methods

2.1. Chemicals. Silymarin was purchased from Microlabs, Bangalore, India. Gallic acid and ellagic acid were purchased from Natural Remedies Pvt. Ltd., Bangalore, India. Diagnostic kits for assaying ALT, AST, ALP, and total protein were purchased from Crest Biosystems, Goa, India.

2.2. Plant Collection and Authentication. Fresh bark of PE was collected from Kem Village, Solapur District of Maharashtra, India, during monsoon season, washed thoroughly with water, and shade-dried. A voucher specimen (No: RMC-1.BSI/WRC/Tech.2012) was identified and authenticated by Botanical Survey of India, Pune.

2.3. Extraction. Powdered dried bark (100 g) was extracted with 250 mL mixture of absolute ethanol and water in the ratio of 7 : 3 using a Soxhlet apparatus. Further, hydroalcoholic extract was evaporated to dryness by distillation under reduced pressure in rotatory evaporator. The yield of PEE was 3 g per 100 g of PE bark. The PEE was then subjected to characterization and phytochemical screening.

2.4. Phytochemical Screening. PEE was subjected to phytochemical evaluation to examine the presence of carbohydrate, flavonoids, tannins, glycosides, saponins, alkaloids, sterols and triterpenoids, and amino acids. Total phenols, flavonoids, and tannins were also quantified in PEE.

2.4.1. Total Phenols. Total phenolic content in PEE was determined based on Folin-Ciocalteu (FC) colorimetric method [21]. In brief, 1 mg/mL of the PEE was prepared and mixed with FC reagent. After 3 min, 3 mL of sodium carbonate (20%) was added to the reaction mixture and allowed to stand for 2 h with occasional shaking. The absorbance of the blue colour was measured at 760 nm using spectrophotometer. Total phenolic content was calculated from the calibration curve of gallic acid and concentration of total phenols expressed as gallic acid equivalent in mg/g of dry PEE.

2.4.2. Flavonoids. Total flavonoid content was determined using the aluminium chloride assay through colorimetric assay method [22]. One millilitre of PEE was diluted with 2 mL of distilled water and, after 5 min, 3 mL of 5% sodium nitrite and 0.3 mL of 10% aluminium trichloride was added. After 6 min, 2 mL of 1 M sodium hydroxide was added and volume was made up to 10 mL with distilled water. After incubation a red coloured complex was formed which was measured at 510 nm. Flavonol content was calculated from the calibration curve of quercetin and was expressed as quercetin equivalent in mg/g of PEE.

2.4.3. Tannins. Total tannin content was determined using modified vanillin assay [23, 24]. PEE (1 mL of 1 mg/mL) was incubated in water bath for a brief period to bring it to temperature equilibrium. The working vanillin reagent (5 mL) was added to the sample and 5 mL 4% HCl was used as blank. Both sample and blank were incubated for 20 min and absorbance was read at 500 nm. Total tannin content was calculated from the calibration curve of catechin and was expressed as catechin equivalents mg/g of PEE.

2.5. Characterization of PEE Using HPTLC Analysis

2.5.1. Instrumentation and Chromatographic Conditions. PEE was standardized using HPTLC analysis in accordance with Jeganathan and Kannan [25] using a solution of the PEE (20 mg/mL) and standard stock solutions of gallic acid and ellagic acid stock (10 μg/mL), prepared in HPLC grade methanol. The stock solutions and PEE were filtered through 0.45 μ syringe filter and then subjected to HPTLC analysis. The analysis was performed by comparing and interpolating the PEE peak area with that of the standard gallic acid and ellagic acid from the calibration curve.

HPTLC aluminium plates precoated with silica gel $F60_{254}$ (10 × 10 cm) with 200 μm thickness (Merck) were used as the stationary phase. The mobile phase used was toluene : ethyl acetate : formic acid (2 : 7 : 2). Ascending development was carried out in a twin trough glass chamber saturated with the mobile phase until the solvent reached a maximum front distance of 160 mm. The plate was saturated for 30 minutes at room temperature (25 ± 2°C) and subsequently allowed to dry at room temperature. The separated bands on the HPTLC plates were scanned over the wavelength of 200–400 nm with a maximum absorbance at wavelength of 280 nm. The peak areas were recorded to obtain the concentrations of gallic acid and ellagic acid.

2.6. In Vitro Antioxidant Activity

2.6.1. Hydrogen Peroxide Scavenging Activity. Briefly, various concentrations of PEE were prepared and mixed with 0.3 mL of 4 mM solution prepared in phosphate buffer (0.1 M, pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm against blank solution containing the PEE without H_2O_2. Ascorbic acid was used as a standard. All measurements were made in duplicate and average of these two observations was considered. The scavenging effect was then calculated according to the following equation:

$$H_2O_2 \text{ scavenging activity} = 1 - \left(\frac{A_s}{A_c}\right) \times 100, \quad (1)$$

where A_c = absorbance of control and A_s = absorbance of extract.

The concentration equivalent to ascorbic acid was calculated by plotting the values of the PEE on standard curve of ascorbic acid. EC_{50} value (mg/mL) is the concentration at which the scavenging activity was 50% [26].

2.6.2. ABTS Radical Scavenging Activity. In this method, ABTS was dissolved in distilled water to achieve concentration of 7 mM. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM of potassium persulfate and the mixture was allowed to stand in the dark at room temperature for 12–16 h before use. The percent scavenging activity of the PEE was determined by calculating the % inhibition by the following formula and results were compared with ascorbic acid as standard:

$$ABTS^{\bullet+} \text{ scavenging } (\%) = 1 - \left(\frac{A_s}{A_c}\right) \times 100, \quad (2)$$

where A_c = absorbance of control and A_s = absorbance of the PEE.

The concentration equivalent to ascorbic acid was calculated by plotting the values of the PEE on standard curve of ascorbic acid [27].

3. Hepatoprotective Activity

3.1. Animals. Male Wistar rats (150–200 g) were kept at National Toxicology Centre (Pune, India). Animals were housed in group of six (three of parallel sex in one cage) in polypropylene cages and acclimatized to standard laboratory conditions (temperature 25 ± 10°C, relative humidity 50 ± 15%) one week prior to the actual commencement of the experiment. Light-dark cycle of 12-12 h was maintained for animals. They were provided with standard food pellets (NAV Maharashtra Chakan Oil Mills Ltd., Pune) and tap water ad libitum. The study and protocol were approved by Institutional Animal Ethical Committee (CPCSEA/IAEC/NTC/P-200/2012).

3.1.1. Ethanol-Induced Hepatotoxicity. Animals were randomized into five different groups containing six animals in each group. Group I served as normal control and was given 1 mL/kg saline p.o. for 30 days. Group II served as disease control and received 70% ethanol (10 mL/kg p.o.) for 30 days. Group III served as positive control and was given silymarin (25 mg/kg p.o.) followed by 70% ethanol (10 mL/kg p.o.) for 30 days. Groups IV and V served as treatment groups and received PEE oral dose of 500 and 1000 mg/kg, respectively, followed by 70% ethanol (10 mL/kg p.o.) for 30 days.

3.1.2. Biochemical Estimation. At the end of 4 weeks, animals were starved overnight and then sacrificed by overdose of anesthesia. Blood was collected and kept for 1 h at room temperature for clotting. Serum was separated by centrifugation at 3000 rpm for 20 mins and the biochemical parameters serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) activities, and total protein content were determined by spectrophotometric procedures, using the ERBA assay kits (ERBA Diagnostics Mannheim GmbH, Mannheim, Germany).

3.1.3. Histopathological Examination. The animals used in the study were sacrificed and liver tissue was examined grossly

and weighed. A small portion of liver tissue of each animal was fixed in 10% neutral buffered formalin processed. Then it was embedded in paraffin wax to obtain 5-6 μm thick hematoxylin and eosin stained sections for examination [28].

Five fields were viewed by blinding for histopathological signs at a magnification of 200. Sinusoidal congestion (in hepatic parenchyma), degenerative changes of hepatocytes, cellular swelling, vacuolar changes with granular cytoplasm of hepatocytes, necrotic changes of hepatic parenchyma, loss of nucleus, fragmentation of nuclei, centrilobular, midzonal necrosis, bile duct hyperplasia, perivascular lymphoid aggregation, infiltration of mononuclear cells (MNC), formation of microgranuloma, fatty liver (lipid deposition in hepatocyte), and steatosis were graded as the following: no abnormality detected (NAD), minimal pathological changes (+), mild pathological changes (++), moderate pathological changes (+++), and severe pathological changes (++++).

3.2. Acute Toxicity Study. The acute toxicity study for PEE was performed using male albino mice. The animals were fasted overnight prior to the experiment and maintained under standard conditions. The study was conducted as per Organization of Economic Cooperation and Development (OECD) Test guidelines 425 on Acute Oral Toxicity [29]. The study was carried out in a stepwise procedure. In step I, three animals were used and given 2000 mg/kg of the PEE. When mortality was found to be unlikely, step II was carried out. In step II, additional three animals were again given the PEE at a dose of 2000 mg/kg and observed for 14 days.

3.3. Statistical Analysis. Data obtained from in vitro experiments was expressed as mean ± SEM. For in vivo experiments and statistical differences between the treatments and the control were evaluated by One Way ANOVA followed by Dunnett's test. A probability value of $p < 0.05$ was considered as significant.

4. Results

4.1. Qualitative Analysis of Phytochemicals Present in PEE. As shown in Table 1, qualitative analysis of PEE revealed the presence of flavonoids, tannins, glycosides, saponins, and alkaloids.

4.2. Quantification of Total Phenolic, Flavonoids, and Tannin Contents Present in PEE. As shown in Table 2, the quantitative phytochemical screening of PEE revealed the presence of total phenols, flavonoids, and tannins.

4.2.1. Total Phenolic Content. The total phenolic content of the PEE was determined from the regression equation of calibration curve ($R^2 = 0.996$) and found to be 99.523 mg/g which was equivalent to 275 μg/mL of gallic acid.

4.2.2. Total Flavonoid Content. The total flavonoid content of the PEE was determined from the regression equation of calibration curve ($R^2 = 0.996$) and found to be 389.33 mg/g which was equivalent to 200 μg/mL of quercetin hydrate.

Table 1: Qualitative analysis of phytochemicals present in PEE.

Phytochemical analyzed	Test method	Inference
Carbohydrate	Molisch's test	−
	Fehling's test	−
Flavonoids	Alkaline reagent test	+
	Ferric chloride test	++
Tannins	Ferric chloride test	++
	Potassium dichromate test	+++
Glycosides	Keller-Kiliani's test	+
Saponins	Foam test	+++
Alkaloids	Mayer's test	+
	Dragendorff's test	+
Sterols and triterpenoids	Salkowski test	−
Amino acids	Ninhydrin test	−

(+++): appreciable amount; (++) moderate amount; (+) trace amount; (−) completely absent.

Table 2: The total phenolic, flavonoids, and tannin contents present in PEE.

Parameters	Hydroalcoholic extract of PE bark
Total phenolic content (mg of GAE/g of PEE)	99.523 ± 1.91
Total flavonoid content (mg of quercetin hydrate/g of PEE)	389.33 ± 1.25
Total tannin content (mg of catechin/g of PEE)	310 ± 0.21

Values are expressed as mean ± SEM of three replicates.

4.2.3. Total Tannin Content. The total tannin content of the PEE was determined from the regression equation of calibration curve ($R^2 = 0.993$) and found to be 310 mg/g which was equivalent to 300 μg/mL of catechin.

4.3. HPTLC Analysis of Gallic Acid and Ellagic Acid Present in PEE. Linearity of the calibration curves of gallic acid and ellagic acid was tested by linear regression analysis and found to be linear in the concentration range 10–60 μg/mL with good correlation coefficient (r^2) of more than 0.99. The peaks of gallic acid and ellagic acid in the PEE were identified by comparing retention time of reference gallic acid and ellagic acid. The amount of gallic acid and ellagic acid in the PEE was estimated to be about 25.05 mg/g of gallic acid and 13.31 mg/g of ellagic acid.

4.4. In Vitro Antioxidant Activity

4.4.1. Hydrogen Peroxide Scavenging Activity. The ability of PEE and ascorbic acid to scavenge hydrogen peroxide radicals is shown in Table 3. PEE was capable of scavenging free radicals in dose dependent manner. Up to concentration of 200 μg/mL, the percentage inhibition of PEE (43.20%) was

Table 3: H_2O_2 radical scavenging activity of PEE ($n = 6$).

Concentration [μg/ml]	% scavenging activity of ascorbic acid	% scavenging activity of PEE
10	6.82 ± 1.106	7.85 ± 2.427
50	14.63 ± 0.571	14.02 ± 5.603
100	28.80 ± 0.7495	23.91 ± 2.998
150	40.82 ± 1.999	32.01 ± 2.248
200	55.39 ± 0.2855	43.20 ± 3.301
250	71.34 ± 0.2498	79.62 ± 2.541

Values are expressed as a mean ± SEM.

Table 4: ABTS radical scavenging activity of PEE ($n = 6$).

Concentration [μg/ml]	% inhibition	Concentration equivalent to ascorbic acid [μg/ml]
50	21.79 ± 0.011	34.0
100	27.52 ± 0.001	55.0
150	31.74 ± 0.011	70.5
200	36.23 ± 0.007	87
250	42.91 ± 0.002	111.5

Values are expressed as mean ± SEM.

almost comparable to that of ascorbic acid (55.39%). However, at 250 μg/mL of PEE, the percentage inhibition of PEE was 79.62%, which was found to be better than ascorbic acid (71.34%). The IC_{50} value of PEE was 188.80 μg/mL while that of ascorbic acid was 177.7 μg/mL.

4.4.2. ABTS Radical Scavenging Activity. The ABTS radical scavenging activity of PEE was found to be concentration dependent. The maximum inhibition of ABTS radical at the concentration of 250 μg/mL was 42.91% which was less effective than that of standard (Table 4). The IC_{50} value of the PEE was 329.20 μg/mL while that of ascorbic acid was 133.96 μg/mL.

4.5. Biochemical Estimation. As shown in Table 5, the ethanol intoxicated group showed significant increase in serum levels of ALT ($p < 0.001$), AST ($p < 0.001$), and ALP ($p < 0.05$) along with decrease in the total protein ($p < 0.001$) compared to normal rats, indicating hepatotoxic effect of ethanol. After administration of PEE at the dose of 500 mg/kg, a statistically significant decrease in the elevated levels of ALT ($p < 0.01$), AST ($p < 0.001$), and ALP ($p < 0.05$) was observed along with improvement in TP content ($p < 0.001$) towards normal. Administration of 1000 mg/kg dose of PEE also restored elevated levels of ALT ($p < 0.05$), AST ($p < 0.01$), and TP ($p < 0.01$) but the effect of ALP was not statistically significant. Effect of PEE at dose of 500 mg/kg on ALT, AST, ALP, and TP content was comparable to that of silymarin, a standard hepatoprotective agent. When tested and standard drug treatment groups were compared with normal control group, there was no significant difference for all the mentioned biochemical parameters. Difference between test and standard drug treatment groups was also statistically nonsignificant.

4.6. Histopathological Examination. Histology of the liver sections of rats of different groups is shown in Figure 1. Examination of the histological activity index (HAI, Table 6) of the liver found that animals of normal control group showed normal histoarchitecture of hepatic parenchyma with hepatocytes arranged in cord like fashion around the central vein. The nucleus and cytoplasm showed normal histological features with intactness and normal morphology of cytoplasm and blood vessels and bile duct in portal triad.

The liver sections from disease control group showed moderate degree of damage to hepatic parenchyma with diffuse cellular swelling, degenerative changes in cytoplasm, and nucleus and focal areas of necrosis in the midzonal area. Many hepatocytes showed accumulation of lipoid material while few areas showed derangement of hepatic cords and necrotic changes of hepatocytes with occasional basophilia of nucleus and vacuolar cytoplasmic changes in hepatocytes. Focal areas of haemorrhages in the degenerative foci of hepatic parenchyma were also seen in a few sections. The overall features indicated a moderate degree of pathological changes leading to condition of fatty liver with degenerative and ensuing necrobiotic changes (Figures 1(a) and 1(b)).

Liver sections from animals of PEE 500 mg/kg group showed minimal to mild degree of histolopathological changes in the hepatic parenchyma. The hepatocytes showed minimal pathomorphological features of hepatocytes with multifocal areas of cellular swelling, vacuolar changes, and focal infiltration of mononuclear cells in hepatic parenchyma. Focal hyperplasia of bile duct in portal triad was also noted in one animal of this group with mild pathological changes in hepatic parenchyma.

As compared to PEE 500 mg/kg group, the histological evaluation from liver sections of PEE 1000 mg/kg group showed only minimal changes in hepatic parenchyma with normal hepatocytes and blood vessels and bile ducts. Focal changes of minimal degree were seen in only a few hepatocytes with less severity without any necrotic features.

The liver sections of SIL 25 mg/kg group showed normal hepatic parenchyma with normal hepatocytes and blood vessels and bile ducts. Focal changes of minimal degree were seen in only a few hepatocytes with less severity without any necrotic features. The observations of SIL 25, PEE 500, and PEE 1000 mg/kg groups were comparable with each other with slight differences.

4.7. Acute Toxicity Study. PEE did not show any significant change in body weight and behavioural pattern. There were no signs and symptoms of toxicity or mortality up to the dose level of 2000 mg/kg (data not shown). The LD_{50} was found to be greater than 2000 mg/kg with a cutoff at 5000 mg/kg.

5. Discussion

Ethanol is a familiar hepatotoxic chemical used for inducing liver damage in animals. Toxicity produced by ethanol is

Table 5: Effect of PEE on ALT, AST, ALP, and TP in ethanol-induced liver damage in rats.

Study groups	Treatment administered	ALT	AST	ALP	TP
Normal control	Saline (1 mL/kg/day)	49.49 ± 4.647	155.4 ± 6.197	270.71 ± 48.66	5.2 ± 0.31
Disease control	70% ethanol (10 mL/kg/day)	$103.9 \pm 16.33^{\#\#\#}$	$301.8 \pm 17.91^{\#\#\#}$	$396.4 \pm 17.07^{\#}$	$3.017 \pm 0.31^{\#\#\#}$
SIL25	Silymarin (25 mg/kg/day)	$56.20 \pm 1.953^{**}$	$230.5 \pm 21.65^{*}$	$278.6 \pm 4.351^{*}$	$4.9 \pm 0.50^{**}$
PEE500	PEE (500 mg/kg/day)	$61.20 \pm 4.693^{**}$	$189.8 \pm 8.84^{***}$	$267.1 \pm 14.15^{*}$	$5.283 \pm 0.25^{***}$
PEE1000	PEE (1000 mg/kg/day)	$69.03 \pm 3.66^{*}$	$210.8 \pm 17.82^{**}$	360.9 ± 44.92	$5.133 \pm 0.35^{**}$

ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, and TP: total protein. All values are expressed as mean ± SEM of at least 6 animals from each experimental group. Data found significant when One Way ANOVA followed by Dunnett's multiple comparison test performed. $p < 0.05$ was considered to be significant. Statistical significance ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$) compared with the normal control group and ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$) compared with the disease control group.

Table 6: Effect of PEE on histopathological activity index in ethanol-induced liver damage in rats.

Study groups	Sinusoidal congestion in hepatic parenchyma	Degenerative changes of hepatocytes, cellular swelling, vacuolar changes with granular cytoplasm of hepatocytes	Necrotic changes of hepatic parenchyma, loss of nucleus, fragmentation of nuclei, centrilobular, midzonal necrosis	Bile duct hyperplasia	Perivascular lymphoid aggregation	Infiltration of mononuclear cells (MNC), formation of microgranu-loma	Fatty liver (lipid deposition in hepatocyte), steatosis	Overall pathological grade (lesion score)
Normal control	+/focal	NAD	NAD	NAD	+/focal	NAD	+/focal	NAD
Disease control	++	+ ++	++	+	+	NAD	++	+++
SIL25	NAD	+/focal	NAD	NAD	NAD	NAD	NAD	+
PEE500	NAD	+/focal	+/focal	+	NAD	+/focal	NAD	++
PEE1000	NAD	+	+	+/focal	NAD	NAD	NAD	+

$n = 4$ per experimental group. No abnormality detected (NAD), minimal pathological changes (+), mild pathological changes (++), moderate pathological changes (+++), and severe pathological changes (++++). Focal and minimal changes may not be significant for alteration of functional capacity of the organ.

related to its oxidative metabolism by enzymes like alcohol dehydrogenase and CYP2E1 [30]. The metabolism of ethanol results in elevated production of superoxide [31] and hydrogen peroxide free radicals [32, 33]. Additionally, an increased level of acetaldehyde also decreases hepatic glutathione content and impairs the defence system of body that neutralizes free radicals and activates phagocytic cells. This overproduction of free radicals ultimately results in an increased level of lipid peroxidation followed by formation of adducts with cellular proteins and nucleic acids. These adducts eventually limit the function of hepatocytes [33].

In the present study, the hepatoprotective effect of PEE was evaluated using alcohol-induced hepatotoxicity model, since it is clinically relevant. Ethanol produces a group of characteristic effects in the liver leading to ALD [34]. In a similar manner, we also found biochemical and architectural perturbations in liver of the ethanol intoxicated rats. Chronic administration of ethanol led to a significant elevation of serum levels of ALT, AST, and ALP. The rise in the ALT level is usually accompanied by an elevation in the levels of AST, which plays a role in the conversion of amino acids to keto acids. Decrease in the levels of total protein (TP) was observed in the alcohol treated rats indicating the destruction in the number of hepatic cells, which may result in a decrease in hepatic capacity to synthesize protein.

Alcohol intoxication-mediated oxidative stress causes peroxidation of cell membrane lipids and alters membrane phospholipid composition and fluidity, thereby increasing the cell membrane permeability. Such an injured cell membrane causes leakage of various enzymes including ALT, AST, and ALT into blood circulation as shown by abnormally high levels of serum hepatic markers in disease control group. The present observations are in line with the previous studies which indicated that ALT, AST, and ALP are normally located in the cytoplasm and released into systemic circulation after hepatic cellular injury [35]. An elevated serum ALP level could be due to defective hepatic excretion or by increased production of ALP by hepatic parenchymal or duct cells. Ethanol-mediated hepatocyte injury is also evidenced in our study where chronic administration of ethanol resulted in

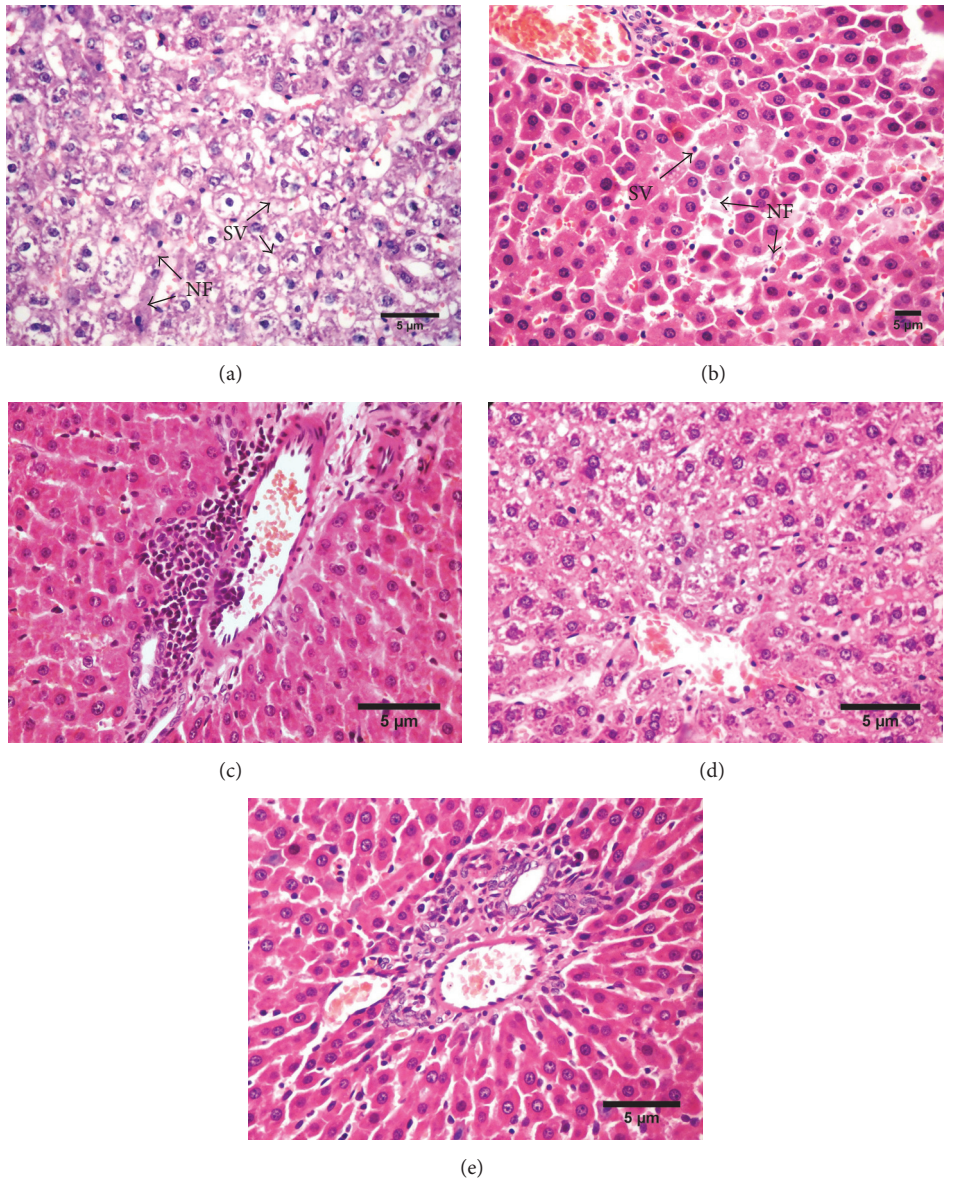

Figure 1: Photomicrographs of liver sections stained with hematoxylin and eosin. (a and b) Ethanol treated rat liver, (c) PEE500 mg/kg + ethanol treated, (d) PEE1000 mg/kg + ethanol treated, and (e) silymarin 25 mg/kg + ethanol treated (200x) [SV: degenerated hepatocytes with cellular swelling and vacuolar changes in cytoplasm; NF: necrotic foci with loss of nucleus of hepatocytes].

accumulation of lipoid material in hepatocytes, derangement of hepatic cords, and necrotic and vacuolar cytoplasmic changes in hepatocytes.

However, oral administration of PEE significantly decreased serum levels of AST, ALP, and ALT at both doses. Compared to 1000 mg/kg dose of PEE, its 500 mg/kg dose was found to be more effective. The hepatoprotective effect observed with PEE at the dose of 1000 mg/kg was not significantly prominent compared to that observed with PEE 500 mg/kg. Obtained hepatoprotection with PEE could be due to restoration of hepatocyte membrane integrity by PEE, which led to the restoration of hepatic enzymes. Additionally, suppression of elevated ALP activities with concurrent increase in the total protein content suggests the stability of biliary dysfunction in rat liver during hepatic injuries with toxicants. Treatment with PEE markedly elevated the total protein level which was comparable with standard drug silymarin. Hepatoprotective activity of PEE was found to be more prominent at the dose of 500 mg/kg than that at 1000 mg/kg compared to control group.

Apart from ethanol-mediated oxidative stress, alcohol is a well-known genotoxic substance that causes genomic instability. This could be another mechanism that contributes to alcohol-induced liver damage. A recent work by Guo and Wang has demonstrated that PE could activate spindle assembly checkpoint and prevent mitotic aberrations and genomic instability in human cells. Therefore, the hepatoprotective potential of PE may also be attributed to the reinforcement of

endogenous mechanisms against alcohol-induced genomic instability [36].

Preliminary phytochemical screening of PEE as well as quantification of the PEE revealed the presence of tannins, flavonoids, and phenols. Additionally, quantitative characterization of PEE by HPTLC method revealed it to be a rich source of ellagic acid and gallic acid. All these measured phytoconstituents in the present study have been reported earlier for their antioxidant potential by many researchers [37–39]. In this context, an antioxidant and hepatoprotective activity of PEE observed in this study could be attributed to its high content of gallic acid and ellagic acid. A large body of evidence shows the hepatoprotective potential of gallic acid and ellagic acid [40–44].

In the present study, results of in vitro antioxidant assays like hydrogen peroxide assay (IC_{50} value: 188.80 μg/mL) and ABTS assay (IC_{50} value: 329.20 μg/mL) noticeably showed dose dependent free radical scavenging potential of PEE. Our study results are in line with the findings of previous studies [45, 46]. As both in vitro methods are widely used because of their relevance to biological systems, in vitro free radical scavenging effect of PEE correlates to its in vivo antioxidant activity. Studies have shown that PE attenuates oxidative stress and related damage by increasing the endogenous antioxidant enzymes. Very recently, Tahir et al. reported that PE leaves extract ameliorates pulmonary fibrosis by elevating the activities of catalase, superoxide dismutase, glutathione peroxidase, and reduced glutathione in the pulmonary samples of rat [47]. Moreover, reports suggest that PE increases the antioxidant enzymes and prevents ethanol-induced toxic effects [48]. Considering the findings of previous studies, the hepatoprotective effect of PEE observed in the present study could be due to restoration and/or increase in the endogenous antioxidant milieu.

Although, silymarin, an isolated hepatoprotective flavonoid, is one of the most studied phytoconstituents in animals as well as humans, PE has many advantages over silymarin. PE is an enriched source of ascorbic acid and other phytoconstituents including linoleic acid, emblicanin A and emblicanin B, gallic acids, chebulic acid, ellagic acid, quercetin, and rutin. Synergistic interactions or multifactorial effects between these phytoconstituents make PE a better hepatoprotective herbal modality. Briefly, phytoconstituents present in PE work in a holistic manner. Although the dose response relationship can be easily established while using silymarin (a single isolated constituent), complete characterization of the PEE can address the limitation of the whole extract of PE. In this study, the hepatoprotective effect of silymarin was marginally better than that of PE as evidenced by biochemical and histopathological parameters. Silymarin and PE share many mechanisms that are responsible for their therapeutic effect including free radical scavenging activity, antioxidant activity, augmentation of endogenous antioxidant enzymes, attenuation of lipid peroxidation and inflammation, and stimulation of protein synthesis.

In summary, the in vitro and in vivo studies confirmed the antioxidant as well as hepatoprotective potential of hydroalcoholic extract of *P. emblica* bark and found it to be comparable with silymarin, a standard hepatoprotective phytoconstituent.

6. Conclusion

The data of the present study demonstrate that PEE possesses potent antioxidant activity against free radicals and provides significant protection against alcohol-induced liver damage. Biochemical analysis showed that PEE efficiently restores the levels of ALT, AST, ALP, and TP, which is further supported by histopathological observations. The resulting antioxidant and hepatoprotective activities of the PEE can be attributed to the presence of gallic acid, ellagic acid, polyphenols, and flavonoids. Although the findings corroborate the therapeutic potential of PE bark, further studies are needed to identify and isolate the polyphenolic compounds responsible for the antioxidant activity of PEE. Further investigation on PEE can then decide its use in patients with alcoholic liver disease.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Apolipoprotein CIII Overexpression-Induced Hypertriglyceridemia Increases Nonalcoholic Fatty Liver Disease in Association with Inflammation and Cell Death

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Nonalcoholic fatty liver disease (NAFLD) is the principal manifestation of liver disease in obesity and metabolic syndrome. By comparing hypertriglyceridemic transgenic mice expressing apolipoprotein (apo) CIII with control nontransgenic (NTg) littermates, we demonstrated that overexpression of apoCIII, independent of a high-fat diet (HFD), produces NAFLD-like features, including increased liver lipid content; decreased antioxidant power; increased expression of TNFα, TNFα receptor, cleaved caspase-1, and interleukin-1β; decreased expression of adiponectin receptor-2; and increased cell death. This phenotype is aggravated and additional NAFLD features are differentially induced in apoCIII mice fed a HFD. HFD induced glucose intolerance together with increased gluconeogenesis, indicating hepatic insulin resistance. Additionally, the HFD led to marked increases in plasma TNFα (8-fold) and IL-6 (60%) in apoCIII mice. Cell death signaling (Bax/Bcl2), effector (caspase-3), and apoptosis were augmented in apoCIII mice regardless of whether a HFD or a low-fat diet was provided. Fenofibrate treatment reversed several of the effects associated with diet and apoCIII expression but did not normalize inflammatory traits even when liver lipid content was fully corrected. These results indicate that apoCIII and/or hypertriglyceridemia plays a major role in liver inflammation and cell death, which in turn increases susceptibility to and the severity of diet-induced NAFLD.

1. Introduction

Hypertriglyceridemia is a common condition caused by multiple environmental and genetic factors [1, 2]. Elevated plasma levels of triglyceride- (TG-) rich remnant lipoproteins are independent risk factors for cardiovascular disease (CVD) [3]. Clinical and experimental studies have shown strong correlations and causal links between plasma TG and apolipoprotein CIII (apoCIII) levels [4, 5]. Plasma apoCIII levels are also increased in individuals with diabetes [6, 7]. Moreover, loss-of-function mutations in the apoCIII gene are associated with low TG levels and a reduced risk of CVD [8, 9]. Therefore, TG levels are causally linked to apoCIII and CVD, and apoCIII inhibitors are already in clinical development to reduce CVD risk [10].

Hypertriglyceridemia and nonalcoholic fatty liver disease (NAFLD) are common features in obesity and metabolic syndrome [11]. The prevalence of NAFLD in western countries ranges from 25 to 35% [12], and liver steatosis is observed in 80% of individuals with obesity [13]. Hepatic insulin resistance and type II diabetes are considered sequelae of NAFLD [14]. Furthermore, persistent steatosis may progress to steatohepatitis (NASH), cirrhosis, and hepatocarcinoma [15].

The two-hit hypothesis [16] has been proposed to explain NAFLD pathogenesis. In this hypothesis, steatosis represents the "first hit." Steatosis increases the vulnerability of the liver to various "second hits" that in turn lead to inflammation, fibrosis and cellular death. Oxidative stress is one such second hit. The inflammatory response, including the production of numerous proinflammatory molecules and adipokines, also

has a key role in the initiation and progression of the disease [17]. Proinflammatory cytokines can cause liver damage either directly or indirectly by increasing oxidative stress; in turn, oxidative stress can impair liver function either directly or indirectly by perpetuating a vicious cycle [18]. The pathways that control oxidative stress and inflammation underlie many cardiometabolic diseases, including obesity, diabetes, and atherosclerosis. Accordingly, recent evidence suggests that the morbidity and mortality associated with NAFLD are not restricted to changes in the liver, as the majority of deaths of patients with NAFLD are related to CVD [19].

We previously demonstrated that hypertriglyceridemic transgenic mice overexpressing apoCIII exhibit increases in hepatic glycerolipid content and liver oxidative stress. The latter was associated with increased NADPH oxidase and xanthine oxidase activities, even when the mice consumed a regular low-fat diet (LFD) [20]. Another recent study reported that apoCIII-overexpressing mice develop NAFLD associated with severe hepatic insulin resistance, increased liver lipid uptake and decreased lipid secretion following consumption of a high-fat diet (HFD) [21].

The present study was designed to investigate whether apoCIII overexpression and/or the resulting hypertriglyceridemia trigger the main events driving the evolution of steatosis to NASH, namely, inflammation and cell death. Furthermore, we tested whether the PPARa agonist fenofibrate, which regulates many genes related to inflammation and lipid metabolism, including apoCIII, could reduce susceptibility to NAFLD.

2. Materials and Methods

2.1. Animals and Treatments. All experimental protocols for this study were approved by the university's Committee for Ethics in Animal Experimentation (CEUA/UNICAMP, protocol number 2436), and the research was conducted in conformity with the Public Health Service Policy. Male mice transgenic for human apoCIII and nontransgenic controls were maintained at the Division of Physiology and Biophysics, Biology Institute, State University of Campinas (São Paulo, Brazil). Human apoCIII transgenic founder mice (line 3707) [22] were donated by Dr. Alan R. Tall (Columbia University, New York, NY) in 1996 and have since been crossbred with wild-type (NTg) C57BL/6 mice (Multidisciplinary Center for Biological Research of the University of Campinas). The apoCIII transgenic mice were screened according to their fasting TG plasma levels (apoCIII mice > 300 mg/dL; control mice < 100 mg/dL) and housed in a room at 22°C ± 2°C with a 12-hour light-dark cycle with free access to water and food. One-month-old male mice (transgenic and NTg littermates) were fed either a LFD or a HFD until 4 months of age. Additional groups of mice fed a HFD were treated with fenofibrate (100 mg/kg bw, Allergan, SP, Brazil, solubilized in 5% Arabic gum) or 5% Arabic gum (control untreated group) during the last 2 weeks of HFD consumption by daily gavage. At 4 months of age, fasted mice were anesthetized via intraperitoneal (IP) injection of ketamine and xylazine (50 and 10 mg/kg) and euthanized by exsanguination through the retro-orbital plexus (see Table 1).

2.2. Biochemical Analyses. Plasma levels of total cholesterol, triglycerides (Chod-Pap; Roche Diagnostic GmbH, Mannheim, Germany), nonesterified fatty acids (Wako Chemical, Neuss, Germany), and liver transaminases (Biotecnica, SP, Brazil) were assayed using enzymatic-colorimetric methods according to the manufacturers' instructions. Leptin, adiponectin (Merck Millipore, Darmstadt, Germany), C-reactive protein (IBL-America, Minneapolis, USA), TNFα, and IL-1β (R&D Systems, Minneapolis, USA) plasma concentrations were determined using ELISA. For analysis of IL-1β expression in liver tissue, 50-mg tissue samples were homogenized in 2 mL Lysis Buffer 2 (R&D Systems, Minneapolis, USA). IL-6 plasma levels were analyzed using a Multiplex Assay (Merck Millipore, Darmstadt, Germany). Protein carbonyl content in the liver was measured using a colorimetric assay kit (Cayman Chemical Company, Michigan, USA).

2.3. Levels of Reduced and Oxidized Glutathione in Liver and Plasma. Liver (50 mg) and plasma (50 μL) levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) were assayed separately according to the fluorometric orthophthaldehyde (OPT) method reported by Hissin and Hilf [23]. This method is based on the fact that OPT reacts with GSH and GSSG at pH 8.0 and pH 12, respectively, to yield a highly fluorescent product that can be activated at 350 nm with an emission peak of 420 nm. GSSG levels were determined after sample treatment with N-ethylmaleimide to ensure complete removal of GSH. The concentrations of GSH and GSSG in samples were calculated according to standard curves individually prepared with GSH and GSSG.

2.4. Liver Triglyceride Content. Liver lipids were extracted using the Folch [24] method. The lipid extracts were resuspended in Triton buffer (20 mL of 0.5 M potassium phosphate, pH 7.4, 0.25 M NaCl, 25 mM cholic acid, 0.5% Triton® X-100), and TG levels were determined using an enzymatic-colorimetric method according to the manufacturer's instructions (Chod-Pap; Roche Diagnostic GmbH, Mannheim, Germany).

2.5. Liver Histological Analysis. Liver tissue samples were taken from the left lobe and incubated in 10% phosphate-buffered formaldehyde at room temperature overnight. The samples were then washed 3 times with phosphate-buffered saline (PBS) and fixed in 70% ethanol. After fixation, the tissues were embedded in paraffin, sectioned to a thickness of 5 μm and stained with hematoxylin-eosin (HE).

2.6. Oil Red O Staining. Liver samples were fixed in 4% formaldehyde, washed with PBS, embedded in Tissue-Tek OCT embedding compound, and frozen. The frozen sections (10 μm thick) were rehydrated, and neutral lipid accumulation was detected by Oil Red O staining. Then, the sections were rinsed with 60% isopropanol and stained for 18 min with prepared Oil Red O solution (0.5% in isopropanol followed by dilution to 60% with distilled water and filtration). Slides were washed twice in 60% isopropanol and distilled water.

Table 1: Diet composition (g/100 g).

Diet	Protein	Fat	Carbohydrate	Fiber	Calories (Kcal/100 g)
Low fat	14	4 (9.5% of calories)	72	5	380
High fat	14	35 (59% of calories)	41	5	536

Note. The basis of both the low-fat and high-fat diets is AIN-93.

Digital images were taken with an Olympus BX51 microscope connected to an Olympus DP72 digital camera.

2.7. Immunofluorescence Microscopy. Liver sections were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature and then washed with PBS. Nonspecific binding was blocked by incubation with 5% bovine serum albumin (BSA) in PBS for 1 h. The sections were then incubated with primary antibodies specific for TNFR1 (1 : 50, Santa Cruz Biotechnology) or IL1β (1 : 100, Cell Signaling) overnight (4°C), followed by incubation with a primary antibody against CD68 (1 : 250, Serotec) for 3 h (room temperature). Next, the tissue sections were incubated with Alexa Fluor-labeled secondary antibodies (Invitrogen) for 1 h (room temperature). Pictures were taken on a Leica DMI600B microscope, and colocalized areas in random fields (1 per section/mouse) were analyzed using ImageJ software.

2.8. Analysis of Apoptosis. Apoptosis was analyzed via the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method using an in situ cell detection kit (Roche Diagnostics). Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI; Sigma Aldrich). Only TUNEL-positive cells that colocalized with DAPI-stained nuclei were considered apoptotic and counted. Random fields (5 per section) of 10 μm were counted for each mouse.

2.9. Oral Glucose Tolerance Test and Insulin Tolerance Test. For the oral glucose tolerance test (OGTT), after 12 hours of fasting, mice received an oral dose of glucose solution (1.5 g/kg body weight). Basal blood samples were collected from the tail tip before (t = 0 min) and 15, 30, 60, and 90 min after glucose ingestion. For the insulin tolerance test (ITT), mice were fasted for three 3 hours, and blood samples were collected immediately before IP insulin injection [0.75 U/Kg body weight of regular human insulin (Eli Lilly Co.)] and at 5, 10, 15, 30, and 60 min after injection for glucose analysis. Blood glucose concentrations were measured using a glucose analyzer (Accu-Chek Advantage, Roche Diagnostic, Switzerland).

2.10. Pyruvate-Derived Glucose Production Test. After 16 hours of fasting, mice were injected with a pyruvate solution (1.5 g/kg body weight). Blood samples were collected from the tail tip before (t = 0 min) and 15, 30, 60, and 90 min after the injection. Blood glucose concentrations were measured using a glucose analyzer (Accu-Chek Advantage, Roche Diagnostic, Switzerland).

2.11. Analysis of Liver Very Low-Density Lipoprotein-Triglyceride Secretion. After 12 h of fasting, basal blood samples were collected from mice via the tail tip (t = 0 min). Then, the mice received an IP injection of Triton WR 1339 (500 mg/kg in saline solution; Sigma) to inhibit lipoprotein lipase activity as well as TG hydrolysis and clearance. Additional blood samples were collected at 120 and 150 minutes after Triton injection. For analysis of very low-density lipoprotein-(VLDL-) TG secretion, plasma TG levels were determined using an enzymatic-colorimetric assay according to the manufacturer's instructions (Chod-Pap; Roche Diagnostic GmbH, Mannheim, Germany).

2.12. RNA Extraction and Real-Time Reverse Transcription PCR. Total liver RNA was extracted from approximately 50 mg of tissue using TRIzol reagent (Invitrogen, Grand Island, NY, USA). RNA integrity was assessed using Tris-borate 1.2% agarose gels stained with ethidium bromide. RNA quantity and purity were measured via optical density readings taken at 260 and 280 nm (Gene Quant, Amersham-Pharmacia Biotech). Genomic DNA contamination was excluded by running polymerase chain reaction (PCR) on the RNA samples. cDNA was prepared in duplicate from 2 μg of total RNA via reverse transcription using an Applied Biosystems High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions. Gene expression was determined using real-time reverse transcription polymerase chain reaction (RT-PCR) (Step One Real-time PCR System, Applied Biosystems, Foster City, CA, USA) with SYBRGreen PCR Master Mix and specific primers. The $\Delta\Delta$CT method was used to quantify gene expression. The threshold cycle was normalized to β-actin and then expressed relative to the control groups (see Table 2).

2.13. Western Blotting. Liver tissue samples were homogenized in urea lysis buffer (2 M thiourea, 5 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1% aprotinin, 2 mM PMSF, and 1% Triton-X 100), and protein concentrations were determined using the Bradford [25] method. Forty-microgram samples of protein lysate were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes and stained with Ponceau S (Sigma) to verify transfer efficiency and equal sample loading. The membranes were blocked with 5% albumin in Tris-HCl pH 7.6 containing 150 mM sodium chloride and 0.1% Tween-20 (TBST) and incubated for 2 hours at room temperature with antibodies against caspase-1 (1 : 500, Abcam), caspase-3 (1 : 100, Santa Cruz Biotechnology), Bcl2 (1 : 1000, Cell Signaling), or Bax (1 : 1000, Cell Signaling). An antibody against

Table 2: Primer sequences used for RT-PCR.

Genes		Primers
β-Actin	Forward	5′ GGACTCATCGTACTCCTGCTT 3′
	Reverse	5′ GAGATTACTGCTCTGGCTCCT 3′
ACC	Forward	5′ AGGCAGCTGAGGAAGTTGGCT 3′
	Reverse	5′ CGCTGCACAGAGCAGTCACG 3′
ACO	Forward	5′ TGTGACCCTTGGCTCTGTTCT 3′
	Reverse	5′ TGTAGTAAGATTCGTGGACCTCTG 3′
Adiponectin receptor-2	Forward	5′ ACGTTGGAGAGTCATCCCGTA T 3′
	Reverse	5′ CTCTGTGTGGATGCGGAAGAT 3′
Apo B	Forward	5′ GCGAGTGGCCCTGAAGGCTG 3′
	Reverse	5′ CCGTGGAGCTGGCGTTGGAG 3′
ATGL	forward	5′ TGTGGCCTCATTCCTCCTAC C 3′
	Reverse	5′ TCGTGGATGTTGGTGGAGCT 3′
ChREBP	Forward	5′ ACTCAGGGAATACACGCCTACAG 3′
	Reverse	5′ TCTTGGTCTTAGGGTCTTCAGGAA 3′
CPT1	Forward	5′ AGTGACTGGTGGGAGGAATA 3′
	Reverse	5′ CTTGAAGTAACGGCCTCTGT 3′
FAS	Forward	5′ GATATTGTCGCTCTGAGGCTGTTG 3′
	Reverse	5′ GGAATGTTACACCTTGCTCCTTGC 3′
MTP	Forward	5′ CATTCAGCACCTCCGGACTT 3′
	Reverse	5′GATACTGCTGTCACTTTTGAAATCCA 3′
PGC1α	Forward	5′ CCTGACACGGAGAGTTAAAGGAA 3′
	Reverse	5′ GATGGCACGCAGCCCTAT 3′
PPARα	Forward	5′ GCAGCTCGTACAGGTCATCA 3′
	Reverse	5′ CTCTTCATCCCCAAGCGTAG 3′
SCD1	Forward	5′ TGGGTTGGCTGCTTGTG 3′
	Reverse	5′ GCGTGGGCAGGATGAAG 3′
SREBP1c	Forward	5′ CCTGGTGGTGGGCACTGAAGC 3′
	Reverse	5′ GCGTCTGAAGGGTGGAGGGGT 3′
TNFα	Forward	5′ CCCTCCTGGCCAACGGCATG 3′
	Reverse	5′ TCGGGGCAGCCTTGTCCCTT 3′
UCP2	Forward	5′ AGCATGGTAAGGGACCAGTG 3′
	Reverse	5′ CAGTTCTACACCAAGGCTC 3′

tubulin was used as an internal control (1 : 20,000, Sigma). Then, the membranes were washed with TBST, incubated with horseradish peroxidase- (HRP-) conjugated secondary antibodies diluted 1 : 1000, and washed again. Reactions were developed using an enhanced chemiluminescence detection system (Pierce ECL Western Blotting Substrate, Thermo Scientific, Rockford, USA). Images were captured using ImageQuant LAS 400 Mini equipment, and band intensities were quantified via optical densitometry using ImageJ software.

2.14. Statistical Analysis. Results are presented as the mean ± standard error for the number of determinations (n) indicated. Statistical analysis was performed using two-way ANOVA followed by Bonferroni correction. Statistical significance was defined as $p \leq 0.05$.

3. Results

3.1. ApoCIII Overexpression Increases Diet-Induced Adiposity. Hypertriglyceridemic apoCIII-overexpressing mice were compared to control nontransgenic (NTg) littermates following consumption of either a LFD or a HFD. Morphometric and plasma biochemical parameters were assessed (Table 3). The HFD increased daily caloric intake, body mass, and white perigonadal adipose tissue mass but decreased relative liver mass in both groups. The apoCIII mice fed the HFD showed greater adiposity accompanied by increased leptin plasma levels. As expected, there was a genotype-dependent but diet-independent hyperlipidemic phenotype in the apoCIII mice, as shown by elevated plasma levels of TG, cholesterol and free fatty acids (Table 3).

Table 3: Food intake; body, liver, and adipose tissue masses; and fasting plasma concentrations of lipids, leptin, and inflammatory markers in NTg and apoCIII mice fed either a low-fat diet (LFD) or a high-fat diet (HFD) for 16 weeks.

	LFD		HFD	
	NTg	CIII	NTg	CIII
Food intake (kcal/mouse/day)	15.41 ± 1.2 (7)	14.6 ± 0.9 (7)	19.6 ± 0.8* (5)	21.8 ± 0.6* (5)
Body mass (g)	23.2 ± 0.3 (17)	24.0 ± 0.3 (16)	29.6 ± 1.1* (8)	29.5 ± 0.9* (6)
Liver (%body weight)	3.7 ± 0.0 (17)	3.8 ± 0.1 (17)	2.9 ± 0.1* (8)	3.2 ± 0.1* (8)
Perigonadal WAT (%)	1.2 ± 0.1 (10)	1.4 ± 0.1 (10)	3.0 ± 0.2* (8)	3.6 ± 0.1*# (8)
Plasma triglycerides (mg/dL)	71.4 ± 5.0 (7)	780.2 ± 53.6# (7)	81.5 ± 3.8 (7)	668.1 ± 52.8# (7)
Plasma cholesterol (mg/dL)	122.0 ± 7.8 (9)	150.3 ± 11.3 (7)	135.1 ± 4.9 (7)	199.5 ± 13.2# (8)
Nonesterified fatty acids (mEq/L)	0.24 ± 0.01 (10)	0.50 ± 0.04# (8)	0.30 ± 0.03 (6)	0.41 ± 0.02# (5)
Plasma leptin (pg/mL)	635.6 ± 156 (9)	526.0 ± 162 (7)	1712.8 ± 618* (6)	3033.4 ± 925* (7)
Plasma IL6 (pg/mL)	5.7 ± 0.6 (6)	6.5 ± 0.9 (6)	13.4 ± 0.9* (7)	21.03 ± 2.1*# (4)
Plasma C-reactive protein (ng/mL)	320.9 ± 31.2 (9)	563.1 ± 73.8# (7)	405.7 ± 45.8 (7)	628.6 ± 39.7# (8)

Mean ± SEM (n). *LFD versus HFD groups; #NTg versus apoCIII mice ($p < 0.05$; two-way ANOVA).

Next, we assessed several indicators related to the natural history of NAFLD, including lipid accumulation, redox imbalance, inflammation, and cell death.

3.2. Overexpression of ApoCIII Promotes Hepatic Steatosis and Liver Dysfunction. Overexpression of apoCIII resulted in increased liver lipid content independent of diet type. This was observed as macrovesicular steatosis in histological analyses performed using HE (Figure 1(a)) and Oil Red O staining (Figure 1(b)) and as increased liver TG content (38% in the LFD group and 28% in the HFD group) (Figure 1(c)). Confirming the presence of liver injury, the apoCIII mice showed increased plasma levels of the hepatic transaminase AST under both diets and of ALT under the HFD (Figures 1(d) and 1(e)).

3.3. Overexpression of ApoCIII Promotes Glucose Intolerance and Increases Hepatic Glucose Production following Consumption of a HFD. Disturbed glucose metabolism is associated with liver steatosis; therefore, we next evaluated glucose homeostasis in the mice. As shown in Figure 2, HFD consumption induced glucose intolerance in both groups, although the effect of the HFD was more potent in the apoC-III mice (Figure 2(a)), which showed a 12% increase in the area under the glycemic curve compared to the NTg group. The ITT results showed no diet or genotype effects (data not shown). However, in determining the pyruvate-derived liver glucose production rate (Figure 2(b)), we showed that the HFD-fed apoCIII mice had increased gluconeogenesis capacity. Together with the glucose intolerance exhibited by these mice, these results indicate that hepatic insulin resistance was present in the apoCIII mice fed the HFD.

3.4. Effects of ApoCIII Overexpression and HFD Consumption on Intracellular Lipid Metabolism-Related Gene Expression and Liver VLDL-TG Secretion. To identify the processes driving the observed increases in liver lipid content, we analyzed the expression of genes related to lipid uptake, synthesis, catabolism, and secretion (Figures 3 and 4). We observed that the mRNA expression of CD36, which is responsible for lipid uptake, was not significantly modulated by diet or genotype (Figure 3). In contrast, mRNA expression of ChREBP (carbohydrate response element binding protein), a transcription factor involved in lipogenesis and activated by glucose, increased only in the livers of the HFD-fed apoCIII mice. The HFD also increased the mRNA levels of SREBP1c (sterol response element binding protein) and one of its targets, ACC (acetyl-CoA carboxylase), independent of genotype. Expression of stearoyl-CoA desaturase-1 (SCD-1) was markedly reduced by the HFD (Figure 3(f)).

Expression levels of genes related to lipid catabolism and secretion are shown in Figure 4. The mRNA level of ATGL (adipose tissue triglyceride lipase), responsible for initial TG hydrolysis, was reduced by HFD consumption independent of genotype. Levels of CPT1 (carnitine palmitoyl acyl transferase) and UCP2 (mitochondrial uncoupling protein-2), which both accelerate fatty acid oxidation, were reduced in the LFD-fed apoCIII mice and in both HFD groups. PGC1α

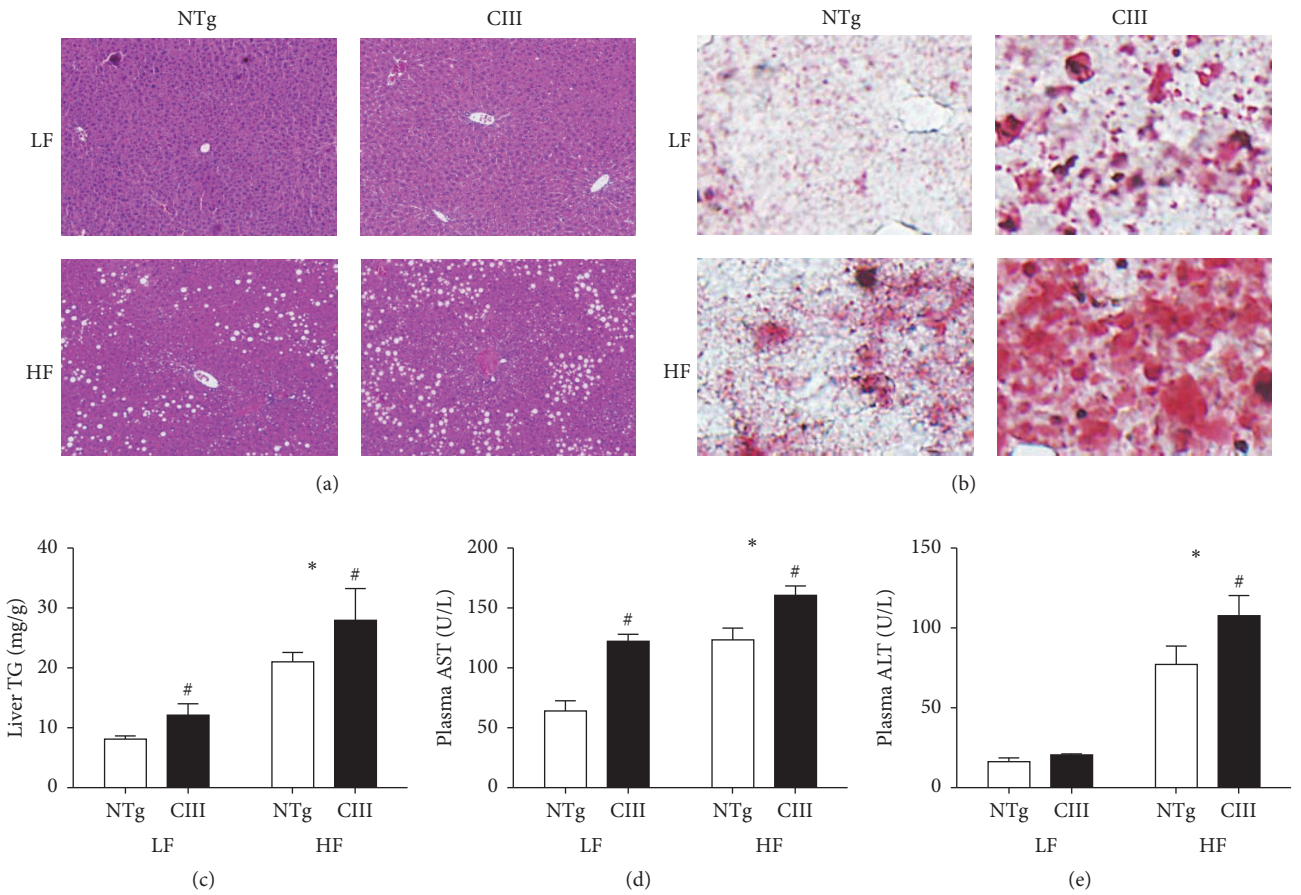

Figure 1: Overexpression of apoCIII promotes hepatic steatosis and dysfunction. NTg and apoCIII mice fed with low-fat (LF) or high-fat (HF) diets for 16 weeks. Representative liver sections isolated from mice stained with (a) HE and (b) Oil Red O. Note the macrovesicular lipid deposits, which appear as white spots in the HE-stained tissues (a), and the presence of lipids, which are stained in red (b). (c) Liver triglyceride content determined by enzymatic assay (n = 7-8). (d) Plasma concentrations of the transaminases ALT and AST (n = 5). Data are expressed as the mean ± SEM. *LF versus HF groups; $^{\#}$NTg versus apoCIII mice ($p < 0.05$; two-way ANOVA).

(PPAR gamma coactivator-1α and inducer of mitochondria biogenesis) levels were reduced by the HFD in both groups. VLDL assembly for subsequent TG secretion is a complex process, involving the association of lipids with apoB, which is mediated by MTP (microsomal triglyceride transfer protein). We observed a reduction in the mRNA abundance of MTP under the HFD in both groups, whereas apoB mRNA was reduced in the apoCIII mice under both diets. In summary, the HFD (independent of genotype) induced the expression of lipogenesis-related genes (SREBP1c and ACC) and decreased the expression of catabolism- and secretion-related genes (SCD1, ATGL, PGC1α, and MTP), while apoC-III overexpression (under the LFD) significantly reduced the expression of the CPT1, UCP2 and apoB100 genes.

To measure the actual liver TG secretion rates under the above experimental conditions, we performed a direct functional assay, as shown in Figure 5. The results show that liver VLDL-TG secretion rates increased in the apoCIII-overexpressing mice regardless of diet and that the HFD reduced VLDL-TG secretion rates in both apoCIII and NTg mice.

3.5. Overexpression of ApoCIII Increases the Ratio of Oxidized to Reduced Glutathione in Liver. Diet-induced NAFLD is associated with cell oxidative stress. Because GSH is one of the most abundant reducing power used to maintain cell redox homeostasis, we measured GSH levels in liver. As shown in Figure 6, overexpression of apoCIII increased the liver GSSG/GSH ratio, independent of diet type, although the HFD exacerbated this effect. Protein carbonylation, which is an indicator of oxidative damage of proteins, was increased by the HFD independent of genotype.

3.6. ApoCIII Overexpression Induces Liver Inflammation. Inflammation is a key event in the pathogenesis of NAFLD. Therefore, we measured a panel of systemic and liver proinflammatory markers in the mice. Increased plasma levels of the cytokine IL6 were present in both HFD groups, although greater increases were observed in the apoCIII mice. Another inflammatory marker, C-reactive protein, was significantly increased in the apoCIII mice, independent of diet type (Table 3). Plasma levels of adiponectin, an anti-inflammatory

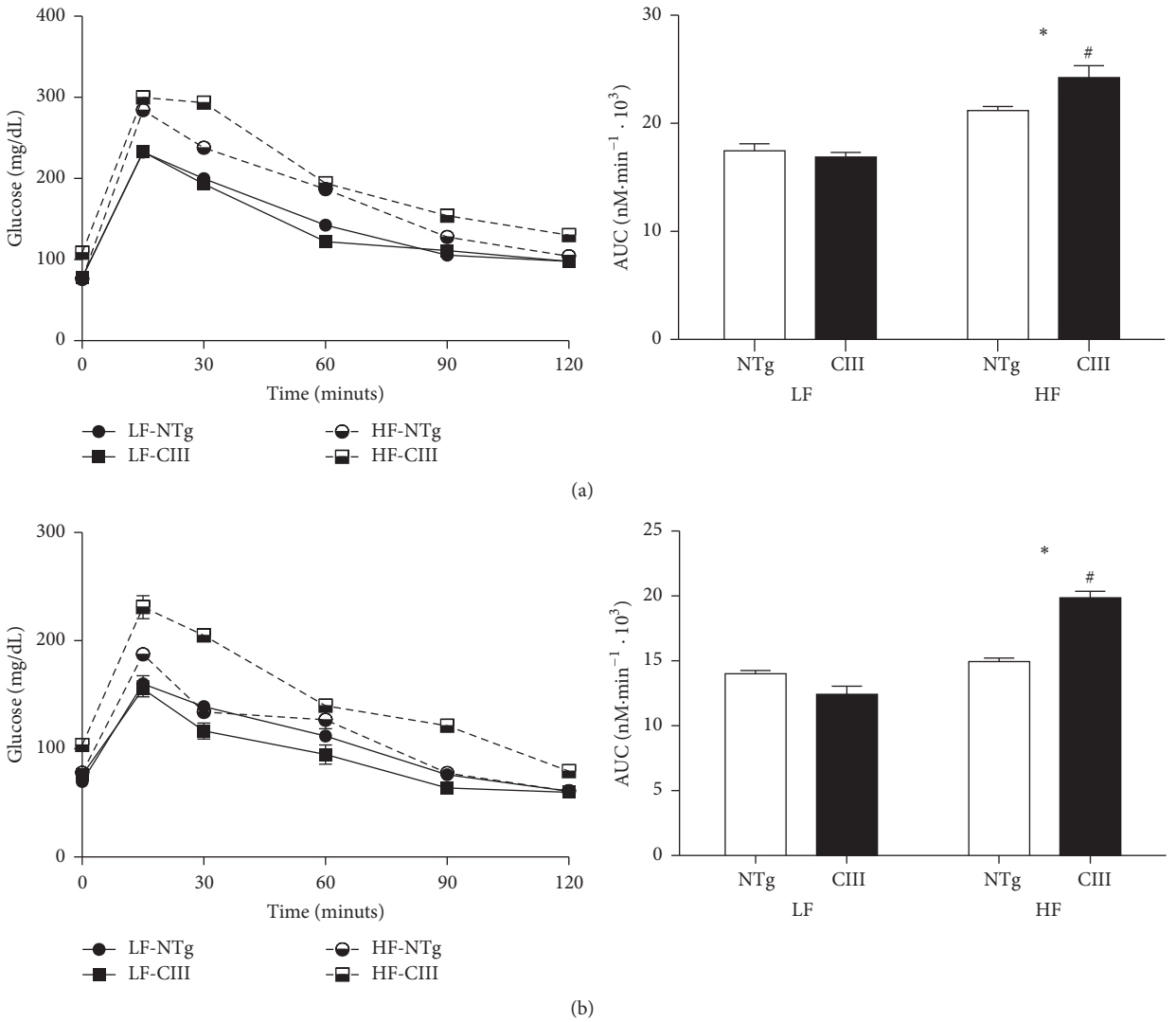

Figure 2: Overexpression of apoCIII promotes glucose intolerance and increases glucose hepatic production in mice consuming a high-fat diet. NTg and apoCIII mice were fed either a low-fat (LF) or high-fat (HF) diets for 16 weeks. (a) Glucose tolerance test results and area under the curve (AUC) (n = 7–9). (b) Pyruvate-derived glucose production and area under the curve (AUC) (n = 6–8). Data are expressed as the mean ± SEM. *LF versus HF groups; #NTg versus apoCIII mice ($p < 0.05$; two-way ANOVA).

mediator, were diminished by the HFD in both genotypic groups (Figure 7(a)). The levels of liver adiponectin receptor 2 were also reduced by the HFD and in the apoCIII mice, independent of diet type (Figure 7(b)). Therefore, adiponectin signaling appears to be hampered both by HFD consumption and apoCIII overexpression.

The HFD induced elevation of plasma TNFα levels, and this effect was more pronounced in the apoCIII mice (Figure 7(c)). Furthermore, liver TNFα mRNA levels were increased in the apoCIII mice, independent of diet type (Figure 7(d)). Immunohistochemical analysis confirmed these findings, showing increased expression of the macrophage marker CD68 and colocalization of macrophages with cell-surface TNFα receptors in the livers of the apoCIII mice, independent of diet type (Figures 7(e) and 7(f)).

The inflammasome pathway appears to be activated in NAFLD. Activation of this pathway involves the formation and activation of a protein multicomplex that contains cysteine-aspartate protease-1 (caspase-1). Activated caspase-1 in turn activates and leads to the subsequent secretion of IL1β. Unlike the LFD, the HFD increased the levels of activated (cleaved) caspase-1, although the apoCIII mice exhibited increases in activated caspase-1 levels regardless of diet (Figure 8(a)). The same pattern was observed for IL1β: the HFD increased liver IL1β levels in both groups, but the apoCIII mice had higher IL1β levels independent of diet (Figure 8(b)). In the plasma, we observed that only the HFD affected IL1β levels (Figure 8(c)). Immunohistochemical analysis revealed that IL1β colocalized with macrophages in the livers of the apoCIII mice, independent of diet type (Figures 8(d) and 8(e)).

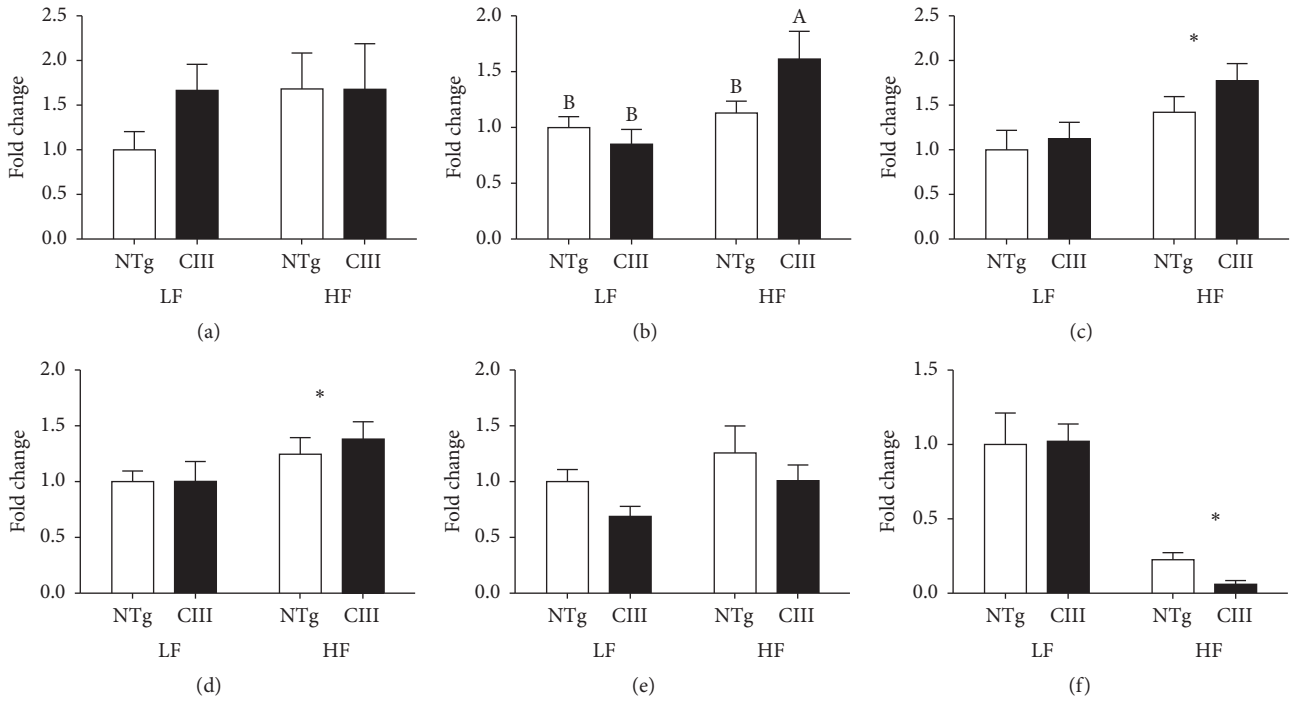

Figure 3: Effects of apoCIII overexpression and high-fat diet consumption on the expression of lipogenesis-related genes. mRNA expression levels for (a) CD36, (b) ChREBP, (c) SREBP1c, (d) ACC, (e) FAS, and (f) SCD1 in the livers of NTg and apoCIII mice fed either a low-fat (LF) or high-fat (HF) diets were analyzed and normalized to β-actin ($n = 6$–8). Data are expressed as the mean ± SEM. *LF versus HF group; $^{\#}$NTg versus apoCIII mice ($p < 0.05$; two-way ANOVA). ^{A,B}Mean values with nonmatching superscript letters are significantly different ($p < 0.05$; ANOVA followed by Bonferroni correction).

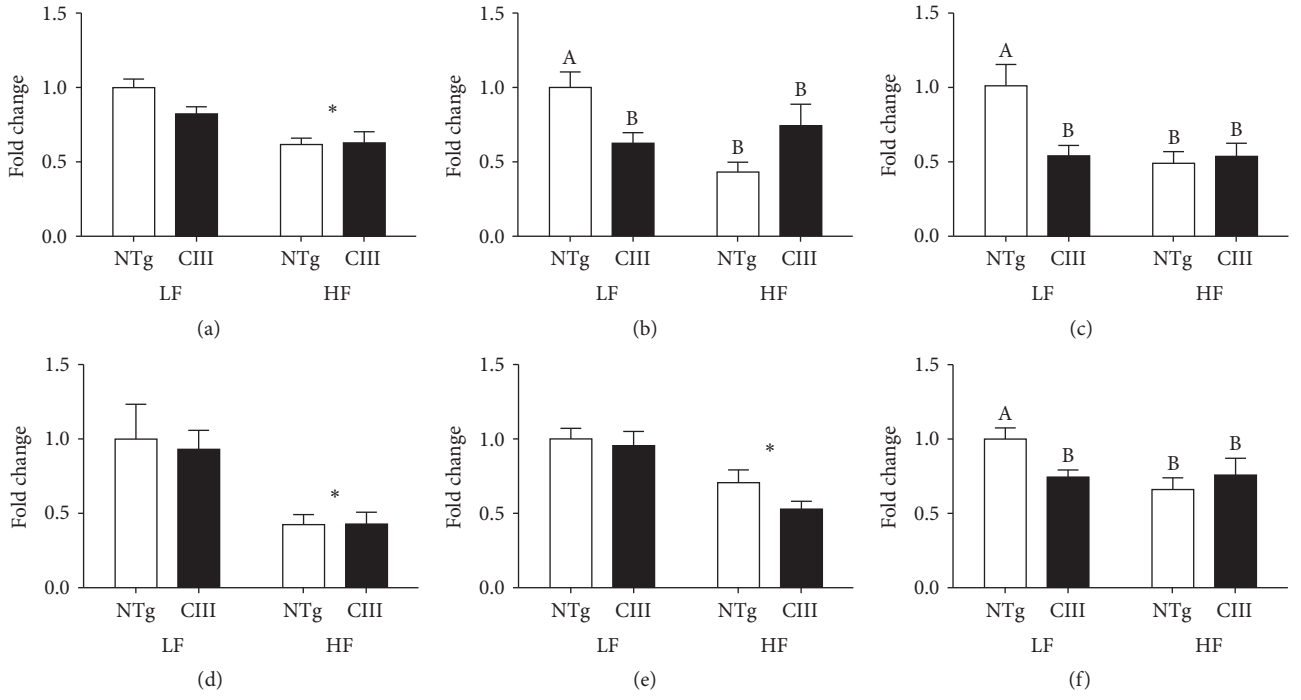

Figure 4: Effects of apoCIII overexpression and high-fat diet consumption on the expression of genes related to lipid catabolism and secretion. mRNA expression levels for (a) ATGL, (b) CPT1, (c) UCP2, (d) PGC1α (d), (e) MTP, and (f) APOB100 in the livers of NTg and apoCIII mice fed either a low-fat (LF) or high-fat (HF) diets and normalized to β-actin ($n = 6$–8). Data are expressed as the mean ± SEM. *LF versus HF group; $^{\#}$NTg versus CIII mice ($p < 0.05$; two-way ANOVA). ^{A,B}Mean values with nonmatching superscript letters are significantly different ($p < 0.05$; ANOVA followed by Bonferroni correction).

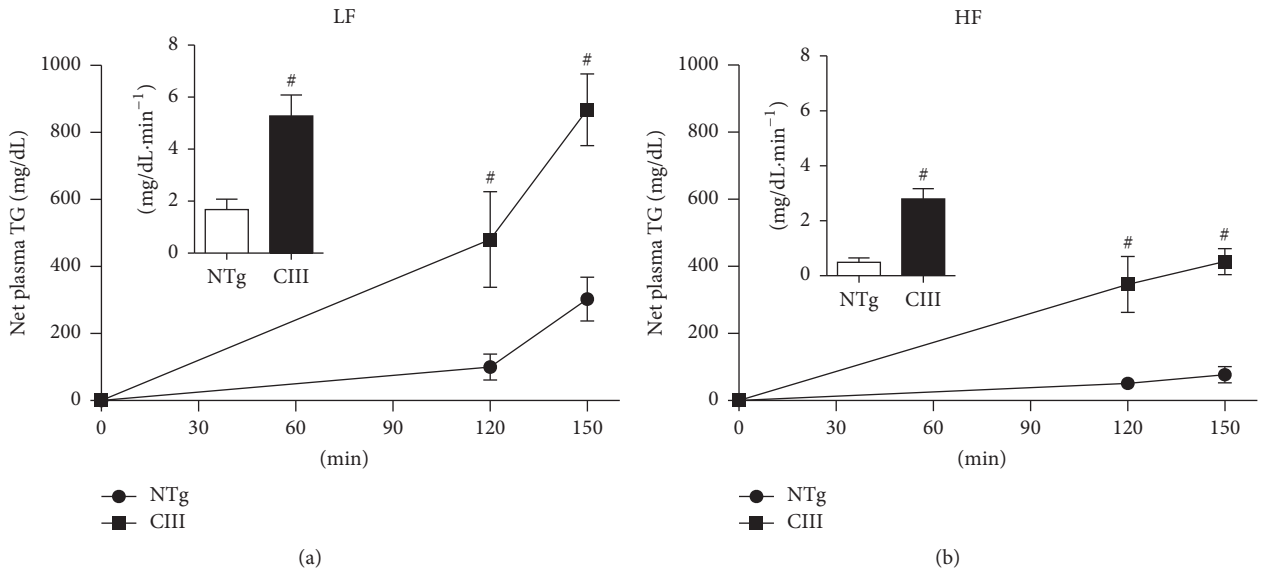

Figure 5: Overexpression of apoCIII increases liver VLDL-TG secretion. Net increases in plasma triglyceride concentrations after intraperitoneal injection of Triton WR 1339 (500 mg/kg) and curve slopes (inserts) for NTg and apoCIII mice fed either a low-fat (LF) or high-fat (HF) diets (n = 3–5). Data are expressed as the mean ± SEM. $^{\#}$NTg versus apoCIII mice ($p < 0.05$; Student's t test).

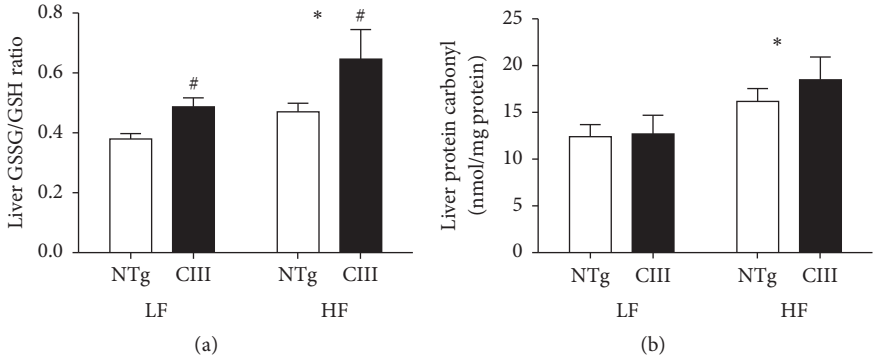

Figure 6: Effects of apoCIII overexpression and high-fat diet consumption on liver oxidative stress and damage. (a) Liver ratio of oxidized to reduced glutathione (n = 7–9) and (b) levels of carbonylated proteins (n = 6–8) in NTg and apoCIII mice fed either a low-fat (LF) or high-fat (HF) diets for 16 weeks. Data are expressed as the mean ± SEM. *LF versus HF group; $^{\#}$NTg versus apoCIII mice ($p < 0.05$; two-way ANOVA).

3.7. ApoCIII Overexpression Induces Liver Apoptosis. Apoptosis is present at a more advanced stage of the disease (steatohepatitis, NASH), which involves both intrinsic and extrinsic cell death pathways. As shown in Figure 9(a), the apoCIII mice exhibited activation of the intrinsic apoptosis signaling pathway, demonstrated by the decreased Bcl2/Bax ratio. The HFD also diminished this ratio in the NTg group. To confirm the occurrence of cell death, we measured the levels of caspase-3, the final death effector, and counted apoptotic cells using a TUNEL assay. Cell death was more prominent in the apoCIII mice (independent of diet) and was further increased by the HFD as evidenced by the high levels of active (cleaved) caspase-3 and high number of TUNEL-positive apoptotic cells (Figures 9(b), 9(c), and 9(d)).

3.8. Fibrate Treatment Resolves ApoCIII-Induced Intracellular Lipid Accumulation but Not Inflammation. We next investigated whether NASH could be reversed to the same extent among the different groups of mice. To accomplish this, mice were treated with fenofibrate during the last 2 weeks of HFD consumption. By activating PPARα, this drug modulates the expression of many genes that regulate lipid metabolism, mainly by decreasing synthesis and increasing catabolism. Fibrates are also known to reduce apoCIII expression. As shown in Table 4, fenofibrate treatment reduced body weight, adipose tissue weight, and leptin plasma levels in both HFD groups. Plasma TG, cholesterol, and FFA levels were not affected by fenofibrate treatment and remained elevated in the apoCIII mice. Fenofibrate did not alter PPARα mRNA

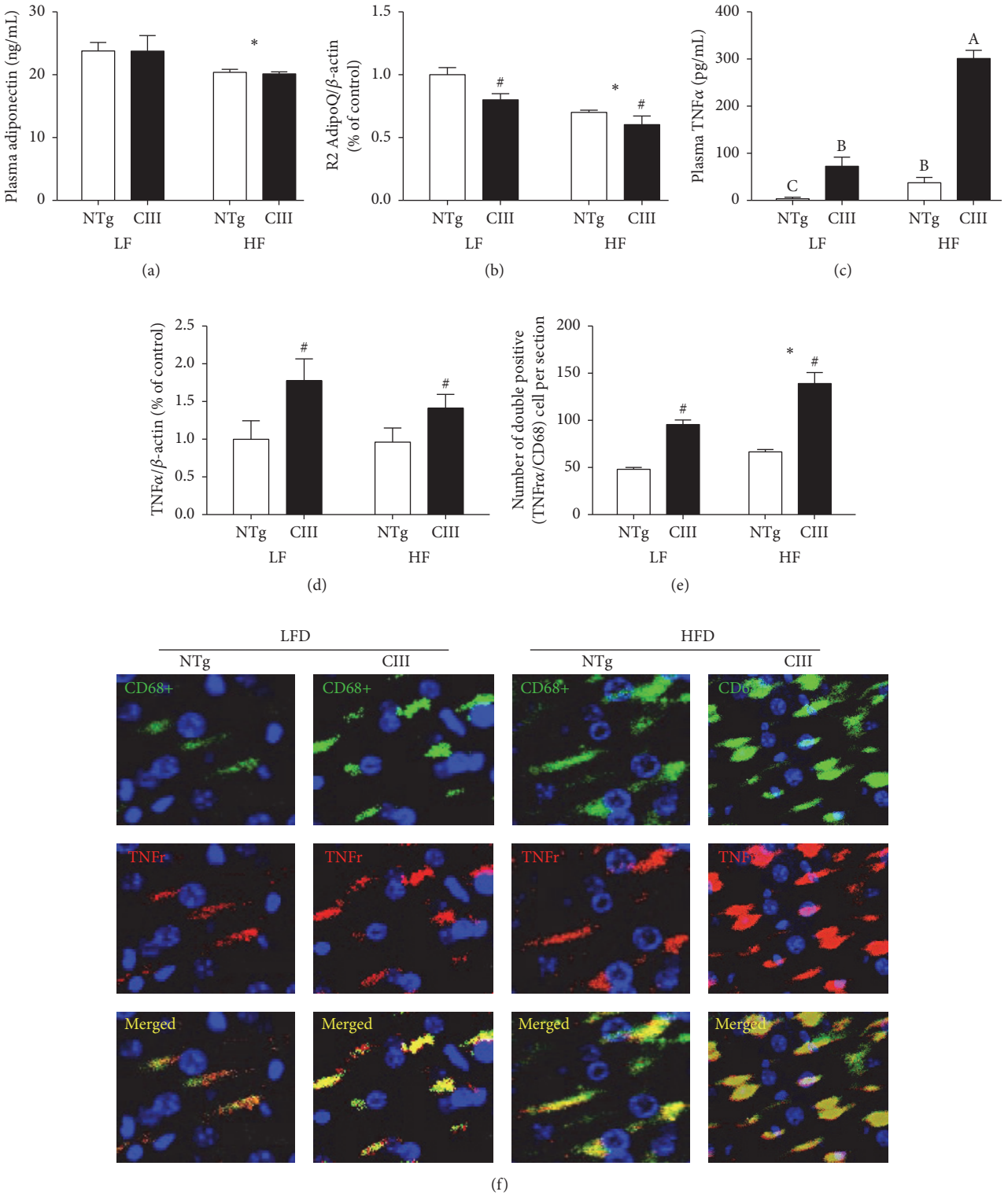

FIGURE 7: Effects of apoCIII overexpression and high-fat diet consumption on plasma and liver inflammatory markers. (a) Plasma adiponectin (n = 7–9), (b) liver adiponectin receptor 2 mRNA (n = 7–9), (c) plasma TNFα (n = 5-6), (d) liver TNFα mRNA (n = 6–8), (e) quantification of TNFα and CD68 colocalized areas (n = 3), and (f) representative immunofluorescent confocal microscopy images of TNFα and CD68 in liver sections from NTg and apoCIII mice fed either a low-fat (LF) or high-fat (HF) diets. Data are expressed as the mean ± SEM. *LF versus HF group; $^{\#}$NTg versus apoCIII mice ($p < 0.05$; two-way ANOVA). ^{A,B,C}Mean values with nonmatching superscript letters are significantly different ($p < 0.05$; ANOVA followed by Bonferroni correction).

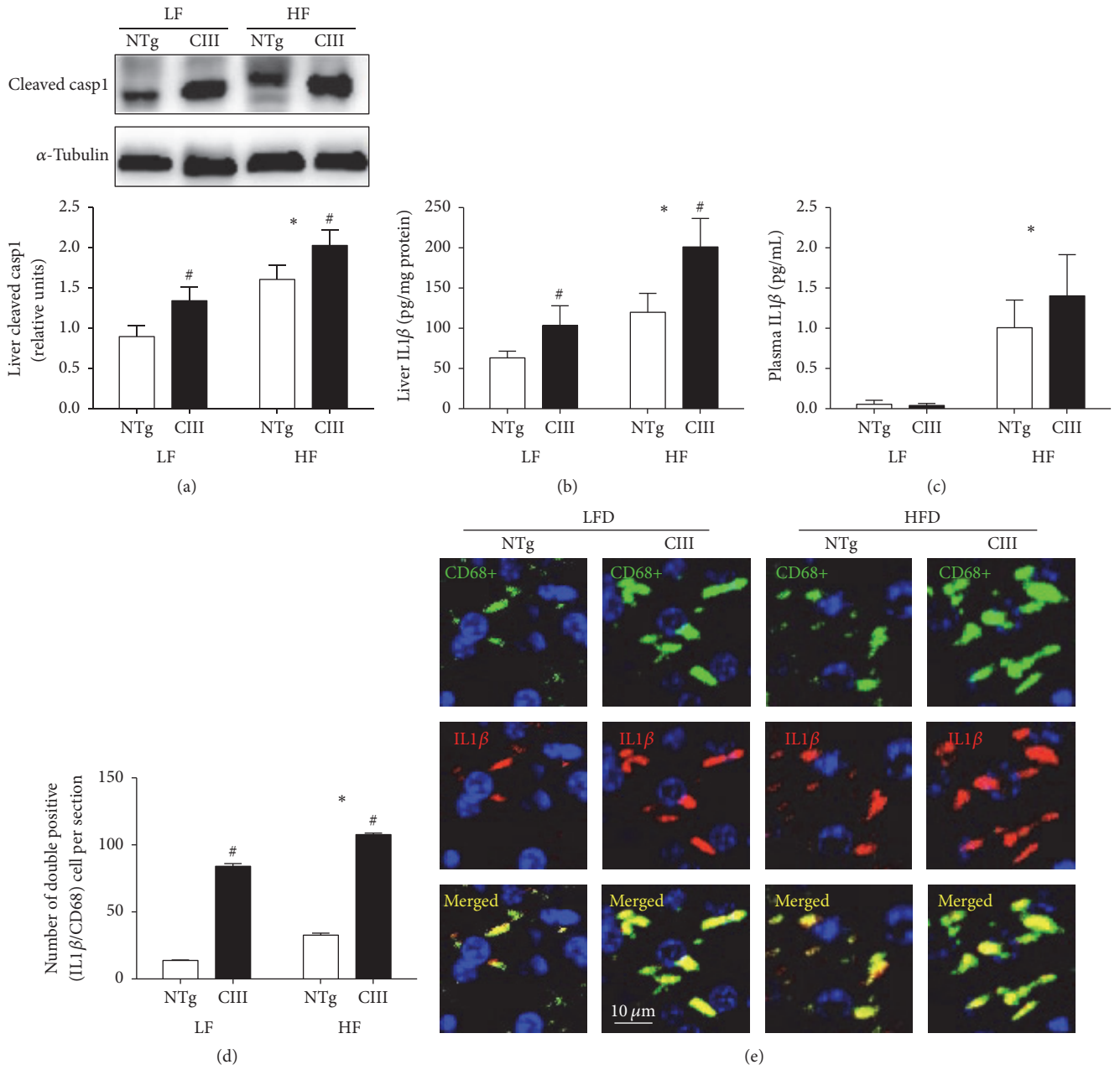

FIGURE 8: Effects of apoCIII overexpression and high-fat diet consumption on plasma and liver inflammatory markers. (a) Liver cleaved caspase-1 ($n = 5$), (b) liver IL1$\beta\beta$ ($n = 4$), (c) plasma IL1$\beta\beta$ ($n = 4$-5), (d) quantification of IL1$\beta\beta$ and CD68 colocalized areas, and (e) representative immunofluorescent confocal microscopy images of IL1$\beta\beta$ and CD68 in liver sections ($n = 3$) from NTg and apoCIII mice fed either a low-fat (LF) or high-fat (HF) diets for 16 weeks. Data are expressed as the mean ± SEM. *LF versus HF groups; #NTg versus apoCIII mice ($p < 0.05$; two-way ANOVA).

expression, but it reduced FAS mRNA expression and increased ACO, CPT1, and UCP2 mRNA expression in both HFD groups (Table 5). Fenofibrate also markedly reduced liver TG content. In addition, fenofibrate decreased plasma levels of the hepatic transaminases AST and ALT, although AST levels remained elevated in the apoCIII mice (Table 5).

Fenofibrate treatment was also effective in attenuating both oxidative stress and inflammation (Table 5). GSSG/GSH ratio and carbonyl content were both reduced by fenofibrate, although the latter remained elevated in the apoCIII mice. Additionally, plasma TNFα levels were reduced by the drug treatment but remained higher in the apoCIII mice. Plasma adiponectin levels were not altered by fenofibrate, whereas the levels of liver adiponectin receptor 2 were significantly increased only in the NTg mice. Fenofibrate also reduced plasma levels of IL6, but not C-reactive protein (Table 5).

Levels of cleaved caspase-1 (Figure 10(a)) in the liver as well as plasma and liver levels of IL1β (Table 5) were diminished by treatment with fenofibrate. However, liver IL1β levels (Figure 10(b)) remained elevated in the apoCIII mice

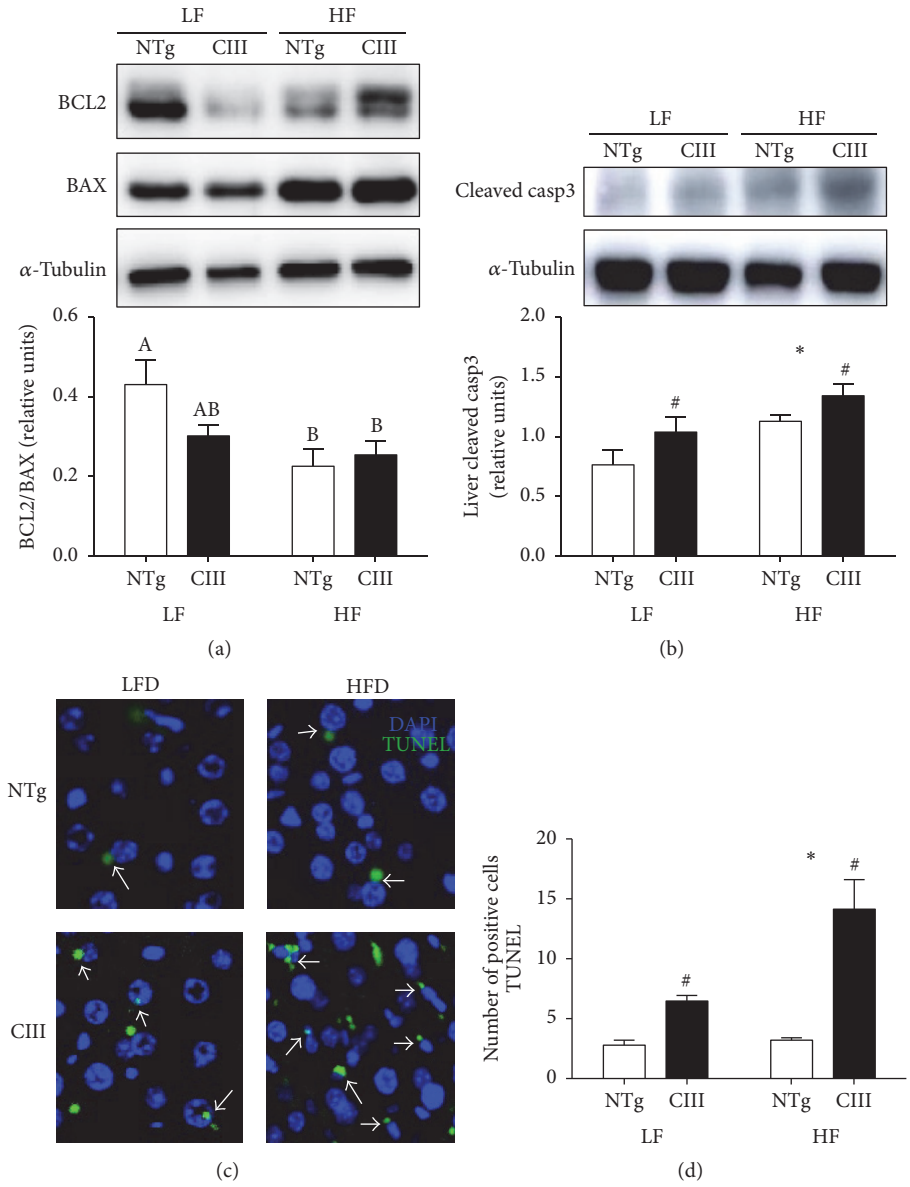

Figure 9: Effects of apoCIII overexpression and high-fat diet consumption on liver cell death. (a) Ratio between the anti- and proapoptotic proteins BCL2 and BAX (n = 7–9), (b) cleaved (activated) caspase-3 (n = 5-6) and number of apoptotic cells (TUNEL-positive cells), and (c) representative images and (d) quantification (n = 3) in NTg and apoCIII mice fed either a low-fat (LF) or high-fat (HF) diets for 16 weeks. Data are expressed as the mean ± SEM. *LF versus HF group; #NTg versus apoCIII mice ($p < 0.05$; two-way ANOVA). A,B,ABMean values with nonmatching superscript letters are significantly different ($p < 0.05$; ANOVA followed by Bonferroni correction).

independent of treatment. Fenofibrate also increased the Bcl2/Bax ratio (Figure 10(c)) and reduced activated caspase-3 levels in both NTg and apoCIII mice compared to nontreated counterparts. Confirming these results, there was a reduction in the number of TUNEL-positive cells in both fenofibrate-treated groups (Figures 10(d) and 10(e)).

4. Discussion

In the present study, we demonstrated that overexpression of apoCIII results in NAFLD, independent of diet. The development of NAFLD was characterized by increases in liver TG content, oxidative stress, inflammation, and cell death. The condition was aggravated and additional NAFLD features were induced, such as impaired glucose tolerance and hepatic insulin resistance, in apoCIII mice consuming a HFD. Furthermore, inflammatory indicators and apoptotic cell numbers were augmented in the HFD-fed apoCIII mice. As expected, fenofibrate treatment reversed several of the effects associated with HFD consumption and apoCIII overexpression. However, it is important to emphasize that fenofibrate did not normalize apoCIII-induced inflammatory traits, such as increased plasma TNFα levels and augmented liver TNFr and IL1β levels. These findings suggest that persistent

Table 4: Food intake; body, liver, and adipose tissue masses; and fasting plasma concentrations of lipids and leptin in NTg and CIII mice fed a high-fat diet (HFD) and treated or not with fenofibrate (HFD/F).

	HFD		HFD/F	
	NTg	CIII	NTg	CIII
Food intake (kcal/animal/day)	19.6 ± 0.8 (5)	20.8 ± 0.6 (5)	20.1 ± 0.7 (4)	20.1 ± 0.8 (4)
Body weight (g)	28.8 ± 0.5 (9)	31.6 ± 2.2 (6)	27.4 ± 0.8$^{\$}$ (6)	28.2 ± 0.3$^{\$}$ (6)
Liver (% body weight)	3.2 ± 0.1 (7)	3.0 ± 0.1$^{\#}$ (8)	3.6 ± 0.1$^{\$}$ (6)	3.4 ± 0.1$^{\#\$}$ (6)
Perigonadal WAT (%)	3.0 ± 0.3 (6)	3.5 ± 0.3 (7)	1.8 ± 0.2$^{\$}$ (6)	2.1 ± 0.4$^{\$}$ (6)
Plasma triglycerides (mg/dL)	83.2 ± 4.9 (7)	693.0 ± 28.3$^{\#}$ (7)	59.0 ± 4.7 (7)	508 ± 59.6$^{\#}$ (7)
Plasma cholesterol (mg/dL)	132.9 ± 5.2 (6)	187.2 ± 14.2$^{\#}$ (6)	154.3 ± 11.6 (6)	232.7 ± 30.6$^{\#}$ (6)
Nonesterified fatty acids (mEq/L)	0.33 ± 0.03 (7)	0.40 ± 0.01$^{\#}$ (8)	0.25 ± 0.02 (6)	0.48 ± 0.05$^{\#}$ (6)
Plasma leptin (pg/mL)	1783.4 ± 143 (6)	2011.5 ± 266 (5)	644.69 ± 57$^{\$}$ (6)	704.1 ± 52$^{\$}$ (6)

Mean ± SEM (n). $^{\$}$HFD versus HFD/F groups; $^{\#}$NTg versus apoCIII mice ($p < 0.05$; two-way ANOVA).

hypertriglyceridemia might be more relevant to liver inflammation than intracellular lipid accumulation.

A previous study showed that increased plasma apoCIII concentrations predispose mice to diet-induced NAFLD [21]. These authors focused on the insulin resistance observed in this mouse model when fed a HFD. Severe hepatic insulin resistance was characterized using the hyperinsulinemic-euglycemic clamp technique and was attributed to increased hepatic diacylglycerol content and protein kinase C-epsilon activation and decreased insulin activation of Akt2 [21]. Here, we confirmed that HFD consumption induces insulin resistance more severely in apoCIII mice than in NTg mice, as shown by the simultaneous glucose intolerance and increased gluconeogenesis that developed in the former group. We also confirmed a recent find from our group that apoCIII overexpression aggravates diet-induced obesity [26]. We have previously shown that apoCIII mice exhibit normal glucose homeostasis and insulin secretion when fed a normal diet, and these parameters were disturbed following acute increases in plasma FFA levels induced by heparin [27] or HFD consumption [28].

We showed that apoCIII overexpression increases VLDL-TG secretion regardless of diet type. However, this secretion was not enhanced enough to avoid the accumulation of lipids in the livers of the apoCIII mice. Reductions in the expression of genes related to lipid catabolism (CPT1 and UCP2) were observed in the apoCIII mice, which may explain, at least in part, their higher net liver lipid accumulation. The HFD reduced VLDL-TG secretion rates in both apoCIII and NTg mice, which explains the exacerbations in lipid accumulation that were observed in mice fed this diet. Lee et al. [21] reported increased FFA and TG liver uptake in apoCIII mice regardless of diet type. Others have found that FFA uptake from plasma is increased in liver and skeletal muscle but decreased in adipose tissue in obese subjects with NAFLD compared to obese subjects with normal intrahepatic triglyceride content [29]. Although we found no differences in FAT/CD36 mRNA expression between the apoCIII and NTg mice, it is likely that FFA uptake increased in the apoCIII mice under both diets due to the greater availability of TG and FFA in the plasma of these mice. Interestingly, we found that the HFD induced the expression of ChREBP mRNA specifically in the apoCIII mice. Hepatic de novo lipogenesis is regulated through activation of both SREBP-1c [30] and ChREBP [31], which transcriptionally activate nearly all genes involved in this process. Thus, lipogenesis may also contribute to liver steatosis in apoCIII mice. In addition to increased total TG content, in a previous study, we compared the liver lipidomes of apoCIII and NTg mice fed a regular diet. We demonstrated higher incorporation of oleic acid in phosphatidylcholine and TGs, higher content of phosphatidylinositol-containing arachidonic acid, and distinct overall FFA profiles in the livers of apoCIII mice, which showed elevated relative abundances of oleic (18 : 1), palmitoleic (16 : 1), arachidonic (20 : 4), margaric (17 : 0), and stearic (18 : 0) acids [32]. It is generally accepted that, in the natural course of liver disease, lipid accumulation, either from diet or from de novo lipogenesis, is the triggering event (first hit of the two-hit hypothesis) that leads to lipotoxicity in hepatocytes [16, 33]. In hepatocytes, excessive lipid storage may directly contribute to organelle failure, including mitochondrial dysfunction and endoplasmic reticulum stress and may play a role in hepatic insulin resistance [34]. We previously examined hepatic oxidative status in apoCIII mice fed a regular diet. We found cell redox

Table 5: Plasma indicators of liver injury and inflammation, liver triglyceride content, oxidative stress indicators, and gene expression related to energy metabolism in NTg and CIII mice fed a high-fat diet (HFD) with or without fenofibrate treatment (HFD/F).

	HFD		HFD/F	
	NTg	CIII	NTg	CIII
Plasma AST (U/L)	123.3 ± 9.8 (5)	160.6 ± 7.7$^{\#}$ (5)	74.5 ± 13.7$^{\$}$ (5)	98.6 ± 11.8$^{\#\$}$ (5)
Plasma ALT (U/L)	77.3 ± 11.4 (5)	107.7 ± 12.5$^{\#}$ (5)	35.7 ± 5.8$^{\$}$ (4)	39.9 ± 10.9$^{\#\$}$ (4)
Plasma adiponectin (ng/mL)	20.2 ± 0.6 (5)	20.13 ± 0.3 (5)	19.86 ± 0.5 (5)	19.12 ± 0.2 (6)
Plasma TNFα (pg/mL)	37,51 ± 11.3 (4)	301.05 ± 17.5$^{\#}$ (4)	13.3 ± 7.0$^{\$}$ (4)	140.1 ± 7.0$^{\#\$}$ (4)
Plasma IL6 (pg/mL)	13.64 ± 1.3^{b} (5)	26.52 ± 2.1^{a} (5)	14.2 ± 1.8^{b} (5)	14.38 ± 1.4^{b} (5)
Plasma C-reactive protein (ng/mL)	415.2 ± 44.1 (5)	582.6 ± 32.5 (5)	586.8 ± 22.1 (5)	530.9 ± 29.0 (5)
Plasma IL1β (pg/mL)	1.00 ± 0.34 (4)	1.40 ± 0.51 (4)	0.53 ± 0.08 (4)	0.54 ± 0.07 (5)
Liver triglycerides (mg/g)	11.36 ± 1.1 (7)	14.0 ± 2.6 (8)	7.10 ± 0.8$^{\$}$ (6)	8.66 ± 1.5$^{\$}$ (6)
Liver GSSG/GSH ratio	0.53 ± 0.01 (6)	0.54 ± 0.02 (6)	0.49 ± 0.01$^{\$}$ (6)	0.47 ± 0.04$^{\$}$ (6)
Liver protein carbonyl (nmol/mg protein)	15.1 ± 1.5 (6)	17.2 ± 2.4$^{\#}$ (7)	7.1 ± 0.5$^{\$}$ (6)	12.2 ± 2.0$^{\#\$}$ (6)
Liver mRNA (% control)				
PPARα	1.0 ± 0.1 (5)	1.0 ± 0.1 (6)	1.1 ± 0.2 (5)	1.1 ± 0.2 (6)
FAS	1.0 ± 0.2 (8)	1.2 ± 0.1 (5)	0.39 ± 0.1$^{\$}$ (5)	0.45 ± 0.1$^{\$}$ (5)
CPT1	1.0 ± 0.1 (8)	0.62 ± 0.1 (8)	1.2 ± 0.2$^{\$}$ (6)	1.1 ± 0.2$^{\$}$ (6)
ACO	1.0 ± 0.3 (6)	1.1 ± 0.1 (6)	1.7 ± 0.1$^{\$}$ (6)	1.4 ± 0.2$^{\$}$ (6)
UCP2	1.0 ± 0.1 (6)	0.9 ± 0.1 (5)	2.0 ± 0.1$^{\$}$ (5)	2.0 ± 0.3$^{\$}$ (5)
Adiponectin R2	1.0 ± 0.1^{b} (8)	0.54 ± 0.05^{c} (6)	1.70 ± 0.2^{a} (6)	0.56 ± 0.1^{c} (6)

mRNA data normalized to beta-actin. Mean ± SEM (n) $^{\$}$HFD versus HFD/F groups and $^{\#}$NTg versus apoCIII mice ($p < 0.05$; two-way ANOVA). ^{a,b,c}Mean values with nonmatching superscript letters are significantly different ($p < 0.05$, ANOVA followed by Bonferroni correction).

imbalance, evidenced by increased total levels of carbonylated proteins and malondialdehydes and elevated GSSG/GSH ratios in the livers of apoCIII mice compared to NTg mice [20]. Moreover, we found that the origin of this oxidative stress was associated with higher activities of two oxidases, NADPH oxidase, and xanthine oxidase, whereas mitochondria actually produced lower amounts of H_2O_2 due to a mild uncoupling adaptation mediated by the opening of ATP-sensitive potassium channels [20, 35]. Here, we confirmed that the livers of apoCIII mice have lower levels of GSH regardless of diet type, while consumption of a HFD induced protein carbonylation in the livers of both apoCIII and NTg mice.

Recent findings have indicated that inflammation is a key process for both the initiation and progression of NAFLD. For instance, knockout of the caspase-1 gene, which blocks the inflammasome pathway, is sufficient to protect mice from diet-induced NASH [36]. Accordingly, the absence of interleukin 1α or 1β (both targets of caspase-1) inhibits the evolution of liver steatosis to steatohepatitis and liver fibrosis [37]. In addition, double knockout of TNFα receptor 1 and 2 protects mice against liver lipid accumulation and other features of NAFLD induced by consumption of a methionine/choline-deficient diet [38], whereas anti-TNFα antibody treatment was shown to decrease lipid content and JNK signaling pathway in HFD-fed ob/ob mice [39]. Huang et al. [40]

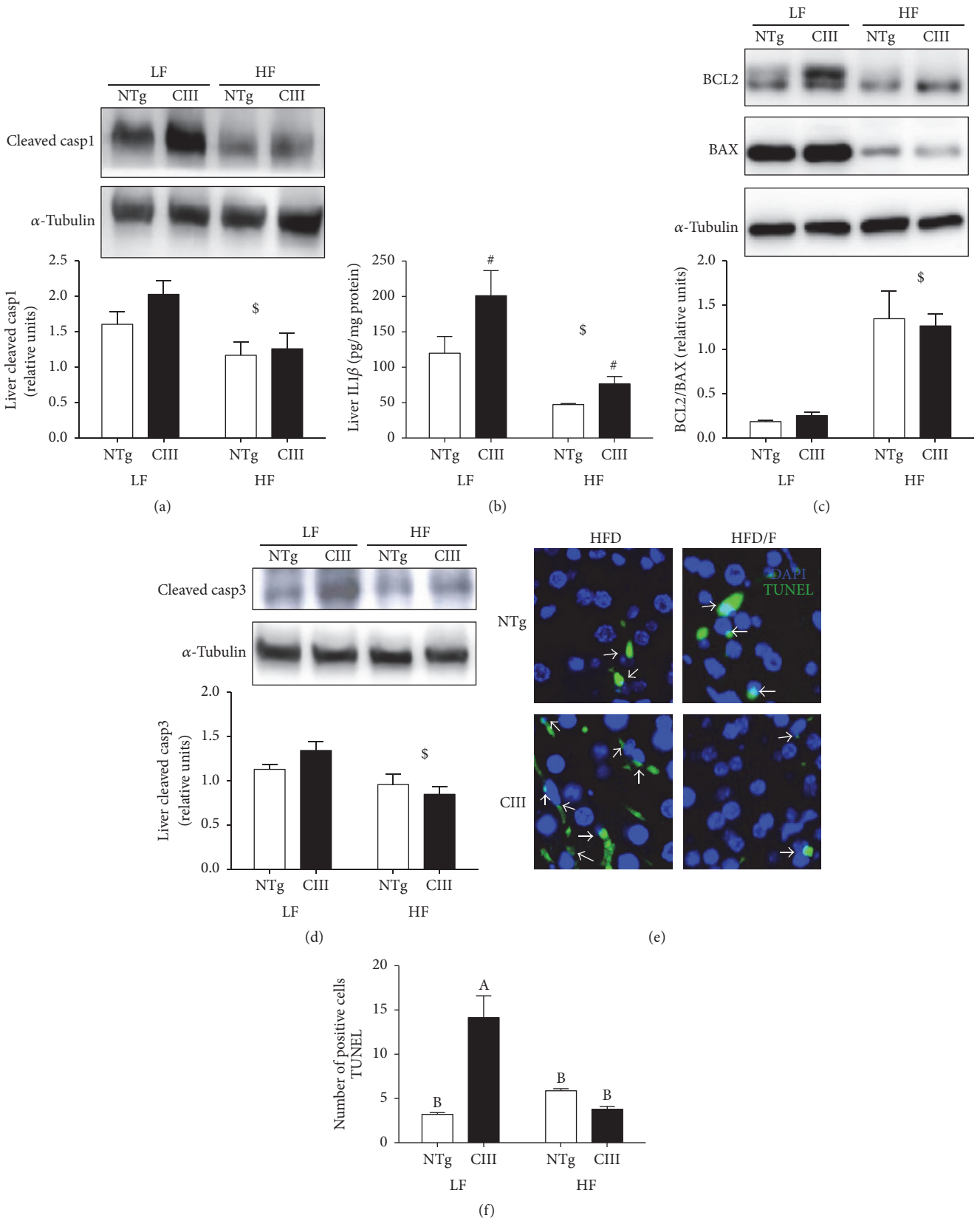

FIGURE 10: Effects of fenofibrate treatment on high-fat-diet-induced inflammation and apoptosis. (a) Liver cleaved caspase-1 ($n = 4$), (b) liver IL1$\beta\beta$ ($n = 4$), (c) liver BCL2/BAX ratio ($n = 7$–9), (d) liver cleaved caspase-3 ($n = 6$) and number of apoptotic cells (TUNEL-positive cells), and (c) representative images and (d) quantification ($n = 3$) from NTg and apoCIII mice fed either a low-fat (LF) or high-fat (HF) diets for 16 weeks. Data are expressed as the mean ± SEM. $^{\$}$LF versus HF group; $^{\#}$NTg versus apoCIII mice ($p < 0.05$; two-way ANOVA). ^{A,B}Mean values with nonmatching superscript letters are significantly different ($p < 0.05$; ANOVA followed by Bonferroni correction).

demonstrated that high-fat or high-sucrose diet-induced steatosis is preventable when the liver is depleted of Kupffer cells, and neutralizing antibodies against TNFα attenuate Kupffer cell-induced alterations in hepatocyte lipid metabolism. Our results support the central role of inflammation in NAFLD. In the present study, apoCIII mice fed a LFD exhibited a broad spectrum of liver inflammation, including activation of the TNFα, inflammasome, and adiponectin pathways. This inflammation persisted after fibrate treatment, which resolved intracellular lipid accumulation but did not correct the levels of proinflammatory effectors (TNFα, TNFα receptor, IL1β, and low adiponectin-R2).

There are likely multiple origins for the proinflammatory state in this model. It should be noted that none of the inflammatory markers/effectors that were measured in this work are tissue specific. They can be produced locally in the liver by activated Kupffer cells but are also derived from activated circulating immune and vascular cells as well as from adipose tissue, particularly enlarged visceral fat, which is an important source of many cytokines. Mild to moderate hypertriglyceridemia in healthy young men has been associated with increased concentrations of several biochemical markers of inflammation and endothelial activation and dysfunction [41]. This hypertriglyceridemia-associated systemic inflammation can be reversed to varying degrees by administration of fibrates, resveratrol or omega-3 fatty acids [42–44]. However, independent of triglyceride levels, apoCIII has inflammatory and cytotoxic effects [45]. For instance, purified human apoCIII enhances the attachment of monocyte-like cells to the human saphenous vein or coronary artery endothelial cells under both static and laminar shear stress conditions via induction of VCAM-1 [46]. In addition to inflammation, elevated circulating apoCIII levels may contribute to beta-cell apoptosis and diabetes [47–49], although this is controversial [50]. Supporting the finding that apoCIII is proapoptotic, we recently demonstrated that apoCIII-overexpressing spleen mononuclear cells present higher rates of apoptosis in vitro and that apoCIII mice have a reduced number of blood circulating lymphocytes. Additionally, cytochrome c release into cytosol and caspase-8 activity were both increased in apoCIII-overexpressing mononuclear cells, indicating that cell death signaling starts upstream of mitochondria but does involve this organelle [51]. Here, we showed that apoCIII mice exhibit marked increases in apoptotic cell numbers in the liver under low-fat (2-fold) and high-fat (5-fold) diets.

In conclusion, our findings show that, in addition to excessive hepatic lipid accumulation and insulin resistance, apoCIII overexpression-induced hypertriglyceridemia is associated with liver inflammation and cell death, which increase susceptibility to and the severity of diet-induced fatty liver disease.

Abbreviations

ACC: Acetyl-CoA carboxylase
ACO: Acetyl-CoA oxidase
AdipoQr2: Adiponectin receptor 2
ALT: Alanine aminotransferase
Apo B: Apolipoprotein B
Apo CIII: Apolipoprotein CIII
AST: Aspartate aminotransferase
ATGL: Adipose tissue triglyceride lipase
Bax: Bcl2-associated X protein
Bcl2: B-cell lymphoma 2
CD68: Cluster of differentiation 68
ChREBP: Carbohydrate response element binding protein
CPT1: Carnitine palmitoyl acyl transferase 1
CVD: Cardiovascular disease
DAPI: Diamidino-2-phenylindole
FAS: Fatty acid synthase
FFA: Free fatty acid
GSH: Reduced glutathione
GSSG: Oxidized glutathione
HFD: High-fat diet
IL-1β: Interleukin-1 beta
IL-6: Interleukin-6
ITT: Insulin tolerance test
LFD: Low-fat diet
MTP: Microsomal triglyceride transfer protein
NADPH: Nicotinamide adenine dinucleotide phosphate
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
NTg: Nontransgenic
OGTT: Oral glucose tolerance test
PBS: Phosphate-buffered saline
PGC1α: PPAR gamma coactivator 1 alpha
PPARα: Peroxisome proliferator-activated receptor alpha
RT-PCR: Real-time polymerase chain reaction
SCD1: Stearoyl-CoA desaturase-1
SREBP1c: Sterol response element binding protein
TG: Triglycerides
TNFr1: Tumor necrosis factor receptor 1
TNFα: Tumor necrosis factor
TUNEL: Deoxynucleotidyl-mediated deoxyuridine triphosphate end labeling
UCP2: Mitochondrial uncoupling protein-2
VCAM-1: Vascular cell adhesion molecule 1
VLDL: Very low-density lipoprotein.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Adriene A. Paiva and Helena F. Raposo contributed equally to this work.

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Research Article

Low-ω3 Fatty Acid and Soy Protein Attenuate Alcohol-Induced Fatty Liver and Injury by Regulating the Opposing Lipid Oxidation and Lipogenic Signaling Pathways

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Chronic ethanol-induced downregulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and upregulation of peroxisome proliferator-activated receptor gamma coactivator 1-beta (PGC1β) affect hepatic lipid oxidation and lipogenesis, respectively, leading to fatty liver injury. Low-ω3 fatty acid (Low-ω3FA) that primarily regulates PGC1α and soy protein (SP) that seems to have its major regulatory effect on PGC1β were evaluated for their protective effects against ethanol-induced hepatosteatosis in rats fed with Lieber-deCarli control or ethanol liquid diets with high or low ω3FA fish oil and soy protein. Low-ω3FA and SP opposed the actions of chronic ethanol by reducing serum and liver lipids with concomitant decreased fatty liver. They also prevented the downregulation of hepatic Sirtuin 1 (SIRT1) and PGC1α and their target fatty acid oxidation pathway genes and attenuated the upregulation of hepatic PGC1β and sterol regulatory element-binding protein 1c (SREBP1c) and their target lipogenic pathway genes *via* the phosphorylation of 5′ adenosine monophosphate-activated protein kinase (AMPK). Thus, these two novel modulators attenuate ethanol-induced hepatosteatosis and consequent liver injury potentially by regulating the two opposing lipid oxidation and lipogenic pathways.

1. Introduction

Alcohol liver disease is a major cause of morbidity and mortality, affecting millions world-wide [1]. Long-term exposure of ethanol causes fatty liver disease or hepatosteatosis [2], which further leads to steatohepatitis, fibrosis, and finally cirrhosis that may result in death [3]. Hepatosteatosis is characterized by the accumulation of lipids, triglyceride and cholesterol, due to an imbalance between hepatic lipid degradation and synthesis, leading to an enlarged fatty liver [3]. Studies have shown that alcohol causes the following: (i) increased mobilization of adipose fat into the liver, due to increased adipose lipoprotein lipase, (ii) decreased fat oxidation due to downregulation of fatty acid oxidation genes, (iii) increased fat synthesis due to upregulation of lipogenic genes, and (iv) impaired synthesis of apolipoprotein B and secretion of very low density lipoprotein (VLDL), the major lipoprotein for the export of hepatic lipids to peripheral tissues [4].

Transcriptional coactivators peroxisome proliferator receptor coactivator 1 alpha (PGC1α) and peroxisome proliferator receptor coactivator 1 beta (PGC1β) as well as sterol regulatory element-binding proteins (SREBPs) play vital roles in regulating the lipid oxidizing and lipogenic genes and thereby control the progression of hepatosteatosis and the consequent onset of fibrosis and other forms of liver injury [5, 6]. Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor super family that are ligand-dependent transcription factors. There are three isotypes, namely, PPARα, PPARβ, and PPARγ. Whereas PPARα is expressed in all tissues controlling the fatty acid oxidation pathway genes, PPARγ is primarily expressed in

adipose tissue and the liver, regulating the lipogenic pathway genes. PPARβ is found in many tissues although mainly in gut, kidney, and heart [7–9]. It is linked to colon cancer [10] but has not been well studied. PGC1α regulates lipid oxidation pathway genes *via* PPARα and PGC1β regulates lipogenic pathway genes *via* the sterol regulatory element-binding proteins SREB1a, SREB1c, and SREBP2 [11]. SREB1c predominantly regulates fatty acid biosynthesis while SREB1a and SREBP2 control cholesterol synthesis [3]. AMP activated protein kinase (AMPK) is known to be activated by phosphorylation to form phosphorylated AMPK (pAMPK), which, in turn, phosphorylates and inactivates acetyl CoA carboxylase (ACC) and the rate-limiting enzyme of lipogenesis [4, 12, 13]. PGC1α is controlled by silence regulator gene 1 (SIRT1), the eukaryotic equivalent of SIR2 gene in prokaryotes, and histone acetyltransferases (HAT) [14]. SIRT1 activates PGC1α by deacetylation while HAT inactivates PGC1α by acetylation [15]. On the other hand, SIRT1 destabilizes SREBP1c by deacetylation while HAT stabilizes SREBP1c by acetylation [16]. PGC1β is upregulated by dietary saturated fat and coactivates SREBP1c and liver X receptor (LXR) families of transcription factors leading to increased lipogenesis, lipoprotein transport, and VLDL secretion [17, 18]. Therefore, any modulator that can either activate PGC1α *via* the interplay between SIRT1 and histone acetyltransferase (HAT) or inactivate PGC1β/SREBP1c should be beneficial in preventing alcoholic hepatosteatosis and consequent liver injury.

Omega-3/6 fatty acids are polyunsaturated fatty acids (PUFA) obtained from fish and plant sources. The most common omega-3 PUFA are eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and alpha-linolenic acid (ALA). Whereas algae and oils from fish such as salmon, mackerel, and herring are rich in EPA and DHA, ALA is found in vegetable oils such as canola, flax seed oil, soybean oil, and nuts such as walnuts [19]. Soy proteins (SP) are found in soybean legume containing all 8 essential amino acids and very low saturated fat [20]. In recent times, both omega-3 PUFA and SP have received increased attention due to their beneficial effects against cardiovascular disease, obesity, type 2 diabetes, and certain cancers, among others [19, 21, 22]. Low omega 3 fatty acids (low-ω3FA) are known to have lipid lowering effects in humans [23] while SP lowers plasma and liver cholesterol and triglycerides in both animals and humans [24]. Studies have shown that SP prevents hyperinsulinemia and reduces the expression of LXRs and SREBP1c mRNAs in obese Zucker rat model [25–27]. However, the molecular mechanisms by which these dietary modulators can control the two transcriptional coactivators are yet to be explored. In this study, we demonstrate the novel actions of low-ω3FA and SP in inhibiting alcoholic hepatosteatosis by regulating two opposing vital pathway genes of lipid degradation and synthesis *via* PGC1α and PGC1β, respectively. Therefore, low-ω3FA and SP are potentially potent dietary modulators that seem to have these profound lipid lowering properties involving lipid catabolic and anabolic pathways. Moreover, low-ω3FA and SP stimulate AMPK phosphorylation and block ethanol-induced increased lipogenesis. Thus, this may be the first time a systematic approach is made to alleviate alcoholic hepatosteatosis by the combined effects of novel natural modulators that promise to intervene with both lipid oxidizing and lipogenic pathways.

2. Material and Methods

2.1. Animals. Wild-type (WT) female Wistar rats (~150 g body weight) from Charles River, Wilmington, MA, were housed in pairs per cage in plastic cages, in a temperature-controlled room, at 25°C with 12-hours light-dark cycle. All animals were fed a pelleted commercial diet (Purina Rodent Chow, number 500, TMI Nutrition, St. Louis, MO) during the first week of acclimation period after arrival. Experiments were performed according to the approved institutional animal care and use committee protocol. Female rats were randomly divided into 4 groups of 5 rats each and were pair-fed Lieber-DeCarli control or ethanol (EtOH) liquid diets (36% total fat calories) with high-ω3FA (14.1% of calories as ω3FA) or low-ω3FA (2.7% of calories as ω3FA) fish oil or EtOH with SP for 4 weeks.

2.2. Diets. The diets are isocaloric and their formulations are according to the modified method of Lieber and DeCarli [28] with the recommended normal nutrients, vitamins, and minerals according to AIN-93 diet [29]. Thus, 36% of the total energy of ethanol diet is from fat, 20% from protein, 36% from EtOH, and the rest from the carbohydrate. The corresponding isocaloric control diet has isoenergetic amounts of dextrin-maltose in place of EtOH. EtOH concentration in the liquid diet was gradually increased starting at 1% level on day 1 and reaching the 5% level over a 7-day period to allow the animals to adapt to EtOH in the diet. These diets are supplemented with 120 IU of tocopherol/L and 200 mg/L of tertiary-butyl hydroquinone as antioxidants as per AIN-93 diet recommendations [28, 29].

2.3. Lipid and Lipoprotein Analysis. Blood samples were collected and centrifuged at 3100 rpm using a Beckman J6M (Beckman Coulter, Indianapolis, IN) for 10 min at 4°C. Separated serum, plasma, and liver samples were frozen at −80°C until assayed. Liver lipids and high density lipoproteins (HDL) were extracted as previously described [30, 31]. Cholesterol was analyzed using Sigma diagnostic kit number 352 (Sigma-Aldrich, St. Louis, MO) according to the method of Allain et al. [32] and triglycerides were analyzed using Sigma diagnostic kit number 339 (Sigma-Aldrich, St. Louis, MO) according to the method of McGowan et al. [33]. All protein concentration determinations were done according to Bradford method [34] with bovine serum albumin (BSA) as the standard.

2.4. Isolation of Plasma HDL and Its Labeling with [^{3}H] Cholesteryl Oleate. HDL was isolated from various pooled groups of rat plasma according to Gidez et al. [30]. Protein concentration was determined colorimetrically using bovine serum albumin (BSA) as a standard [34]. HDL cholesterol content was measured according to Zlatkis and Zak [35]. HDL labeling with [^{3}H] cholesteryl oleate was performed according to Basu et al. [36], and the specific activity is expressed as dpm/mg HDL cholesterol.

2.5. Quantification of Hepatosteatosis by Oil Red O. Livers from various experimental groups were cut into small pieces and washed immediately with ice cold PBS and mounted on optimum cutting temperature (OCT) embedding compound in peel-a-way embedding molds (Electron Microscope Sciences, Hatfield, PA). Liver tissues were cryosectioned and stained with oil red O to measure accumulation of lipid using an automated histometric system (Image-Pro Plus 6.1, Media Cybernetics, Bethesda, MD) as described previously [37]. The data are expressed as average oil red O percentage area of lipid staining. Values are means ± SEM.

2.6. RNA Isolation and Real-Time RT-PCR. The total RNA was isolated from each liver using the Tri-Reagent (Molecular Research Center, Cincinnati, OH) as manufacturer's instructions. Isolated total RNA was reverse transcribed by in vitro transcription as described by the manufacturer (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed using a Bio-Rad iCycler using the SYBR green PCR mix (Bio-Rad, Hercules, CA). Typical real-time PCR reaction mixture included same amount of cDNA templates from RT, 10 pM of each primers, 10 μM of dNTPs, 3 mM of $MgCl_2$, 10x buffer, and 2 μ of high fidelity Taq DNA polymerase in a reaction volume of 50 μL with 0.1x SYBR Green I. The PCR conditions were 3 min at 95°C followed by 40 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 min. Each primer pair was first tested by regular PCR to be highly effective and specific for amplification. β-Actin was used as the standard housekeeping gene. Ratios of specific mRNA and actin mRNA expression levels were calculated by subtracting the threshold cycle number (Ct) of the target gene from the Ct of actin and raising 2 to the power of this difference. Ct values were defined as the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold. Target gene expressions were expressed relative to β-actin expression. The various primer pairs for indicated rat genes and transcription factors are listed in Supplemental Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/1840513.

2.7. Western Blot Analysis. Liver extracts from each experimental group were diluted into SDS-PAGE sample buffer [50 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 15 mM 2-mercaptoethanol, and 0.25% bromophenol blue] and electrophoretically resolved in Novex (Life Technologies, San Diego, CA) 4–20% denaturing polyacrylamide gels. Proteins are electrophoretically transferred to PVDF membrane and processed for immunodetection using the corresponding polyclonal primary antibodies for each of the above factors. After thorough washing, the primary antibody was detected with horse radish peroxidase conjugated secondary antibody specific to IgG of the respective primary antibody. Protein bands were visualized by chemiluminescence and quantified using FluorChem Imager (Alpha Innotech, CA). The nuclear extracts from each group were analyzed for the level of SIRT1, PGC1α, and PGC1β and the mature form of SREBP1c in the respective groups using the respective specific antibodies, while total protein extracts were analyzed for the levels of ACC, c-Met, AMPK, and pAMPK using respective specific antibodies. To determine the levels of acetylated-PGC1α, the liver nuclear extract from each group was initially immunoprecipitated with anti-PGC1α followed by immunoblotting with acetylated lysine antibody. The polyclonal antibodies for all the above transcription factors were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Cayman Chemicals (Ann Arbor, MI), and UpState Cell Signaling Solutions (Lake Placid, NY). The specificity of each antibody was verified before use for the above analyses.

2.8. Immunoprecipitation Analysis. Immunoprecipitation was performed as previously described [38]. To determine the levels of acetylated-PGC1α, the liver nuclear extract from each group was initially immunoprecipitated with anti-PGC1α (Abcam, Cambridge, MA), followed by immunoblotting with acetylated lysine antibody (Cell Signaling Technology, Danvers, MA).

2.9. Statistical Analysis. Experimental data were statistically analyzed, employing the paired and unpaired "*t*" tests on the control and the experimental values. The appropriate data were analyzed by one-way or two-way analysis of variance (ANOVA) at $p < 0.05$ followed by Tukey contrast to evaluate the true correlation between various parameters.

3. Results

3.1. Effects of Chronic Ethanol, Low-ω3FA, or SP on Serum and Liver Lipids and Hepatic Lipid Score. Serum cholesterol (Figure 1(a)) and triglycerides (Figure 1(b)) were significantly increased in EtOH group by 1.8-fold ($p < 0.05$) and 1.2-fold ($p < 0.05$), respectively, compared to control. Similarly, total liver cholesterol (Figure 1(c)) and triglycerides (Figure 1(d)) were also markedly increased in EtOH group by 3.9-fold ($p < 0.05$) and 4.1-fold ($p < 0.05$), respectively, compared to control. In contrast, dietary low-ω3FA or SP feeding to EtOH-fed groups significantly decreased serum and liver cholesterol and triglycerides to the level closer to that of the control group. Furthermore, the hepatic accumulation of lipids as measured by oil red O staining is markedly increased in EtOH group by 7.5-fold ($p < 0.001$) as compared to the control. This effect is significantly reduced after dietary administration of low-ω3FA and SP in the EtOH-fed group by 93% ($p < 0.05$) and 45% ($p < 0.05$), respectively (Figure 1(e)).

3.2. Effects of Low-ω3FA and SP on EtOH-Mediated Alterations in the Lipid Oxidation Pathway. Chronic EtOH leads to a significant decrease in fatty acid oxidation (48.7 ± 5.8 nmoles/g/h, $p < 0.05$) as compared to control (100 ± 8.6 nmoles/g/h). We further investigated whether the mechanisms of action of low-ω3FA and SP on EtOH-induced decrease in fatty acid oxidation are mediated *via* the regulation of the transcriptional coactivator PGC1α, SIRT1, and the downstream pathway. Figure 2(a) showed that low-ω3FA and SP treatment restored chronic EtOH-mediated 32% ($p < 0.05$) downregulation in SIRT1 mRNA by 85% ($p < 0.05$) and 80% ($p < 0.05$), respectively, as compared to EtOH group. EtOH also significantly downregulated PGC1α mRNA

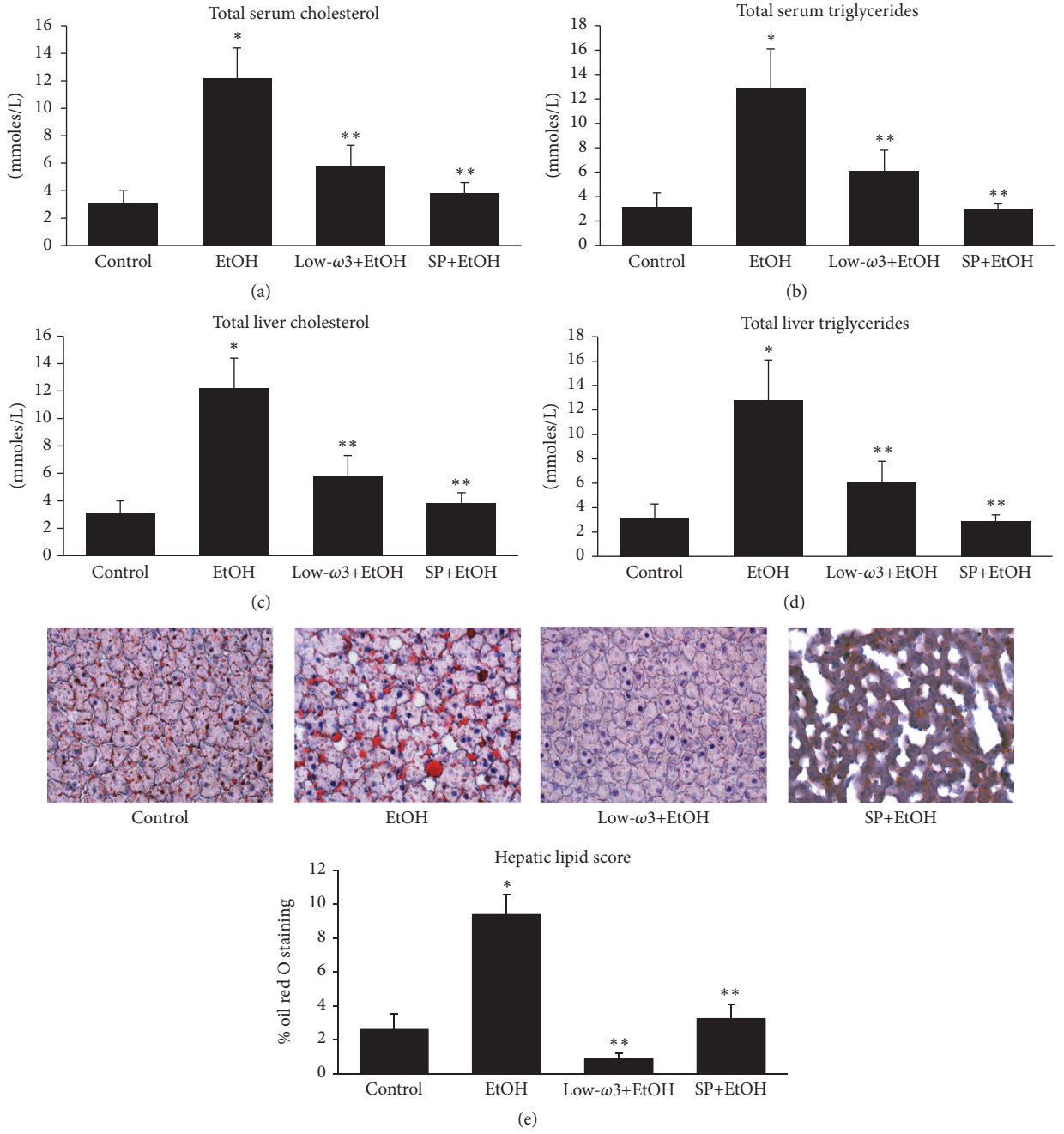

Figure 1: Influence of EtOH, low-ω3FA, and SP on (a) total serum cholesterol, (b) serum triglycerides, (c) total liver cholesterol, and (d) liver triglycerides. Each value is mean ± SD of 3 samples/group. (e) shows the representative medium-power (20x) photomicrographs of liver sections stained with oil red O as described in Section 2 and the plot of the mean hepatic lipid scores of all samples in each group ± SE of 3 samples/group. $^{*}p < 0.05$ versus control; $^{**}p < 0.05$ versus EtOH.

by 40% ($p < 0.05$) that was restored to 1.5-fold ($p < 0.05$) and 2-fold ($p < 0.05$) over the control level by low-ω3FA and SP treatment, respectively (Figure 2(b)). Additionally, CPT1 mRNA was also markedly downregulated by chronic EtOH (24%, $p < 0.05$) which was restored to 1.5-fold ($p < 0.05$) over the control level by these dietary modulators (Figure 2(c)). Similarly, chronic EtOH markedly decreased the nuclear protein expression of SIRT1 and PGC1α by 38% ($p < 0.05$) and 35% ($p < 0.05$), respectively, which was restored over the control levels by low-ω3FA and SP treatment (Figures 2(d) and 2(e)). PPARα, a ligand-activated transcription factor, involved in the regulation of hepatic fatty acid oxidation [39], was also evaluated. EtOH significantly decreased PPARα protein levels by 50% ($p < 0.05$) that was restored by 1.8-fold ($p < 0.05$) and 1.6-fold ($p < 0.05$) by low-ω3FA and SP treatment, respectively (see supplementary materials, Figure S1). Thus, low-ω3FA and SP are effective modulators in correcting the decreased fatty acid oxidation caused by

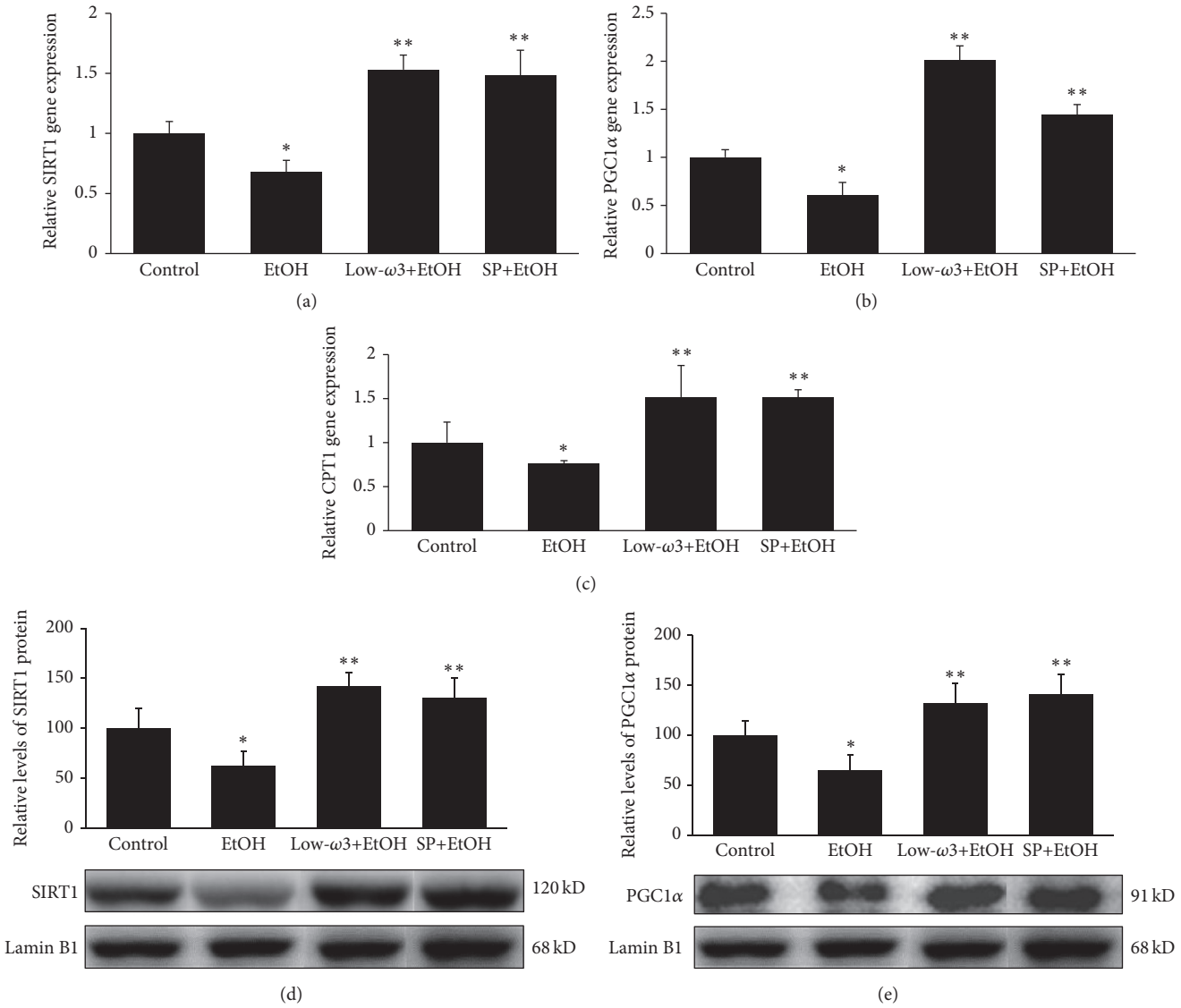

Figure 2: Influence of EtOH, low-ω3FA, and SP on lipid oxidation pathway. Total RNA from each animal was reverse transcribed and used in the qRT-PCR analysis using gene specific primers as described in Section 2 for (a) SIRT1, (b) PGC1α, and (c) CPT1. Each gene was normalized to β-actin mRNA. Nuclear protein was extracted from each animal and used for Western Blot analysis using specific antibodies as described in Section 2 for (d) SIRT1 and (e) PGC1α. Values are means of triplicate experiments ± SD of 3 samples/group and were corrected for difference in loading after reprobing with an antibody to Lamin B1. $^{*}p < 0.05$ versus control; $^{**}p < 0.05$ versus EtOH.

chronic EtOH *via* the regulation of SIRT1, PGC1α, CPT1, and PPARα.

In order to test whether the action of low-ω3FA and SP on hepatic lipid catabolism was mediated through the active or inactive forms of PGC1α *via* the modulation of SIRT1, we determined the level of acetylated PGC1α in the liver tissue of various groups. Figure 3 shows that chronic EtOH increased the hepatic acetylated (inactive) form of PGC1α by 40% ($p < 0.05$) because of EtOH-mediated decrease in SIRT1 by 38% ($p < 0.05$) as compared to the control (Figure 2(d)), thereby accounting for decreased fatty acid oxidation. In contrast, low-ω3FA and SP decreased the inactive form of PGC1α by 37% and 25%, respectively, as compared to EtOH group (Figure 3) *via* the upregulation of SIRT1 (Figures 2(a) and 2(d)), thereby accounting for restoring the decreased fatty acid caused by chronic EtOH to the control level. Thus, low-ω3FA and SP may lower alcoholic hepatosteatosis by augmenting the relative levels of active form of PGC1α; that in turn effectively restored hepatic lipid catabolism that is impaired by chronic alcohol exposure.

3.3. Effects of Low-ω3FA and SP on Chronic EtOH-Mediated Alterations in the Lipogenic Pathway. Figure 4(a) shows that chronic EtOH markedly upregulated PGC1β mRNA level by 52% ($p < 0.05$) as compared to the control, and low-ω3FA and SP downregulated the EtOH effect by 61% ($p < 0.05$) and 55% ($p < 0.05$), respectively. Similarly, Figure 4(b) shows a marked 50% ($p < 0.05$) upregulation in SREBP1c mRNA that

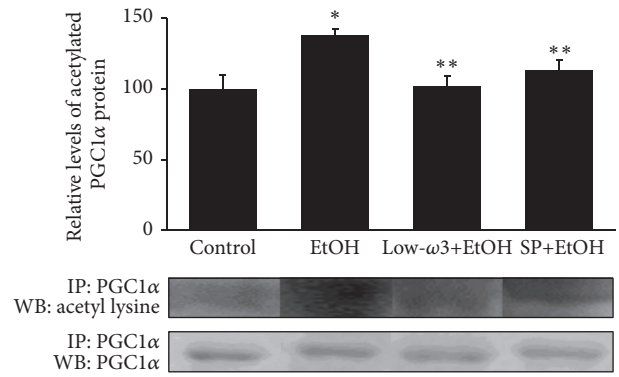

Figure 3: Effect of EtOH, low-ω3FA, and SP on hepatic acetylated-PGC1α levels. Western blot analysis was performed with an acetyl lysine-specific antibody of a nuclear protein extract immunoprecipitated (IP) with an antibody to PGC1α as described in Section 2. Values are means of triplicate experiments ± SD of 3 samples/group. $^{*}p < 0.05$ versus control; $^{**}p < 0.05$ versus EtOH.

was reduced to 30% and 50% ($p < 0.02$) of the control value by low-ω3FA and SP treatment, respectively. Chronic EtOH also markedly upregulated the mRNA expression levels of ACC, which regulates fatty acid synthesis by 2-fold ($p < 0.05$) and this was significantly suppressed by 50% ($p < 0.05$) in the low-ω3FA-EtOH group and by 70% ($p < 0.05$) in SP-EtOH group (Figure 4(c)). In contrast, as shown in Figure 4(d), the mRNA expression levels of c-Met were significantly downregulated by 35% ($p < 0.05$) after chronic EtOH administration, and low-ω3FA and SP treatment significantly restored EtOH-induced downregulation of c-Met mRNA level to 86% ($p < 0.05$) and 95% ($p < 0.05$), of the control value, respectively. These results were confirmed by measuring the nuclear or total protein expression of the above genes relative to those of the corresponding subcellular marker proteins. Figure 4(e) shows that low-ω3FA and SP fed rats showed suppressed EtOH-mediated increase (60%, $p < 0.05$) in the relative nuclear expression of PGC1β by 68% ($p < 0.05$) and 63% ($p < 0.05$), respectively. Similarly, as shown in Figures 4(f) and 4(g), the relative nuclear protein expressions of SREBP1c and ACC were also markedly increased in chronic EtOH group by 30% ($p < 0.05$) and 50% ($p < 0.05$), respectively, compared to the control group. Administration of dietary low-ω3FA and SP reversed these EtOH-mediated effects by decreasing SREBP1c protein expression by 50% ($p < 0.05$) and 56% ($p < 0.05$), respectively (Figure 4(f)), and ACC protein expression by 85% ($p < 0.05$) and 60% ($p < 0.05$), respectively (Figure 4(g)). On the other hand, c-Met expression was decreased in the EtOH group by 25% ($p < 0.05$), which were restored in low-ω3FA and SP groups by 35% ($p < 0.05$) and 45% ($p < 0.05$), respectively, as compared to the EtOH group (Figure 4(h)).

Since chronic EtOH increases hepatic ACC activity and lipogenesis by decreasing the phosphorylation of AMPK (pAMPK), a known inhibitor of ACC, we tested whether low-ω3FA or SP can counteract these effects of chronic EtOH by modulating the phosphorylation status of AMPK. As shown in Figures 5(a) and 5(b), although the level of total AMPK was unaffected in all groups, low-ω3FA and SP restored the hepatic level of pAMPK that was decreased by 50% ($p < 0.05$) in EtOH group. This increase in pAMPK could also account for decreased ACC activity and lipogenesis after low-ω3FA or SP treatment.

These findings are consistent with the ability of low-ω3FA or SP to (i) inhibit chronic EtOH-induced increase in lipogenic pathway genes and (ii) restore ethanol-mediated decreased intracellular transport of hepatic triglycerides to the blood compartment due to impaired VLDL assembly and secretion. This would lead to the low-ω3FA or SP-mediated reduction in fatty liver caused by chronic alcohol abuse.

4. Discussion

Our results show that low-ω3FA and SP exert their hypolipidemic action by upregulating primarily the lipid oxidizing genes *via* SIRT1 and PGC1α signaling pathway that are suppressed by chronic ethanol and downregulating the lipogenic pathway genes predominantly *via* the PGC1β and SREBP1c signaling pathway. Our data also support the alternative possibility that low-ω3FA and SP could prevent alcohol-induced activation of ACC activity by phosphorylating it *via* pAMPK.

SIRT1 is an NAD-dependent deacetylase (histone deacetylase (HDAC)) that has been linked to many beneficial effects of cellular processes including gene silencing, insulin resistance, glucose homeostasis, fatty acid metabolism, and aging, while HAT catalyses the opposite reaction [40]. Thus, SIRT1 activates PGC1α by deacetylation while HAT inactivates PGC1α by acetylation. On the other hand, SIRT1 destabilizes SREBP1c by deacetylation while HAT stabilizes SREBP1c by acetylation. You et al. [16] and Lieber et al. [41] have elegantly shown that both long chain and medium chain saturated fatty acids in the diet restore the expressions of SIRT1 and PGC1α that are downregulated by long chain polyunsaturated fatty acids (PUFA) in chronic ethanol-fed animals. However, PPARγ was unaffected by chronic ethanol. Previously, Fischer et al. [42] have shown in mice that ethanol leads to PPARα dysfunction resulting in impaired fatty acid oxidation and consequent onset of fatty liver that is overcome by a PPARα agonist. Similarly, other studies [43, 44] have shown that alcohol-mediated fatty liver and injury are prevented by PPARγ agonist presumably by activating c-Met and blocking alcohol-mediated induction of TNFα. We recently showed that compared to high fish oil control liquid diet, feeding of the same high fish oil liquid diet containing 5% (w/v) ethanol for 8 weeks significantly downregulated hepatic SIRT1, and PGC1α with the concomitant decreased hepatic rate of fatty acid oxidation [37]. Nanji et al. [45], Ronis et al. [46], and Song et al. [47] have demonstrated that saturated fatty acids protect against chronic alcohol-induced liver injury as compared to high levels of polyunsaturated fatty acids. In addition, Huang et al. [48] demonstrated that low levels of omega 3 polyunsaturated fatty acids, mainly docosahexaenoic acid, suppressed ethanol-induced hepatic steatosis. Similarly, Wada et al. [49] also demonstrated that low levels of fish oil fed prior to ethanol administration

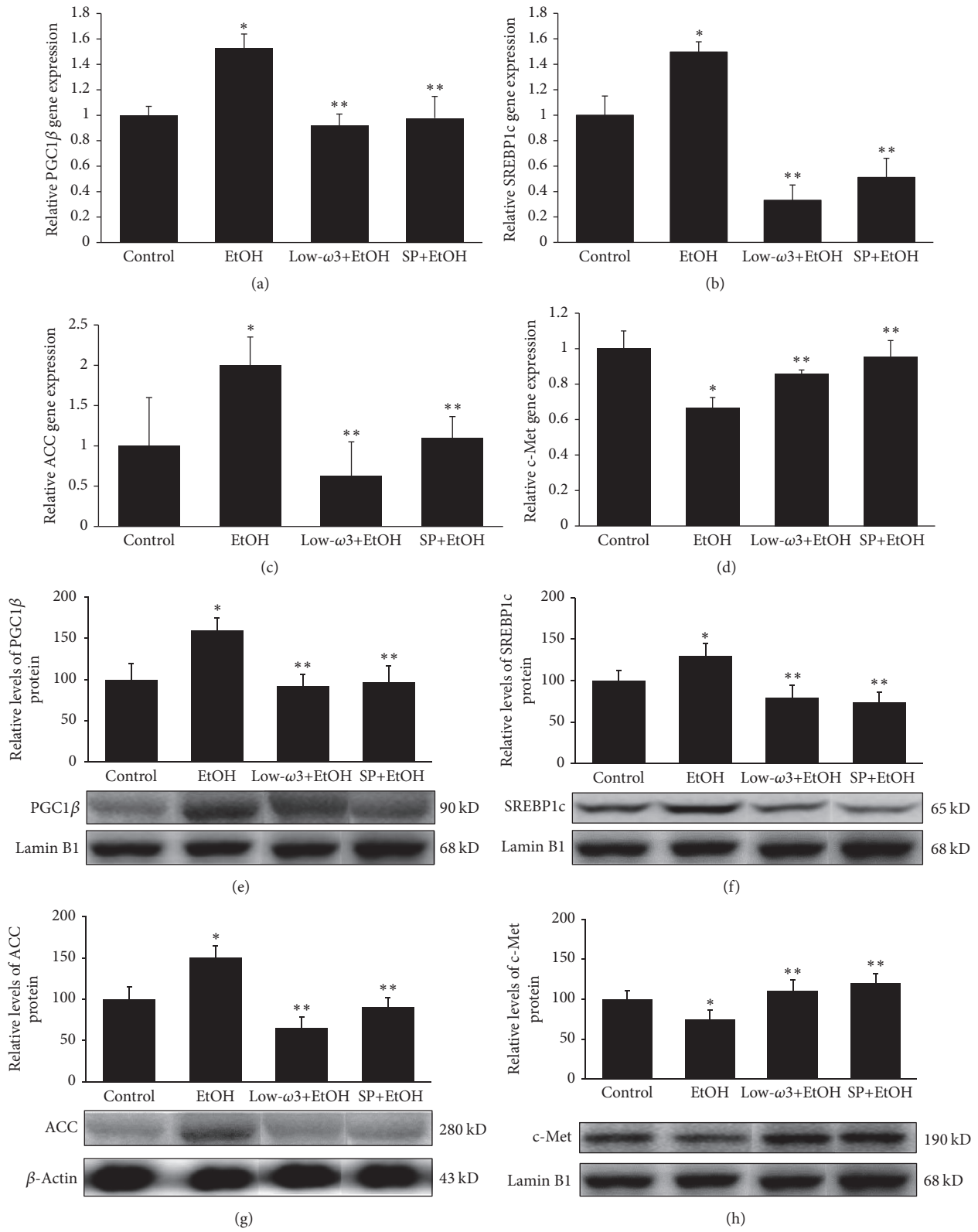

Figure 4: Influence of EtOH, low-ω3FA, and SP on lipogenic pathway. Total RNA from each animal was reverse transcribed and used in the qRT-PCR analysis using gene specific primers as described in Section 2 for (a) PGC1β, (b) SREBP1c, (c) ACC, and (d) c-Met. Each gene was normalized to β-actin mRNA. Nuclear or total protein was extracted from each animal and used for Western Blot analysis using specific antibodies as described in Section 2 for (e) PGC1β, (f) SREBP1c, (g) ACC, and (h) c-Met. Values are means of triplicate experiments ± SD of 3 samples/group and were corrected for difference in loading after reprobing with an antibody to Lamin B1 or β-actin for nuclear or total protein, respectively. $^{*}p < 0.05$ versus control; $^{**}p < 0.05$ versus EtOH.

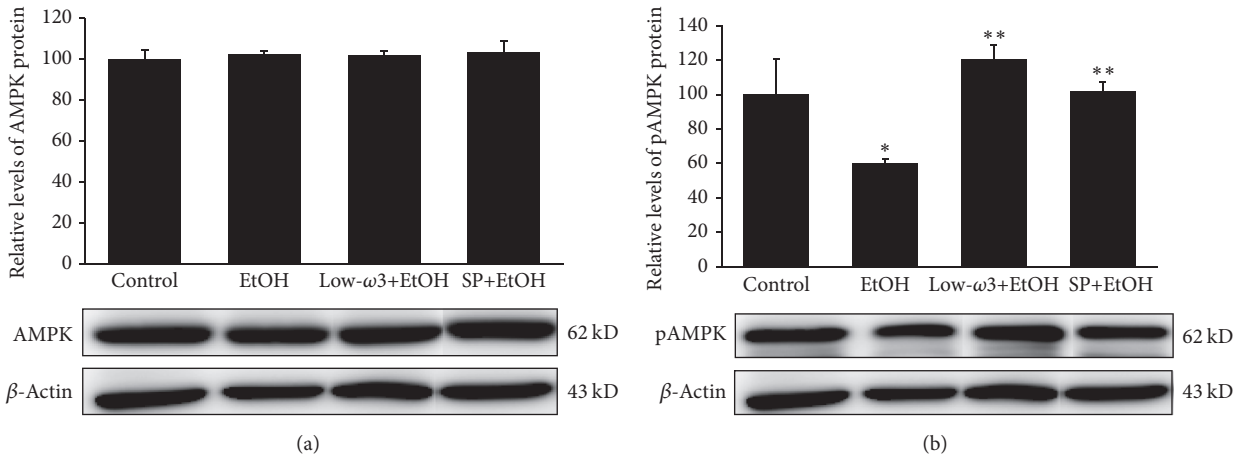

Figure 5: Effect of EtOH, low-ω3FA, and SP on hepatic (a) AMPK and (b) pAMPK was determined. Total protein was extracted from each animal and used for Western Blot analysis using specific antibodies as described in Section 2. Values are means of triplicate experiments ± SD of 3 samples/group and were corrected for difference in loading after reprobing with an antibody to β-actin. $^{*}p < 0.05$ versus control; $^{**}p < 0.05$ versus EtOH.

prevent acute ethanol-induced fatty liver in mice. In agreement with these studies, the present study shows that dietary low level of ω3FA (2.7%), but not high level of ω3FA (14.1%), restores the expression of SIRT1 and PGC1α that are downregulated by chronic ethanol. Our results also show that chronic alcohol exposure upregulates PGC1β, ACC, c-Met, and SREBP1c. Activation of SREBP1c by ethanol feeding in rats had been already associated with increased expression of hepatic lipogenic genes as well as the accumulation of triglyceride in the livers [50]. However, Ki et al. [51], Lu et al. [52], and Zeng et al. [53] demonstrated that SREBP-1c-mediated lipogenesis pathway was not affected by ethanol or even suppressed after chronic ethanol intake in rats. While the inability of these studies to show chronic ethanol-mediated upregulation of SREBP-1c-mediated lipogenic pathway may be due to different dietary fat compositions compared to the present study, we consistently find that the upregulation of this pathway with the high-ω3FA diet was markedly attenuated by low-ω3FA diet. Thus, it is important to point out that different dietary conditions, particularly the amount and the type fat in the diet, do affect SREBP activation pathway in different ways.

Low-ω3FA have an inherent property of attenuating chronic alcohol-mediated hepatosteatosis by upregulating PGC1α and downstream lipid degradation pathways while SP downregulates PGC1β, SREBP1, and downstream lipid synthetic pathways and by controlling the active/inactive forms of AMPK. Recently, Phillipson et al. demonstrated the lipid lowering effects of fish oil rich in ω3FA in humans [23]. However, chronic ethanol-induced liver damage in rats fed a high fat diet (36% fat calories) was exacerbated [45, 46, 54–56] with polyunsaturated FA from either vegetable oil (ω6 family) or fish oil (ω3 family) as evidenced by increased serum aspartate aminotransferase and alanine aminotransferase as well as by histopathology. In this study, fish oil constituted 36% of the total calories in the diet, which amounted to 14.1% of the total dietary calories as ω3FA. In contrast, we showed [57] that the inclusion of only 2.7% of total dietary calories as ω3FA resulted in lower plasma and liver lipids in chronic alcohol-fed animals. Furthermore, the same low level of dietary ω3FA restored the decreased ApoE content in HDL. Thus, a low level of ω3FA has beneficial effects [58–60], whereas a significant increase in ω3FA seems to have a detrimental effect on the liver [45, 46, 54–56]. It is possible that increased ethanol consumption in the intragastric model could have also caused the deleterious effects when PUFA-rich diet was fed [55]. Significantly, PUFA-containing lecithin diet was shown to prevent alcohol-induced hepatic fibrosis in baboons [61]. We showed [57] that low-ω3FA caused decreased VLDL production and serum lipids resulting in lipid-deficient ApoE, which can be easily sialylated and be associated with HDL. This would be consistent with the effects of low-ω3FA in reversing ethanol-mediated decrease in HDL-ApoE. Our previous work [58] also demonstrated that HDL from low-3FA-fed animals were more efficient in carrying out reverse cholesterol transport (RCT) function compared to the control animals regardless of whether the animals were on alcohol or control diet. We found [62] that cholesterol uptake by Hep-G2 cells from reconstituted HDL was stimulated by sphingomyelin (SPM). HDL phospholipid acyl chain composition is known to influence cholesterol efflux [63]. We also showed that chronic ethanol preferentially decreased SPM concentration in HDL of alcoholics leading to its impaired RCT function [64].

The present study shows that SP downregulated ethanol-mediated overexpression of PGC1β, SREBP-1, and its target lipogenic genes such as ACC (Figure 2), whereas it restored ethanol-mediated downregulation of SIRT1, PGC1α, and lipid oxidizing genes such as CPT1 (Figure 4). Overall, our results suggest that the relative hypolipidemic effects of SP compared to low-ω3FA in regulating alcoholic hepatosteatosis were more due to alteration in the lipogenic pathway, whereas that of low-ω3FA compared to SP was more due to alteration in the lipid oxidizing pathway.

In summary, this study has demonstrated the following. (1) Low-ω3FA and SP reduced alcoholic hyperlipidemia as well as hepatic lipid accumulation as evidenced by decreased liver cholesterol and triglycerides as well as hepatic histological lipid scores. (2) Low-ω3FA and SP prevented alcohol-mediated downregulation of SIRT1 and PGC1α and their target fatty acid oxidation pathway genes. (3) Low-ω3FA and SP attenuated alcohol-mediated upregulation of PGC1β, SREBP1c, and its target lipogenic pathway genes. (4) Low-ω3FA and SP decreased the liver nuclear SREBP1c level that was increased by chronic ethanol treatment. (5) Low-ω3FA and SP restored the hepatic level of pAMPK that was decreased by chronic alcohol treatment.

5. Conclusion

Unlike high dietary ω3FA, low dietary ω3FA protects against chronic alcohol-induced liver injury. We have demonstrated that low-ω3FA and SP could potentially upregulate SIRT1/PGC1α and downregulate PGC1β/SREBP1c signaling pathways in alleviating alcoholic hepatosteatosis and liver injury. Thus, our study opens this field to explore other new therapeutic agents targeted on PGC1α and PGC1β pathways for protection against not only alcoholic liver diseases but also metabolic syndrome and obesity, the major world-wide health problems, especially when superimposed in alcohol abusers.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Insights into the Role and Interdependence of Oxidative Stress and Inflammation in Liver Diseases

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The crucial roles of oxidative stress and inflammation in the development of hepatic diseases have been unraveled and emphasized for decades. From steatosis to fibrosis, cirrhosis and liver cancer, hepatic oxidative stress, and inflammation are sustained and participated in this pathological progressive process. Notably, increasing evidences showed that oxidative stress and inflammation are tightly related, which are regarded as essential partners that present simultaneously and interact with each other in various pathological conditions, creating a vicious cycle to aggravate the hepatic diseases. Clarifying the interaction of oxidative stress and inflammation is of great importance to provide new directions and targets for developing therapeutic intervention. Herein, this review is concerned with the regulation and interdependence of oxidative stress and inflammation in a variety of liver diseases. In addition to classical mediators and signaling, particular emphasis is placed upon immune suppression, a potential linkage of oxidative stress and inflammation, to provide new inspiration for the treatment of liver diseases. Furthermore, since antioxidation and anti-inflammation have been extensively attempted as the strategies for treatment of liver diseases, the application of herbal medicines and their derived compounds that protect liver from injury via regulating oxidative stress and inflammation collectively were reviewed and discussed.

1. Introduction

Liver disease, a broad spectrum of disease ranging from early steatosis to severe hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC), has high prevalence worldwide. Hepatic diseases could be triggered by various risk factors including obesity, virus, alcohol, drugs, and other toxins [1]. As liver is a central organ detoxification and nutrients metabolism, it is more vulnerable to oxidative stress and inflammation produced from toxins and metabolites in the body [2]. A huge number of animals studies and clinical trials have demonstrated that sustained oxidative stress and inflammation in the liver are crucial in the initiation and development of hepatic illness, regardless of the etiology [3]. Both of them are considered to be the key elements in the pathogenesis of acute and chronic liver diseases. Oxidative stress causes hepatic damage by provoking alteration of biological molecules such as DNA, proteins, and lipids and, notably, modulating biological pathways associated with genes transcription, protein expression, cell apoptosis, and hepatic stellate cell activation [2]. Regarding inflammation, it is an essential component of the immune response and manifested as infiltration of inflammatory cells to primarily liver for fighting against pathogens invasion; however, once the stimuli exist persistently or overwhelmingly, they in turn lead to cellular injury and lipid accumulation associated with increased risk of severe liver diseases such as steatohepatitis, fibrosis, and cancer [4–6].

Of great interest, investigations focusing on the relationship and interaction of oxidative stress and inflammation have attracted great attention as accumulated evidences indicated that they are tightly correlated and orchestrated to drive the pathophysiological procedure of liver diseases [7–9]. At the early stage of liver diseases, either of them may solely present, but both should participate in the pathogenesis of various liver diseases together at later stages. Besides, a number of reactive oxygen species (ROS) or reactive nitrogen species (RNS) can augment proinflammatory gene expression by provoking intracellular signaling cascade. On the other hand, inflammatory cells could produce more ROS/RNS, resulting in exaggerated oxidative stress at inflammatory lesion [7, 10]. The close interplay of oxidative stress and

inflammation thus creates a vicious cycle that promotes the pathogenesis of liver diseases. In general, antioxidative therapy showed unsatisfied outcomes in many clinical trials, though numerous studies demonstrated actual involvement of oxidative stress in the pathogenesis in the disease. This has been called as the phenomenon of antioxidant paradox in medical science [11, 12]. Some researchers proposed that it is the complex and tight related interdependence between oxidative stress and inflammation that are responsible for the failure of antioxidant treatment [7]. For example, antioxidant that only improves oxidative stress pathways but aggravates inflammatory cascades is more likely to get failure in treating liver diseases. Therefore, clarifying the interaction of oxidative stress and inflammation is of great importance for the selection of antioxidants that block oxidative and inflammatory pathways simultaneously. In this review, we will briefly summarize the roles of oxidative stress and inflammation in highly prevalent liver diseases. Then, emphasis will be put upon the discussion on the relationship and interdependence of oxidative stress and inflammation in liver diseases including alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), fibrosis, and HCC. More importantly, as anti-inflammatory immune suppressor cells also show linkage with oxidative stress [13], based on data as far now, potential mechanism by which immune suppression mediated by oxidative stress regulating inflammation is proposed herein might offer new inspiration for the treatment of liver disease. Furthermore, many herbal medicines have displayed antioxidant and anti-inflammatory activity for treatment of liver diseases by a vast body of studies [8]. Herbal medicines or purified compounds such as berberine (BBR), curcumin, lipoic acid (LA), and epigallocatechin gallate (EGCG) which protect liver from injury via regulating oxidative stress and inflammation collectively will be briefly summarized and discussed in this review.

2. Oxidative Stress and Inflammation in Hepatic Lesion

2.1. Targets and Participation of Oxidative Stress in Liver Diseases. Oxidative stress is defined as the imbalance between productions of ROS/RNS generated in the aerobic metabolism and their elimination by antioxidant defense including enzymes such as superoxide dismutases (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) and nonenzymes particles of electron receptors such as glutathione (GSH) and vitamin C/E, which could be triggered in the liver by diverse factors such as obesity, virus, drugs, alcohol, and other toxins [2]. Under sustained exposure to the above factors, ROS/RNS are generated overwhelmingly, thus leading to substantial damage to cell structure and functions. Targets of these reactive species that cause cellular and tissue injury include lipids, DNA, proteins, and related signaling pathways. Free radicals react with lipids to produce hydroperoxides and endoperoxides, which may suffer fragmentation to generate reactive intermediates, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), to cause irretrievably covalent adducts with proteins, DNA, and phospholipids, resulting in cell death [14]. Reactive stress induced DNA damage primarily occurs in the mitochondrial genome because (i) mitochondrion is open and circular without histone protection and (ii) close to reactive species that is generated heavily inside mitochondrion compared to nuclear region [15]. Cysteine with the thiol group is a reactive amino acid of proteins in cells, and the formation of disulfide by oxidizing two thiols can result in the alteration of protein function. By targeting thiols of proteins, ROS can alter signaling pathways such as cellular kinases, phosphatases, and transcription factors, which have insightful impacts in cell proliferation, differentiation, and apoptosis leading to hepatocytes injury by stimulating oxidant-induced hepatocyte apoptosis or by restraining cell survival signaling cascades [14]. Cellular kinases, particularly in the mitogen-activated protein kinase (MAPK) family such as extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK, have a critical role in transducing a multitude of extracellular stimuli by phosphorylating and activating downstream transcription factors. Modulation of protein expression in response to oxidative stress occurs mainly via the activation of redox-sensitive transcription factors such as nuclear factor κB (NF-κB), activator protein-1 (AP-1), early growth response protein 1 (EGR-1), and G proteins [15, 16]. It is worth noting that the fate of hepatocytes mainly depends on intensity and duration of the stimuli, which determines the degree and duration of the activation/inactivation of these redox-sensitive cascades, especially the relative level of activation of NF-κB, ERK1/2, and JNK.

Involvement of oxidative stress in liver diseases has been extensively explored and its crucial impact on the pathogenesis of a variety of liver diseases such as ALD, NAFLD, fibrosis, and HCC has been revealed. In ALD, during the metabolism process of alcohol in the liver, reactive intermediate acetaldehyde, which can react with DNA and proteins to form adducts, causes tissue injury. Activated cytochrome P450 2E1 (CYP2E1) responsible for alcohol breakdown in the case of chronic alcohol exposure commits the generation of ROS, resulting in fatty acids deposition and the progress of hepatic steatosis [17, 18]. Additionally, defense system to remove reactive species is also altered by alcohol, such as peroxisome proliferator activated receptor gamma-(PPAR-γ-) coactivator 1 α, which can induce activation of various ROS-mediated detoxifying enzymes [19–22]. ROS may lead to excessive alcoholic liver fibrosis and cirrhosis via rebuilding of stellate cells and the extracellular matrix within the liver. With regard to NAFLD, substantial hepatic ROS is produced by excessive angiotensin II and activated CYP2E1, ultimately leading to impaired beta-oxidation and fatty liver [23]. Fibrosis, a wound-healing response to hepatocytes injury, is caused by the overproduction of collagen I. The elevated oxidative stress contributes to fibrogenesis via provoking generation of collagen from activated hepatic stellate cells and release of other profibrogenic cytokines, growth factors, and prostaglandins [24–26]. Concerning liver cancer, ROS generated by hypoxia along the edge of tumor growth presents a dual role: (i) it promotes carcinogenesis by activating NF-κB and HIF1α, which favor cancer cell survival, angiogenesis, and tumor expansion; (ii) on the other hand,

after mitochondrial GSH depletion, it shifts hypoxia from a cancer-promoting to a cancer-killing environment [27–29]. It is proposed that the role of oxidative stress in cancer depends on the time course: destructive role in the early phase of carcinogenesis and protective role in late phase.

2.2. Participation of Inflammation in Liver Diseases Spectrum. When the liver is challenged by exogenous and endogenous stimuli like virus, allergens, toxins, or obesity, inflammation usually occurs to protect the liver from injury with characteristic of leukocytes infiltration such as neutrophils, monocytes, and lymphocytes. However, once this process is excessive, prolonged, or dysregulated, pathological inflammation and tissue injury will occur, which is critical for the initiation and development of hepatitis, fibrosis, cirrhosis, and liver cancer [30–32]. Hepatic inflammation has dual roles in the liver; they are essential to maintain tissue healthy and act as critical drivers of the liver pathology when persisted or out of control. The infiltration of leukocytes is a complicated process involving participation of many receptors, adhesion molecules, and chemokines, such as selectins, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and corresponding leukocyte receptors and monocyte chemoattractant protein 1 (MCP-1). In addition to hepatocyte death-mediated leukocytes infiltration, infiltrated inflammatory cells also produce soluble mediators, such as metabolites of arachidonic acid, cytokines, and chemokines, which activate related signal transduction cascades and change transcription factors, to further recruit inflammatory cells to the injured site. Then, liver injury is deteriorated because of increased cytokines and ligands [6, 33].

Similar to oxidative stress, inflammation is generally sustained and participated in the whole spectrum of liver diseases from initial to advanced stage, which is known as inflammation-fibrosis-cancer axis [8]. With the progression to chronic liver diseases, both innate and adaptive immune response are triggered by leukocytes infiltration, activation of Kupffer cells (KCs), and upregulation of inducible nitric oxide synthase (iNOS) [34]. Leukocytes and KCs produce huge amounts of nitric oxide and cytokines, such as potent profibrogenic cytokine, TGF-β4, and inflammation modulator, TNF-α. Plentiful inflammatory mediators including inflammatory cytokines, chemokines, and Toll-like receptors (TLRs) are involved in the regulation of hepatic fibrogenesis [34–36]. Particularly, TLRs, a family of pattern recognition receptors serving as important innate immune response factors, play a vital role in the pathogenesis of liver disease [37]. As Toll-like receptors are redox-sensitive receptor proteins and have been involved in cellular response to oxidative stress, the effect of lipid peroxidation caused by oxidants on TLRs is worth to be explored in the future. As a matter of fact, NF-κB and JNKs are considered as the most key signaling pathways linking inflammation and fibrosis. Hyperactivation of NF-κB in hepatocytes or infiltrated inflammatory cells fosters hepatic inflammation by increased generation of proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6 [16]. Mediators like IL-1β and TNF-α could activate NF-κB in hepatic stellate cells (HSCs) and promote survival of HSCs and fibrogenesis. Recently, it was discovered that ubiquitin-editing protein A20, an important regulator of inflammatory signaling to block NF-κB activation, prevents the development of chronic hepatic inflammation and cancer by protecting hepatocytes from death [38]. JNK is involved in multiple signaling cascades related with hepatocellular injury, as well as regulating hepatic steatosis and inflammatory gene expression. It has direct profibrogenic role by stimulating platelet-derived growth factor (PDGF), TGF-β, and angiotensin II-induced proliferation, α-smooth muscle actin (α-SMA) expression, and collagen production [6, 39, 40]. Moreover, inflammation is intensively implicated in carcinogenesis of HCC [41]. Chronic inflammation is involved in the process of cellular transformation, promotion, proliferation, invasion, angiogenesis, and metastasis of carcinogenesis [42]. The generation of proinflammatory cytokines like cyclooxygenase-1 (COX-1), COX-2, TNF-α, IL-1, IL-26, IL-8, IL-18, and macrophage inflammation protein-1 (MIP-1α) via activation of NF-κB alters the hepatic microenvironment and leads to fibrosis and carcinogenesis [24, 42].

3. The Relationship and Interdependence between Oxidative Stress and Inflammation in Various Liver Diseases

Extensive research has revealed that oxidative stress and inflammation are tightly interrelated in many diseases, as displayed in Figure 1. They seem to occur simultaneously and further promote each other in injury site. On one hand, continued oxidative stress can lead to chronic inflammation. Many transcription factors and receptors such as NF-κB, activator protein-1 (AP-1), p53, hypoxia-inducible factor 1-alpha (HIF-1α), PPAR-γ, β-catenin/Wnt, and erythroid 2-related factor 2 (Nrf2) are activated by oxidative stress, which could regulate the expression of many genes, including those inflammatory cytokines and anti-inflammatory molecules [43]. For example, a pattern recognition receptor, NOD-like receptor protein 3 (NLRP3) that triggers innate immune response through promoting maturation of proinflammatory cytokines like IL-1β and IL-18, can be activated by oxidative stress and thus leads to inflammation [7, 43, 44]. Of note, NF-κB signaling plays a key role in oxidative stress-mediated inflammation response [16]. Apart from the direct activation of NF-κB via oxidative stress, damaged DNA induced by ROS also results in inflammation through NF-κB pathway [16]. Recently, it was found that the release of oxidized peroxiredoxin-2 (PRDX2) and substrate thioredoxin from macrophages could alter the redox status of cell surface receptors and induce inflammation via TNF-α production in an oxidative cascade [45], which provides a new linkage of inflammation and oxidative stress. On the other hand, sustained inflammation could induce oxidative stress. Infiltrated immune cells and activated phagocytic cells such as neutrophils and macrophages produce large amounts of ROS and RNS like superoxide, hydrogen peroxide, hydroxyl free radical, peroxynitrite, and nitric oxide. These reactive species, which are generated to invade agents, can induce localized oxidative stress when diffused out of the phagocytic cells

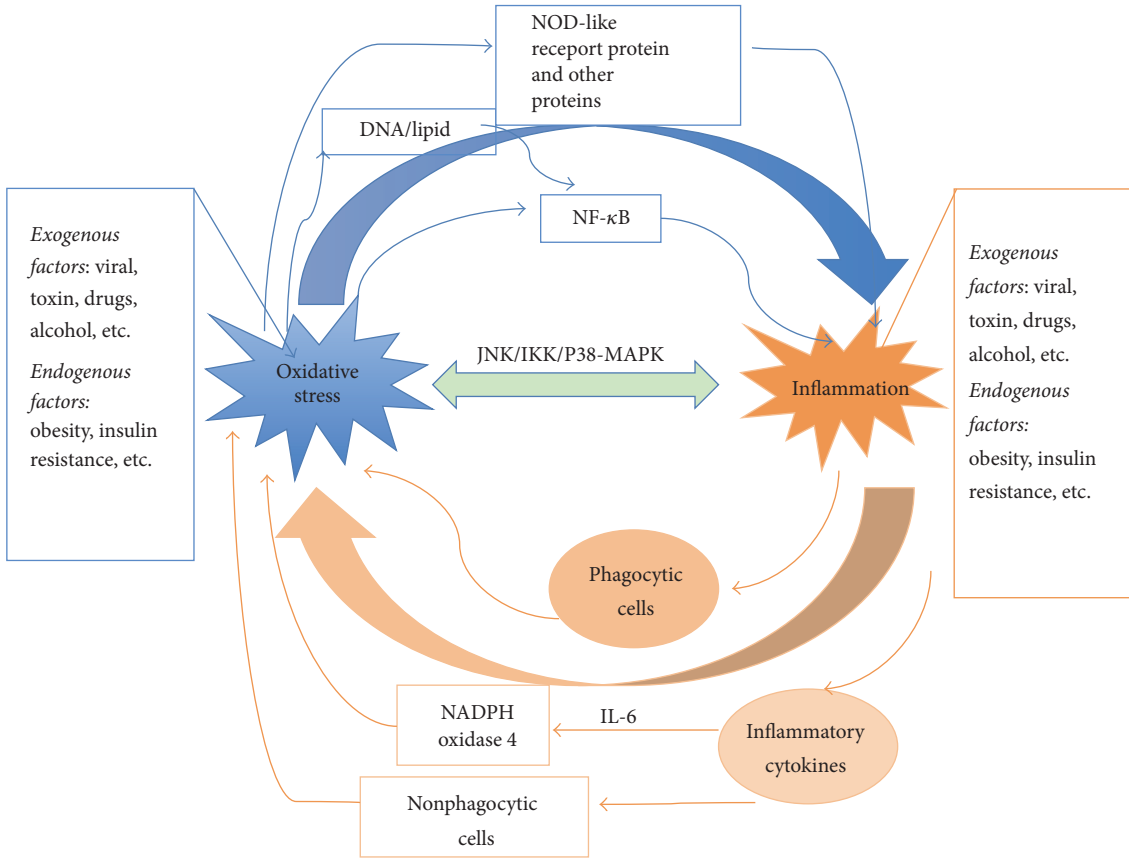

Figure 1: Potential interaction of oxidative stress and inflammation.

[44, 46]. In addition to direct production of ROS/RNS by phagocytic cells, in response to proinflammatory cytokines such as IL-6 and interferon-gamma (IFN-γ), the nonphagocytic cells can also produce reactive species. Redox-sensitive signal transduction pathways such as JNK and p38 MAPK also play vital roles in the interaction between inflammation and oxidative stress [14].

3.1. Alcoholic Liver Disease. As mentioned before, both generation of ROS/RNS and activation of inflammation are critical for hepatic damage induced by alcohol. The oxidative microenvironment created by ROS/RNS via alcohol metabolism process activates stress-related proteins, facilitates adduct formation, and induces endoplasmic reticulum stress, resulting in hepatocellular damage [47]. Particularly, the contributions of oxidative stress in promoting adaptive immune responses in alcoholic liver disease (ALD) have been reported [21, 48]. Oxidative stress has been considered as a trigger for adaptive immune responses in patients of alcoholic liver disease. The adaptive immune responses to MDA adducts indicates that oxidative stress may represent an important stimulus for the development of immune responses associated with advanced ALD [21]. The infiltration of inflammatory cells, activation of macrophage, and the proinflammatory mediators like LPS, TNF-α, IL1β, and IL6 induced by various pathways, such as alcohol-regulated CD14/Toll-like receptor 4 (TLR4) pathway, could provoke cellular injury and apoptosis, resulting in ALD [18, 19]. The activation of KCs by lipopolysaccharides (LPS) via TLR4 is critical to the onset of alcohol-induced hepatic damage, which could enhance the production of inflammatory cytokines and ROS which contribute to ALD [31, 49, 50]. MicroRNA-155, an important regulator of inflammation, has been demonstrated to play a vital role in alcohol-induced steatohepatitis and fibrosis. It was found that miR-155 knockout mice are protected from alcohol-induced steatosis and inflammation, which might be attributed to increased peroxisome proliferator-activated receptor response element (PPRE) and PPAR-α binding and decreased MCP1 production [51]. In the past, the role of oxidative stress and inflammation in the pathophysiological process of ALD was investigated individually. However, of note, with better understanding of this disease, recent studies have begun to explore the interconnected relationship between oxidative stress and inflammation [10].

Several signaling pathways have been identified to be involved in oxidative stress-mediated aggravated inflammation in ALD as shown in Figure 2. The IκB kinase (IKK) kinase activation that leads to the production of proinflammatory cytokines could be accentuated by oxidative stress in ALD, for example, ROS-mediated molecular chaperones such as Hsp90 [31, 52–54]. Notch1 and NF-κB that have been intensively implicated in inflammatory diseases were found to be important mediators in oxidative stress-induced

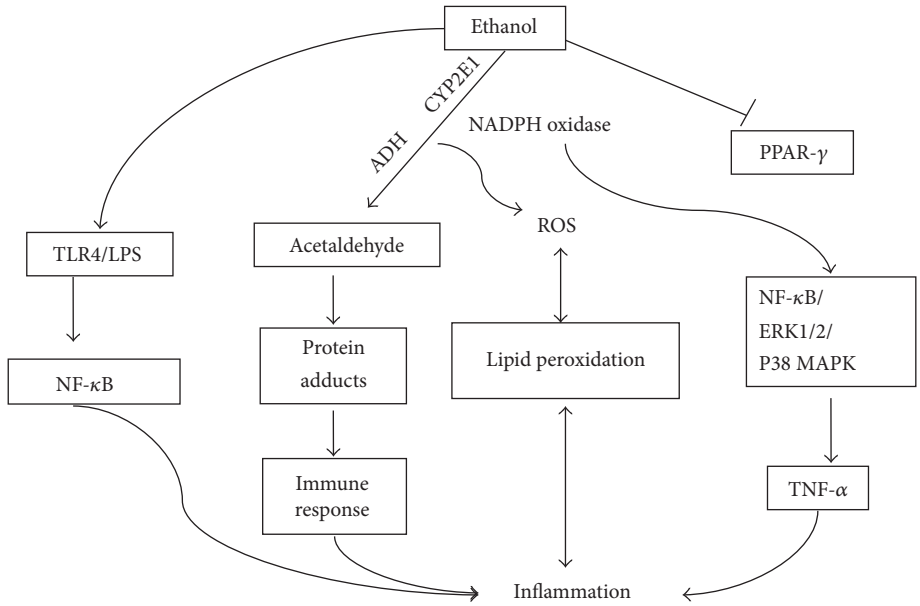

Figure 2: Several signaling pathways involved in oxidative stress-mediated aggravated inflammation in ALD.

alcoholic steatohepatitis [55]. NADPH oxidase, a source of ROS in ALD, can increase NF-κB activation and phosphorylate ERK1/2 and p38 MAPK kinases that intensify the production of TNF-α from KCs [53]. In previous study, the role of alcohol-induced oxidative stress in modulating proinflammatory cytokines production in alcoholic steatohepatitis was determined. It was found that LPS improved TNF-α, macrophage inflammatory protein-alpha (MIP-1α), MCP-1, and cytokine-induced neutrophil chemoattractant 1 (CINC-1) in KCs-SV40, whereas TNF-α upregulated CINC-1, IFN-γ-inducible protein 10 (IP-10), and MIP-2 expression in H4IIEC3 hepatoma cells in a dose-dependent matter. When stimulated by combination of hydrogen peroxide with LPS or TNF-α, KCs-SV40 and hepatocytes increased production of proinflammatory cytokines through NF-κB activation and histone H3 hyperacetylation. But regarding LPS-treated KCs-SV40, 4-hydroxynonenal showed inhibitory effect on cytokine production via significantly enhancing mRNA degradation of cytokines like TNF-α, MIP-1α, and MCP-1 and decreased the MCP-1 protein level by diminishing the phosphorylation of mRNA binding proteins [56]. This study indicates that the role of oxidative stress in regulating inflammatory cytokine production is dependent on cell type, while NF-κB signaling, histone acetylation, and mRNA stability are implicated in this regulation process [56]. Interferon regulatory factor 3 (IRF3) and signal transducer and activator of transcription 3 (STAT3) have also been proposed to increase hepatic proinflammatory cytokines in ALD in association with oxidative stress, which needs further study to confirm. These signaling pathways are of great value to be specifically targeted for ALD treatment.

3.2. Nonalcoholic Fatty Liver Disease. Nonalcoholic fatty liver disease (NAFLD), which is characterized by excessive lipid accumulation in the liver, is similar to ALD that may progress from simple steatosis to steatohepatitis, fibrosis, cirrhosis, or even HCC [57, 58]. However, unlike ALD which is induced by heavy alcoholic consumption, the major etiology of NAFLD is obesity, insulin resistance (IR), dyslipidemia, or diabetes [52, 59]. Due to high caloric diet and life style, NAFLD has an increasing prevalence in Western society and even in the world. In the regard of pathogenesis of NAFLD, two hit hypotheses are widely proposed. It is believed that steatosis resulted from accumulated triglycerides and free fatty acid in the liver because of obesity or insulin resistance is the first hit, which might be a benign and stable pathology [60, 61]. There exist other pathological "hits" including oxidative stress and hepatic inflammation which may promote simple steatosis to further complications like fibrosis or cirrhosis [31]. Therefore, the importance of oxidative stress and inflammation in the pathogenesis of NAFLD is beyond doubt.

In nonalcoholic steatohepatitis (NASH), ROS are generated via a variety of ways [62]. β-Oxidation of overload fatty acid is regarded as the major source of reducing equivalents responsible for increased ROS production. Also, TNF-α and lipid peroxidation products, which inhibit the electron-transport chain of the mitochondria, could induce mitochondrial dysfunction and increase the production of ROS [63]. Mitochondrial damage will result in secondary inhibition of lipids β-oxidation and further increase the level of steatosis. In the cytosol, xanthine oxidase, a key enzyme for the degradation of purine, catalyzes the reaction of hypoxanthine to uric acid in which large amounts of superoxide anions are generated. Increased xanthine oxidase activity induced by 4-HNE may cause further hepatic damage via ROS [64]. The microsomal cytochromes CYP2E1 and CYP4A are involved in the lipooxygenation of long chain fatty acids with generation of ROS [65]. Moreover, CYP2E1 can be upregulated by free fatty acids as well as insulin resistance, which could enhance NADPH oxidase enzyme, leading to increased

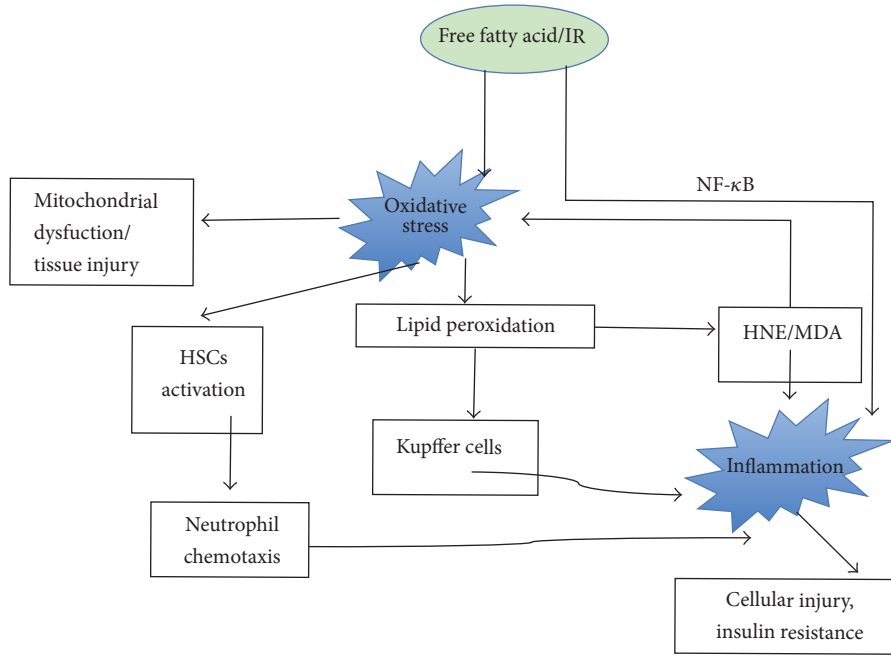

Figure 3: Potential interactions between oxidative stress and inflammation in the context of NAFLD.

production of superoxide [66, 67]. In terms of inflammation, infiltration of various inflammatory cells like neutrophils, macrophages, T helper cells, natural killer T (NKT) cells, and natural killer (NK) cells and upregulation of inflammatory mediators such as TNF-α, IL-6, and IL-1β occur in steatohepatitis, which contributes to insulin resistance as well as other metabolic dysregulations, and play a key role in promoting the progression of steatohepatitis to fibrosis, cirrhosis, and cancer [32]. For example, lipid peroxidation-activated KCs could induce production of TNF-α. In the initial phase of NAFLD, resident immune cells such as KCs and dendritic cells respond to early signs of hepatocellular damage by generating plentiful proinflammatory cytokines like IL-1β and chemokines like chemokine ligand 2 (CCL2), which further promote inflammatory cell infiltration, thus leading to a vicious cycle [64, 68]. It was reported recently that early depletion of KCs using liposomal clodronate could block the development of NASH [69]. In addition, several mediators have been demonstrated which play a positive role in regulating inflammation in the context of NAFLD. For example, IL-15 upregulates expression of chemokines like CCL2, CCL5, and CXCL10 and increases infiltration of mononuclear cells, promoting inflammation in NAFLD [70]. Tumor necrosis factor receptor-associated factor 1 (TRAF1), an important adapter protein, is extensively implicated in mediating immunity/inflammation and cell death. It was reported recently that TRAF1 functions as a positive regulator of inflammation and hepatic steatosis in NAFLD through the activation of ASK1-P38/JNK axis [71]. Moreover, component C5, a central mediator of inflammation, was also found to contribute to liver steatosis and inflammation in NAFLD [72].

Accumulating evidences showed tight interaction between oxidative stress and inflammation in the context of NAFLD (Figure 3). Firstly, ROS can induce lipid peroxidation process in which HNE and MDA are generated, which can freely diffuse into the extracellular space to affect nucleotide and protein synthesis and thereby increase proinflammatory cytokine and activate hepatic stellate cells, ultimately leading to inflammation and the progression of NASH [73, 74]. It was found that the levels of markers of lipid peroxidation and oxidative DNA damage, such as HNE and 8-hydroxydeoxyguanosine, are correlated with the severity of necroinflammation and fibrosis in patients with NAFLD [61, 75]. Lipid peroxidation products developed from phospholipids oxidation can induce adaptive immune responses by forming immunogenic adducts through the interaction with cellular proteins. High titres of antibodies against lipid peroxidation-derived antigens are associated with increased hepatic inflammation and advanced fibrosis in patients with NAFLD [76, 77]. Specifically, ROS and lipid peroxidation induce inflammation through promoting the release of proinflammatory cytokines, resulting in neutrophil chemotaxis and lesions of NASH [23]. For example, ROS activate NF-κB signaling pathway, leading to the synthesis of TNF-α and the upregulated TGF-β, IL-8, IL-6, and Fas ligand. Recently, Satapati et al. reported that the induction of biosynthesis via hepatic anaplerotic/cataplerotic pathways is amplified by increased oxidative metabolism and further contributes to oxidative stress and inflammation during NAFLD [78]. Furthermore, TGF-β, IL-8, and HNE are chemoattractants of human neutrophils, which may result in more neutrophil infiltration. On the other hand, infiltrated neutrophils and other immune cells produce more ROS in hepatic lesion. Moreover, increased proinflammatory mediators such as TNF-α also cause the increase of ROS, leading to mitochondrial dysfunction [74, 79, 80].

3.3. Liver Fibrosis. Liver fibrosis, a reversible multicellular wound healing process that results from chronic liver injuries

with excessive collagen and extracellular matrix (ECM), is characterized by perpetuation of parenchymal necrosis, infiltration of inflammatory cells, and activation of HSCs, macrophages, and KCs, regardless of the etiology [25, 81, 82]. Various growth factors, inflammatory cytokines and chemokines, accumulated ECM, and oxidative stress have been proposed to play a role in fibrogenesis.

Oxidative stress-related molecules and pathways can modulate tissue and cellular events involved in the pathogenesis of liver fibrosis [24]. The presence of oxidative stress and decreased antioxidant defenses caused by stimulus has been detected in almost all settings of fibrosis and cirrhosis in clinical and animal model. The disruption of lipids, proteins, and DNA caused by oxidative stress will induce necrosis and hepatocytes death and intensify the inflammatory response, resulting in the initiation of fibrosis [83]. Those ROS can stimulate the production of profibrogenic mediators from infiltrated inflammatory cells. Remarkably, ROS can interact directly with HSCs, which are the main executors of fibrogenesis to generate ECM. The cellular redox environment could regulate the entry of quiescent HSCs into activated cycle [24, 84]. Redox-sensitive transcription factors such as NF-κB are important to regulate the activities of antioxidant enzymes that mediate ROS signaling. Compared with activated HSCs, the expression of NF-κB in quiescent HSCs is lacking, implying that a redox-sensitive activation of NF-κB can regulate expression of related genes, delivering a proper cellular redox microenvironment for quiescent HSCs into the proliferative cycle [24, 35, 85]. Phagocytosis of apoptotic bodies by HSCs induces NADPH with production of oxidative radicals, which is implicated in liver fibrosis in vivo [86]. Furthermore, ROS can also interact with HSCs to activate fibrosis by DNA methylation, histone modifications, and gene silencing by noncoding RNA species. The adaptive immune response induced by oxidative stress like lipid peroxidation products like MDA and 4-HNE was also implicated in the development from fatty liver to fibrosis. It was suggested that the adaptive immune reactions triggered by oxidative stress could be an independent predictor of progression of NAFLD to advanced fibrosis [76].

In the initial phase of hepatic injury, inflammation triggered by initial cell death contributes to the removal of cellular debris and promotes liver regeneration, ensuring restoration of hepatic architecture and function after acute liver injury [87, 88]. However, once underlying disease cannot be eliminated and the stimuli sustains, chronic inflammation and progressive liver fibrosis will be induced [40]. As a matter of fact, cell death and persisted overwhelming inflammation are the characteristics of chronic liver diseases that progress to fibrosis. A variety of inflammatory mediators and pathways could regulate the activation of HSCs and their survival after activation [89]. It has been found that inflammatory cytokines like IL-1β, TNF-α, and IL-17/20/33, chemokines like MCP-1 and CXCL10, and TLR pathway are intensively involved in the regulation of hepatic fibrogenesis [90–93]. T cells response also plays a vital role in regulating the progression of fatty liver to advanced-stage liver disease [94–96]. It has been indicated that hepatic recruitment of T helper cells and cytotoxic T cells contributes to hepatic inflammation, leading to the development from simple fatty liver to steatohepatitis [97]. T helper cells and cytotoxic T cells infiltration was correlated with the progression of NASH, paralleling the worsening of parenchymal injury and lobular inflammation [96]. CD4+ T helper cells may promote hepatic inflammation through upregulation of IFN-γ and CD40 ligand [98]. But the underlying mechanism of CD8+ cytotoxic T cells in promoting NASH progression is still unclear.

Thus, oxidative stress and inflammation interact with each other in multiple ways, thereby creating a favorable microenvironment for fibrogenesis (Figure 4). In addition to direct interplay of ROS and inflammation, the activated HSCs are a vital bridge to link them together. Both oxidative stress and inflammatory mediators play a role in the activation of HSCs, and, conversely, activated HSCs could in turn enhance cellular oxidative stress and inflammation. On one hand, redox-sensitive pathways influenced by oxidative stress regulate the status of HSCs in the initiation phase and stimulate inflammatory signaling via cytokines, chemokines, and TLR ligands [25, 99]. On the other hand, HSCs activated by inflammatory mediators could also inhibit antioxidant defense and increase the generation of ROS. They work tightly to form a vicious cycle in the process of liver fibrosis. Although there are no highly effective antifibrogenic agents currently available, the potential candidates that can reduce inflammation and oxidative stress as well as ECM are considered to be promising for the prevention and treatment of liver fibrosis. Combination therapy that blocks HSCs activation via antioxidant and anti-inflammatory pathways would be effective to inhibit the progression of the pathogenesis of liver fibrosis.

3.4. Liver Cancer. The initiation and progression of many cancers have been linked to oxidative stress and inflammation, as demonstrated by the excessive sustained ROS and inflammatory mediators. Primary liver cancer, the fifth most common malignancy worldwide, has no exception. Sustained inflammation is associated with persistent hepatic injury and concurrent regeneration, leading to sequential development of advanced stage of liver disease including fibrosis, cirrhosis, and eventually HCC [100]. The perpetuation of a wound-healing response activated by hepatocytes death and the following inflammatory cascade response is a common denominator for HCC initiation. Chronic inflammation has been linked to multiple steps involved in carcinogenesis, such as cellular transformation, promotion, invasion, angiogenesis, and metastasis [100, 101]. For example, in context of hepatic steatosis induced by obesity or other factors, excessive free fatty acids increase proinflammatory cytokines and adipokines that can further promote release of TNF-α and IL-6 from KCs, leading to activation of downstream signaling molecules like STAT3 in hepatocytes, which might result in hepatocarcinogenesis [102–105]. Meanwhile, ROS are produced over a long time under a sustained environmental stress and contribute to cellular structure damage and induce DNA damage and mutations in protooncogenes and tumor-suppressor genes, leading to neoplastic transformation. Under hypoxic conditions, excessive RNS are generated through the mitochondrial respiratory chain. The role of

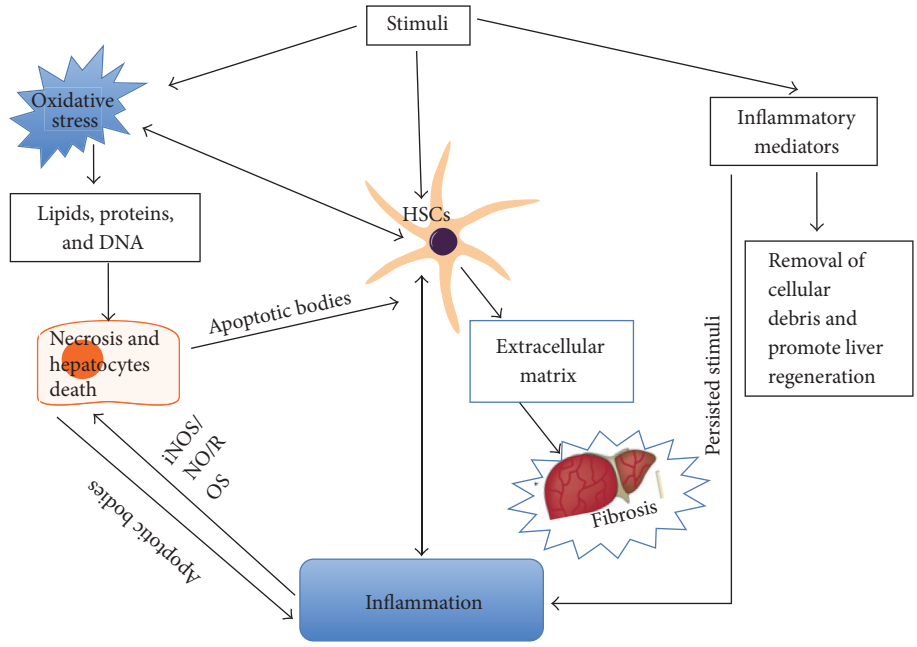

Figure 4: Oxidative stress and inflammation interact with each other in multiple ways to promote fibrogenesis.

oxidative stress in modulating inflammation-induced carcinogenesis in different stage has been systemically reviewed by Reuter et al. Briefly, under an inflammatory stimulus, progression of carcinogenesis mediated by ROS may be direct through oxidation or nitration of DNA or mediated by the cross-talk signaling pathways related to oxidative stress and inflammation. The sustained inflammatory/oxidative microenvironment forms a vicious circle, which can damage healthy cells like neighboring epithelial and stromal cells, ultimately resulting in carcinogenesis.

Of note, the participation of ROS in carcinogenesis is complicated. ROS are regarded as tumorigenic due to their ability to increase cell proliferation, survival, and cellular migration as well as gene mutations. However, ROS can also function as antitumorigenic agents via induction of cellular senescence and cell death. Whether ROS promote tumor cell survival or induce cancer cell death remains as a complex issue, which majorly depends on the location of ROS production, the concentration of ROS, and many other factors such as carcinogenesis stage [15, 29]. For example, cytoglobin (*Cygb*), expressed in HSCs, plays a protective role in controlling ROS/RNS in the inflamed liver. Deficiency of *Cygb* promoted HCC development in CDAA-fed $Cygb^{-/-}$ mouse via upregulating prooxidative genes and downregulating antioxidative genes [28]. However, ROS produced by NOX2 could inhibit proliferation of cancer stem cell-like phenotype and diminish tumor growth of HCC by PPAR-γ agonists, suggesting their protective role in the context of cancer.

4. Immune Suppression: A Potential Linkage of Oxidative Stress and Inflammation

4.1. Immune Suppressive Cells to Resolve Inflammation. As aforementioned, hepatic inflammation has dual roles; they are essential to maintain tissue healthy and act as critical drivers of the liver pathology when persisted or out of control. Therefore, resolving inflammation appropriately and timely is of great significance to maintain liver homeostasis. In this regard, several basic mechanisms in resolution of inflammation were proposed. First, soluble anti-inflammatory mediators are released in order to fight against the innate immune response [39]. The second mechanism mainly involves the activation and induction of immune suppressive cells like regulatory T cells (Tregs). Additionally, the loss of cell-cell mediated costimulation on lymphocytes or antigen presenting cells (APC) and the activation of programmed cell death in those cells are also regarded as potential mechanisms to resolve inflammation. In particular, recently, the activation and induction of immune suppressive cells in resolving inflammation have been intensively studied and attracted great attention [107]. Herein, immune suppressive cells including Tregs, M2-macrophage, and myeloid-derived suppressor cells (MDSCs) are particularly discussed with emphasis on anti-inflammatory effects and linkage to oxidative stress on liver diseases.

4.1.1. Regulatory T Cells. Tregs, a subset of T lymphocytes that primarily works at sites of inflammation in maintaining peripheral tolerance, have been implicated in several inflammatory liver diseases. Tregs are equipped with a wide range of mechanisms of immune suppression, including the removal of target cells, the regulation of APC, the disturbance of metabolic pathways, and the generation of anti-inflammatory cytokines [108]. Increasing evidences showed that the reduced frequency and defective function of Tregs facilitate inflammation in various liver diseases, such as drug induced liver injury, autoimmune hepatitis, and NASH.

Accumulating evidence indicates that Tregs are vital inhibitory mediators of inflammation. It has been demonstrated that, in the initial phase of acute liver injury, intrahepatic Tregs diminished promptly through apoptosis, which may facilitate inflammation and tissue injury, while in the healing phase, Tregs are generated through matrix metalloproteinase (MMP) cascade-dependent activation of TGF-β to terminate inflammation [109]. Development from NAFLD to NASH is marked by an increased frequency of Th17 cells in the liver, which is a subset of proinflammatory T helper cells with the production of IL-17 and is tightly related to Tregs because the signals for Th17 cells differentiation could inhibit Tregs. The ratios of Th17/resting Tregs in peripheral blood and in the liver were also increased [96]. Furthermore, they inhibit the profibrogenic inflammatory milieu through suppressing the infiltration of profibrogenic CD8+ and IL-17+ T cells [110]. In regard of migration of Tregs, it was found that local proinflammatory cytokines lead to the secretion of CXCL9 and CXCL10 by sinusoidal and parenchyma cells, which could recruit $CXCR3^{high}$ circulating Tregs into the liver. Subsequently, CCR4 guides the migration of these Tregs within the inflamed liver. These infiltrated Tregs respond to CCL17 and CCL22 released by activated DCs, thus resulting in their accumulation in the liver with dendritic cells-rich inflammatory infiltrates. The reason why inflammation persists in the presence of Tregs infiltration might be due to their dysfunctional suppressive effects by programmed death receptor-1 in the specific microenvironment [111].

Therefore, modulation of Tregs by immune regulatory agents or adoptive transfer is of great importance in inflamed liver diseases. For instance, dietary n-3 polyunsaturated fatty acids (PUFA) protected mice from Con A-induced hepatitis by enhancing Tregs generation via upregulated expression of PPAR-γ and TGF-β, which might be a promising potential therapeutic agent having anti-inflammatory and immunoregulatory effects for inflammatory diseases [112]. The population of Tregs in the liver significantly decreased after reperfusion, and adoptive transfer of induced Tregs (iTregs) that come from TGF-β-induced CD4+CD62L+T cells before ischemia reperfusion could attenuate liver injury as indicated by reduced proinflammatory cytokines. In vitro study showed that iTregs could suppress expression of IL-1β and TNF-α, promote transcription of IL-10, and increase phosphorylation of mothers against decapentaplegic homolog 3 (SMAD3) in KCs. Furthermore, inhibition of TGF-β signaling by anti-TGF-β abolished the effects on KCs [113]. In acetaminophen-(APAP-) induced liver injury, the depletion of Tregs amplified proinflammatory cytokines and aggravated liver injury, while adoptive transfer of Treg cells showed protective effect [114].

However, Tregs have also been proposed as foe in several types of liver diseases such as vital hepatitis and HCC, in which persisted immune responses are expected to eliminate exogenous infection and neoplasm [115–118]. Failure in immune regulation by Tregs leads to viral persistence and tumor growth. Therefore, appropriate strategy in regulating population and function of Tregs particularly in liver diseases should be highlighted according to different conditions.

4.1.2. Alternatively Activated (M2) Macrophage. There are two major phenotypes of macrophages: classically activated (M1) subset with prototypic macrophage functions which acts as proinflammatory mediator and an alternatively activated (M2) subset involved in wound healing with anti-inflammatory ability. Therefore, macrophage polarization plays a critical role in inflamed liver diseases [119]. In patients with minimal liver damage and steatosis, there is higher hepatic expression of M2 genes compared to patients with more severe liver lesions. A complicated interplay between M1 and M2 types of macrophages expressing a wide range of molecules and receptors is involved in many liver diseases [120].

In ALD, both activated M1 and M2 macrophages are present in the liver [121]. The polarization of KCs toward M1 phenotype initiated the inflammatory process, and it was indicated that promoting anti-inflammatory M2 phenotype polarization protects liver from alcoholic-induced liver injury through mechanism that relies on apoptotic effects of M2 subset towards their M1 counterparts [121]. In addition, M2 macrophages could trigger hepatocyte senescence mediated by IL-6 and enhance alcohol-induced hepatocyte senescence by oxidative stress, which exhibit functional resistance to apoptosis, thus leading to an early protective effect against ALD [122]. In mice with chronic alcohol treatment, genes related with M1 phenotype such as TNF-α, MCP1, and IL-1β and genes associated with M2 macrophages such as Arg1, Mrc1, and IL-10 as well as the population of CD206(+)CD163(+) M2 macrophages were improved in the liver. Alcohol could promote M2 phenotype and the expression of Krüppel-like factor 4 (KLF4), a regulator of macrophage polarization, whereas the intermediate metabolite of alcohol, acetaldehyde, decreased KLF4 and promoted M1 macrophage, which may justify the increased M1 and M2 macrophages in ALD [123]. M2 macrophages expressing CD163 in liver sinusoids of ALD are abundant; IRF-4, which is related to IL-4 production, and M2 polarization were also observed in AH, suggesting that M2 phenotype plays a role in AH pathogenesis [120]. In TAA-induced liver injury model, the double labeling of CD68(+) and CD163(+) macrophages was found, which indicated that macrophage immunophenotypes are interchangeable in injured sites [124].

Pharmacologic interventions targeting M2 polarization during the early stages of liver disease may signify a striking strategy to alleviate liver injury [125, 126]. IL-6, a pleiotropic interleukin that commonly associated with proinflammatory effect, is also necessary for inflammation resolution because of its role in promoting polarization of M2 macrophage [119]. Despite the fact that it is associated with the perpetuation and development of inflammatory disease, the potential benefit of IL-6 in polarization of M2 macrophage should also be considered [119].

4.1.3. Myeloid Derived Suppressor Cells (MDSCs). The myeloid-derived suppressor cells (MDSCs) are a heterogeneous population from bone marrow with remarkable immunosuppressive properties. In the context of tumor setting, MDSCs help tumor cells escape from immunosurveillance, thereby endorsing tumor growth [127]. Both innate immune and

adaptive immune responses could be suppressed by MDSC via several mechanisms including production of large amounts of nitric oxide (NO) as well as ROS and arginine depletion. However, their functional significance in the immune system has been appreciated in recent years [128–131].

Under most of the inflammation conditions, MDSCs infiltrate into the liver and play a protective role in hepatitis models. In models of acute hepatitis mediated by Con A and α-GalCer, farnesoid X receptor (FXR) activation drives the accumulation of MDSCs to liver via upregulation of S100A8 and augments the suppressive function of MDSCs through upregulation of receptor paired immunoglobulin-like receptor B (PIR-B) by binding the PIR-B promoter [132]. MDSCs function as an important negative feedback loop and reduction in this cell population facilitates inflammatory hepatic damage [133]. In the context of immune-mediated hepatic injury, mTOR negatively regulates the recruitment of MDSC, which is critically required for protection against hepatic injury. The inhibition of mTOR by rapamycin or other inhibitor treatments promotes the expansion of MDSCs that protect liver from persistent inflammation [134, 135].

Intervention targeting the expansion and activation of MDSCs is a potential therapeutic approach because deficient or unsuitable activity of this cell population may contribute to the pathogenesis of inflammatory liver. It was proposed that expansion of MDSC could be divided into two processes regulated by different signal transduction pathways. The first process is the expansion induced by various cytokines and growth factors produced in response to chronic stimulation, which involves factors like IL-6 and VEGF, and signaling pathways such as STAT3 and NF-κB, which prevents maturation of myeloid cells. Then, a second activating signal provided by proinflammatory molecules to drive upregulation of arginase and iNOS and production of immune suppressive cytokines is required for their activation [130, 131]. In addition to above proposed pathways, it is reported that activated human HSCs during chronic inflammation induced mature peripheral blood monocytes into MDSCs, and, subsequently, excessive liver injury might be prevented by local induction of MDSCs. However, of note, in liver cancer patients, the expansion of HSC-induced MDSCs plays an opposite role to facilitate tumor growth by immune suppression [130, 131, 136].

4.2. Oxidative Stress Mediating Failure of Immune Suppression Response to Promote Inflammation. After unraveling the role of immune suppression in mediating inflammatory liver diseases, the question that whether oxidative stress, the essential partner of inflammation, participates in this process has been put forward. As oxidative stress and inflammation tightly interplay and drive coherently the progression of liver disease together, it is reasonable to propose that oxidative stress plays a critical role to promote inflammation in terms of negatively regulating the immune suppression. That is, downregulated immune suppression might be another potential linkage between oxidative stress and inflammation. As a matter of fact, increasing evidence demonstrated that oxidative stress could mediate the dysfunction of immune suppression, thus leading to the failure of inflammation resolving.

In a study of high-fat fed mice model, it was indicated that the increased oxidative stress in a fatty liver caused the apoptosis of Tregs and reduces the population of hepatic Tregs, resulting in lowered suppression of inflammatory responses. The treatment with an antioxidant Mn(III)tetrakis(4-benzoic acid)porphyrin chloride decreased the apoptosis of Tregs, increased the number of hepatic Tregs, and thus improved hepatic inflammation in mice with NAFLD. This scenario might be one of the underlying mechanisms that enable the conversion of simple steatosis into steatohepatitis [137]. In another study, it was indicated that monocytic MDSCs are induced by catalase-mediated exhaustion of hydrogen peroxide from mature CD14+ monocytes, and the frequency of MDSC inversely correlated with hydrogen peroxide level. The oxidative stress in many liver diseases with decreased activity of catalase and increased hydrogen peroxide might inhibit the expansion and activation of hepatic MDSCs that are expected to resolve overimmune response [138]. Therefore, oxidative stress might serve as a mediator of impeding immune suppression response to promote inflammation. This mechanism also probably explains the potential benefit of oxidative stress in tumor microenvironment. Therapy of antioxidant that activates immune suppression to resolve inflammation might be promising for treatment of various inflamed liver diseases.

5. Potential Therapeutic Approach on Liver Diseases

Resolving the vicious cycle between inflammation and oxidative stress is of great clinical importance to treat many chronic diseases. It was proposed that identification of primary abnormality is important to break this cycle. That is, in the case where oxidative stress presents as primary event, antioxidants rather than anti-inflammatory agents would be effective therapeutic strategy [7]. Conversely, if inflammation appears as the primary abnormality, anti-inflammation should be considered as a primary therapeutic target. It seems to be a key factor to explain the puzzling phenomenon of medical science, where, in certain case of oxidative stress-related disease, antioxidant treatment showed unsatisfactory outcome [7, 12]. However, despite promising potential for clinic practice, unraveling the primary abnormality is difficult as once the process has been initiated, both inflammation and oxidative stress show up to promote each other and to cause progressive injury. With regard to liver diseases, few researches were dedicated to study the causation of oxidative stress and inflammation in specific disease settings. Therefore, application of agents that modulate both oxidative stress and inflammation is still regarded as the mainstream choice for the prevention and treatment of liver diseases.

Many medicinal herbs show striking abilities in protecting the liver due to their remarkable anti-inflammatory and antioxidative effects. In recent years, traditional Chinese medicine (TCM) has received broad attention from the public due to its long-lasting curative effects and mild complications in treating a variety of liver diseases [178]. The applications of TCM in liver diseases have been systematically reviewed in our previous papers [2, 8]. In Table 1, agents including herbal

Table 1: Herbal medicines or derived compounds targeting oxidative stress and inflammation in various liver diseases.

Materials	Models	Effects	Mechanisms	Refs.
Phyllanthin	CCl4-induced hepatic toxicity	↓ oxidative stress and hepatic fibrosis	↓ TNF-α/NF-κB and profibrotic factor TGF-1 mediating inflammatory signaling	[139]
Gomisin A	CCl4-induced acute liver injury in rats	↓ oxidative stress and inflammatory response; ↓ fibrogenesis	↓ NF-κB and proinflammatory mediators	[140]
Dillenia suffruticosa leaves	CCl4-induced hepatic damage in rats	Impedes hepatic damage	↓ oxidative stress and inflammatory markers	[141]
Diallyl disulfide	CCl4-induced liver damage in rats	↑ phase II/antioxidant enzymes; ↓ inflammatory mediators	↑ Nrf2 pathway; ↓ NF-κB activation	[142]
Folic acid and melatonin	CCl4-induced liver injury in rats	↑ liver function; ↓oxidative stress and inflammation in rats	Restore the oxidative stability and lipid profile; ↓ inflammatory cytokines and cell survival Akt1 signals	[143]
Ursolic acid	CCl4-induced liver injury in mouse	Inhibiting oxidative stress and inflammation	↓ JNK, p38 MAPK, ERK, and NF-κB	[144]
Aloin	Chronic alcoholic liver injury in mice	↓ chronic alcoholic liver injury	↓ lipid accumulation, oxidative stress, and inflammation	[145]
Wild bitter gourd	Chronic alcohol-induced liver injury in mice	↓ oxidative stress and inflammatory responses	↑ antioxidant defence system; ↓ MDA and proinflammatory cytokines; ↓ CYP2E1, SREBP-1, FAS, and ACC protein expression	[146]
Lutein	Alcohol-induced liver injury in rats	↓ oxidative stress and inflammation	↓ inflammatory proteins and cytokines; ↑ Nrf2 levels; ↑ antioxidant enzymes	[147]
Fucoidan	High-fat diet-induced NAFLD in rats	↓ steatohepatitis and insulin resistance	↓ oxidative stress and inflammatory cytokines	[148]
Epigallocatechin gallate	High-fat diet-induced nonalcoholic fatty liver disease in rats	↓ fibrosis, oxidative stress, and inflammation	Modulating the activities of TGF/SMAD, PI3 K/Akt/FOXO1, and NF-κB	[149]
Magnolia extract	High-fat diet-induced liver damage in mice	↓ fibrosis in the liver	Inhibition of lipid accumulation, inflammation, and oxidative stress	[150]
Brown alga *Ecklonia cava* polyphenol extract	High-fat diet-induced obese mice	↓ hepatic lipogenesis, oxidative stress, and inflammation	↑ AMPK and SIRT1	[151]
Chicoric acid	High-fat diet-fed mice	↓ hepatic steatosis, inflammation, and oxidative stress	↑ antioxidant defense system; ↑ adipocytokines and adipogenesis-associated proteins	[152]
Ghrelin	High-fat diet-induced NAFLD in rats	↓ inflammation, oxidative stress, and apoptosis	Restoration of LKB1/AMPK and PI3 K/Akt pathways	[153]
Curcumin, silybin phytosome, and R-lipoic acid	TAA-induced chronic hepatitis in rat model	↓ MDA, GSH depletion, and collagen deposition	↓ macrophage activation, NF-κB, TNF-α, and IL6	[154]
Carvacrol	TAA-induced liver injury in rats	Abrogation of oxidative stress, inflammation, and apoptosis	↓ NF-κB	[155]
Fermented rooibos extract	LPS-induced liver injury in rats	Attenuated liver injury	↓ oxidative stress and proinflammatory cytokines	[156]
α-Lipoic acid	LPS-induced liver injury	Antioxidant, anti-inflammatory, and antiapoptotic activities	↓ iNOS, COX-2, TNF-α, NF-κB, IL-1β, and IL-6	[157]
Paeoniflorin	A mouse model of hepatic ischemia/reperfusion injury	↓ hepatic ischemia/reperfusion injury	Via antioxidative, anti-inflammatory, and antiapoptotic pathways	[158]

Table 1: Continued.

Materials	Models	Effects	Mechanisms	Refs.
Agaricoglycerides	Hepatic ischemia/reperfusion injury in mouse	Protect against hepatic injury	↓ inflammatory response, oxidative stress, and expression of NF-κB	[159]
Cannabidiol	Hepatic ischemia/reperfusion injury in mouse	↓ oxidative stress and inflammation and cell death	↓ key inflammatory pathways, independent of classical CB1/2 receptors	[160]
Dioscin	Hepatic ischemia/reperfusion injury in rats	Inhibition of oxidative-nitrative stress, inflammation, and apoptosis	↓ IL-1A, IL-6, TNF-α, intercellular adhesion molecule-1, MIP-1α, MIP-2, Fas, and FasL; ↓ NF-κB, AP-1, COX-2; ↓ JNK, ERK, and p38 MAPKs phosphorylation; ↑ Bcl-2 and Bcl-x	[161]
Delta(8)-tetrahydrocannabivarin	Hepatic ischemia/reperfusion injury in mice	Prevents hepatic injury and inflammation	Cannabinoid CB2 receptors	[162]
Green tea extract	Hepatic injury after hemorrhage/resuscitation in rats	↓ apoptosis, oxidative stress, and inflammation	↓ JNK and NF-κB	[163]
Astaxanthin and *Corni fructus*	Streptozotocin-induced diabetic rats	↓ glucose concentration; ↓ ROS and lipid peroxidation	↓ advanced glycation end product formation and anti-inflammation	[164]
Resveratrol	Streptozotocin-induced type 1 diabetic rats	↓ oxidative stress and inflammation	↑ Mn-SOD; ↓ NF-κB and IL-1β;	[165]
α-Lipoic acid	Aflatoxin B-1-induced liver damage in broilers	↓ oxidative damage and inflammatory responses	↓ hepatic proinflammatory cytokines; ↓ NF-κB	[166]
Niacin	HepG2 or human primary hepatocytes stimulated with palmitic acid	↓ fat accumulation, oxidative stress, and inflammatory cytokine IL-8	↓ hepatocyte DGAT2 and NADPH oxidase activity	[167]
Quercetin	Tripterygium glycosides-induced acute liver injury in mice	↓ liver injury	↓ oxidative stress and inflammation	[168]
Troxerutin	2,2′,4,4′-Tetrabromodiphenyl ether-induced liver inflammation in mouse	↓ liver inflammation	↓ oxidative stress-mediated NAD^+-depletion	[169]
Geraniol	2-Acetylaminofluorene-induced liver injury in rats	↓ oxidative stress, inflammation, and apoptosis	↓ caspase-3 and caspase-9, COX-2, NF-κB, PCNA, iNOS, VEGF, and disintegration of DNA	[170]
Thymoquinone and curcumin	Gentamicin-induced liver injury in rats	↓ deleterious effects on liver function and histological integrity	↑ antioxidant defense system; ↓ inflammation and apoptosis	[171]
Chrysin	Cisplatin-induced hepatic damage in rats	↓ hepatotoxicity	↓ oxidative stress and inflammatory response	[172]
Bazhen Decoction	Acetaminophen-induced acute liver injury in mice	↓ ALT, AST, ALP, LDH, TNF-α, IL-1β, ROS, TBARS, GSH depletion, and loss of MMP	↑ SOD, CAT, GR, and GPx; ↓ inflammatory mediators; ↓ Bax/Bcl-2 ratio and caspase-3, caspase-8, and caspase-9	[173]
Probiotic *Lactobacillus casei* Zhang	Endotoxin- and D-galactosamine-induced liver injury in rats	Antioxidative and anti-inflammatory effects	TLR4 signaling	[174]
Lutein	Guinea pigs fed a hypercholesterolemic diet	↓ oxidative stress and inflammation	↓ NF-κB DNA binding activity	[175]
Galangin	Fructose-induced liver damage in rat	↓ oxidative damage	↓ inflammatory pathway	[176]
Ganoderma applanatum terpenes	Benzo(alpha)pyrene-induced liver damage in mouse	Decreased oxidative stress and inflammation	↑ antioxidant enzymes and suppressing inflammatory response	[177]

medicine and derived compounds targeting oxidative stress and inflammation in various liver diseases were summarized. Particular emphasis herein is put upon several pure compounds from herbal plants with strong anti-inflammatory and antioxidative ability. Furthermore, several medicines that prevent liver disease by mechanism of regulating immune suppression response, antioxidant, and anti-inflammation were reviewed.

Berberine (BBR), an alkaloid isolated from *Coptidis Rhizoma*, has been reported with several pharmacological activities including antitumoral, antimicrobial, glucose- and cholesterol-lowering, and immunomodulatory properties by us and others [179–181]. Importantly, it exhibited antisteatotic, anti-inflammatory, and antioxidative effect via regulating AMPK and low-density lipoprotein receptor expression by ERK and JNK pathways [8]. BBR treatment significantly attenuated hepatic inflammation, lipid peroxides, and fibrosis in NAFLD model, which may be a therapeutic strategy to prevent the progress of hepatic steatosis to NASH [182]. In another study, it was shown that daily administration of BBR at the dose of 50 mg/kg for three weeks relieved oxidative stress, inflammation, hyperglycemia, hyperlipidemia, hyperinsulinemia, and the neurotoxicity related with NASH [183]. Pseudoberberine, a berberine analogue, displayed antioxidant and anti-inflammatory activities in diabetic mice with fatty liver. Due to poor bioavailability of BBR via oral administration, pseudoberberine might be a new oral hypoglycemic agent for NAFLD [184]. It has been reported that BBR ameliorated the liver fibrosis in mice with CCl4 administration in a dose- and time-dependent manner. The underlying mechanism of its protective effect on chronic liver fibrosis might be due to reduced oxidative stress, inhibition of TNF-α, COX-2, and iNOS expression, and activation of AMPK pathway [185, 186]. The effect of BBR on the drug isoniazid-induced hepatotoxicity has been conducted. The results showed that BBR protected liver from injury through upregulation of PPAR-γ and subsequently suppression of NF-κB and iNOS and release of the proinflammatory cytokines [187]. In addition to isoniazid, BBR also alleviates cyclophosphamide-induced hepatotoxicity by modulating antioxidant defense and inflammatory mediators [188].

Curcumin, a polyphenolic antioxidative constituent in many plants, turmeric in particular, showed remarkable hepatoprotective effects. In a rat model with CCl4-caused liver injury, curcumin could effectively protect liver from fibrosis. The results showed that curcumin attenuates oxidative stress by upregulating hepatic glutathione level, leading to the reduction of lipid hydroperoxide. Additionally, curcumin intensely suppresses inflammation by diminishing contents of inflammatory cytokines, including IFN-γ, TNF-α, and IL-6. Furthermore, curcumin inhibits HSC activation by increasing the level of PPAR-γ and reducing platelet-derived growth factor and TGF-β as well as their receptors and type I collagen [189]. In rat model with thioacetamide- (TAA-) induced chronic hepatitis, hepatic levels of MDA, collagen deposition, inflammatory mediators, and liver function were improved by curcumin treatment [154]. The effect of curcumin on a drug gentamicin-induced liver injury was evaluated in a study. The results showed that liver function indicated as aspartate aminotransferase (AST), alanine transaminase (ALT), and lactate dehydrogenase (LDH) activities, liver histological alterations, antioxidant defense, inflammatory mediators like TNF-α level, proapoptotic proteins caspase 3 and Bax expression were significantly improved by curcumin [171]. Curcumin also displayed protective effects on tetrachloro-p-benzoquinone- (TCBQ-) induced hepatotoxicity in mice, which was demonstrated by the improved AST and ALT activities, and histopathological changes including centrilobular necrosis and inflammatory cells infiltration. The elevated TBAR level and the inhibited activities of SOD and catalase and upregulated iNOS, COX-2, IL-1β, IL-6, TNF-α, and NF-κB induced by TCBQ were effectively reduced by curcumin through the activation of Nrf2 signaling [190].

Epigallocatechin gallate (EGCG) is a type of catechin in many natural plants such as green tea. As a polyphenol, it has a wide range of health benefits. For instance, EGCG could significantly relieve bile duct ligation-induced hepatic damage in mice via improving mitochondrial oxidative stress and inflammation. EGCG reduced gene expression of profibrotic markers like collagen, fibronectin, α-SMA, and connective tissue growth factor (CTGF), cell death marker like DNA fragmentation and PARP activity, mitochondrial oxidative stress, NF-κB activity, and proinflammatory cytokines such as TNF-α, MIP1-α, IL-1β, and MIP2 in bile duct ligation mice. Mitochondrial electron transport chain complexes and antioxidant defense enzymes like GSH-Px and SOD were improved by EGCG administration [191]. In NAFLD model, treatment with EGCG improved fatty score, necrosis, and inflammatory foci, restored liver function, and reduced fibrogenesis with downregulation of nitrotyrosine formation and proinflammatory markers such as iNOS, COX-2, and TNF-α. The activity of TGF/SMAD, PI3K/Akt/FOXO1, and NF-κB pathways could be counteracted by EGCG, suggesting that EGCG is beneficial in the prevention of NAFLD [149]. In addition, EGCG effectively weakened the severity of CCl_4-induced liver injury and the progression of liver fibrosis by mechanism of the reduction in oxidative stress and the inflammatory response [192]. Furthermore, it was demonstrated that, even in healthy rats, EGCG extends lifespan by reducing liver function damage and attenuating age-associated inflammation and oxidative stress. EGCG inhibited NF-κB signaling and increased the expressions of longevity factors including silent mating type information regulation 2 homolog 1 (SIRT1) and forkhead box class O 3a (FOXO3a) [193].

Lipoic acid (LA) is an organosulfur compound derived from octanoic acid, which has two sulfur atoms connected by a disulfide bond and thus can be oxidized, and the molecule presents as two enantiomers (S)-(−)-lipoic acid (SLA) and (R)-(+)-lipoic acid (RLA) and as a racemic mixture (R/S)-lipoic acid (R/S-LA). Its effect on many liver diseases has been widely studied. LA protects liver from aflatoxin B-1 induced injury via downregulating mRNA level of IL-6 and decreasing the protein expressions of both NF-kB p65 and iNOS in broilers [166]. In liver injury induced by LPS, a key inflammatory component of Gram-negative bacteria which contributes to the development of hepatic failure,

administration of LA following LPS treatment effectively prevented oxidative stress and hepatic inflammation [194]. In another study, the protective role of LA in LPS/D-galactosamine- (D-GalN-) induced fulminant hepatic failure in mice was studied. It was found that ROS and TBARS were eliminated, while activity of hepatic CAT and GPx was increased by LA treatment. Additionally, pretreatment with LA significantly decreased LPS/D-GalN-induced expression of inflammatory mediators such as TNF-α, NF-κB, iNOS, COX-2, IL-6, and IL-1β [157]. In rat model with TAA-induced chronic hepatitis, hepatic levels of MDA and liver function were improved by RLA treatment. The depletion of GSH, macrophage activation, collagen deposition, and expression of NF-κB, TNF-α, and IL-6 were significantly decreased in response to RLA administration [154]. In addition, it was found that R/S-LA coadministration could effectively disrupt a vicious pathogenic circle constituting oxidative stress, insulin resistance, and inflammation induced by fructose in rats model [195].

6. Conclusion

The critical roles of oxidative stress and inflammation involved in the pathogenesis of liver diseases have been highlighted for decades, and accumulating evidence showed that a vicious cycle could be created by oxidative stress and inflammation, which participates tightly in the progression of liver diseases. Oxidative stress could elevate proinflammatory gene expression by signaling pathways such as NF-κB, while infiltrated inflammatory cells and cytokines like IL-6 and IFN-γ could produce more oxidative stress. Their tight interactions make the hepatic pathological process complicated. More key interplayed molecules and targets are expected to be discovered in future studies. Importantly, as immune suppressive cells including Tregs, M2 macrophage, and MDSCs are of great importance to resolve inflammation, the failure of immune suppression leads to the sustained inflammation. Increasing evidence showed that immune suppression might be a link between oxidative stress and inflammation as indicated by the increased oxidative stress mediating inhibition of immune suppression in inflamed liver. Intervention mediating immune suppression might be promising to alleviate both inflammation and oxidative stress. Application of agents modulating both oxidative stress and inflammation is still considered to be mainstream choice for the prevention and treatment of liver diseases. Many medicinal herbs or derived compounds show striking abilities in protecting the liver due to their remarkable anti-inflammatory and antioxidative effects in animal studies. In future study, the related translational research should also be further conducted and improved to realize the application of these agents in treating and prevention of liver diseases. Factors such as the duration of treatment, dosage, bioavailability in human body, mode of administration, and rigorous clinical study design are needed to be considered. In short, intensive efforts should be made to address the vicious pathological cycle forming by oxidative stress and inflammation through interacted pathways or immune suppression.

Competing Interests

The authors declare that there are no competing interests.

Authors' Contributions

Sha Li drafted the manuscript; Ming Hong, Hor-Yue Tan, and Ning Wang revised the manuscript. Yibin Feng conceived, designed, revised, and finalized the manuscript. All authors discussed and confirmed the final manuscript.

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Research Article

Redox Control of Antioxidant and Antihepatotoxic Activities of *Cassia surattensis* Seed Extract against Paracetamol Intoxication in Mice: In Vitro and In Vivo Studies of Herbal Green Antioxidant

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The therapeutic potential of *Cassia surattensis* in reducing free radical-induced oxidative stress and inflammation particularly in hepatic diseases was evaluated in this study. The polyphenol rich *C. surattensis* seed extract showed good in vitro antioxidant. *C. surattensis* seed extract contained total phenolic content of 100.99 mg GAE/g dry weight and there was a positive correlation ($r > 0.9$) between total phenolic content and the antioxidant activities of the seed extract. *C. surattensis* seed extract significantly ($p < 0.05$) reduced the elevated levels of serum liver enzymes (ALT, AST, and ALP) and relative liver weight in paracetamol-induced liver hepatotoxicity in mice. Moreover, the extract significantly ($p < 0.05$) enhanced the antioxidant enzymes and glutathione (GSH) contents in the liver tissues, which led to decrease of malondialdehyde (MDA) level. The histopathological examination showed the liver protective effect of *C. surattensis* seed extract against paracetamol-induced histoarchitectural alterations by maximum recovery in the histoarchitecture of the liver tissue. Furthermore, histopathological observations correspondingly supported the biochemical assay outcome, that is, the significant reduction in elevated levels of serum liver enzymes. In conclusion, *C. surattensis* seed extract enhanced the in vivo antioxidant status and showed antihepatotoxic activities, which is probably due to the presence of phenolic compounds.

1. Introduction

There is currently much interest in the therapeutic potential of traditional and herbal therapy as an antioxidants in reducing free radical-induced oxidative stress and inflammation particularly in hepatic diseases [1]. The liver is the primary site for metabolism of almost all drugs because it is relatively rich in a large variety of metabolizing enzymes. Drug induced liver injury (DILI) is one of the most frequent causes of liver injury that poses a major clinical problem and challenge to drug regulators [2]. Drug induced liver injury makes up a total of 5% of all hospital admissions and 50% of all acute liver failures [3]. Paracetamol-induced hepatotoxicity has been linked with a number of cirrhosis, hepatitis, and suicide attempts cases. Paracetamol, if taken in overdose, can cause severe hepatotoxicity that leads to liver failure and nephrotoxicity depletion [4]. The toxic dose of acetaminophen caused the depletion of total glutathione (GSH) by as much as 90 percent, leading to accumulation of toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) which then covalently binds to cysteinyl sulfhydryl groups in hepatic protein through the 3-position of the benzene ring, forming NAPQI-protein adducts [5–7].

This causes the generation of ROS such as hydrogen peroxide (H_2O_2) and hydroxyl (OH^-) radicals that affect the cellular membrane and induce lipid peroxidation by eliminating hydrogen from a polyunsaturated fatty acid and subsequent liver damage or necrosis [8–10]. The toxic metabolite formation and protein binding also cause the dysfunction of mitochondria that leads to adenosine triphosphate (ATP) depletion and oxidant stress [11, 12]. Moreover, numerous mitochondria are found in human liver cells, with about 1000–2000 mitochondria per cell, making up 1/5 of the cell volume [13]. Given the role of mitochondria as the cell's powerhouse, there may be some leakage of the high-energy electrons in the respiratory chain to form reactive oxygen species. This was thought to result in significant oxidative stress in the mitochondria with high mutation rates of mitochondrial DNA (mtDNA) [14]. A vicious cycle was thought to occur, as oxidative stress leads to mitochondrial DNA mutations, which can lead to enzymatic abnormalities and further oxidative stress. Therefore, liver cells with numerous mitochondria are found more vulnerable to free radical oxidation than any other cells in the body. Hence, medicinal plants with hepatoprotective activity are likely to make a considerable contribution for the liver protection against the paracetamol toxicity.

Medicinal plants are an important source of natural antioxidant agents because of the less toxic nature and being free from side effects compared to synthetic antioxidant [15, 16]. Most of these medicinal plants are rich in polyphenol which have the ability to scavenge-free radicals which are generated endogenously [17]. Polyphenols are not synthesized by human being and present only in plants and some microorganisms. Various studies exposed the antioxidant properties of polyphenols towards human pathologies [18]. *Cassia surattensis* is one of the medicinal plants that is rich in polyphenol with health benefits. The genus *Cassia* is well known for its diverse biological and pharmacological properties, comprises about 600 species, and is vastly distributed worldwide [19]. The genus *Cassia* has been used as a potential medicinal plant since long ago [20, 21]. *C. surattensis* belongs to the family Fabaceae, distributed throughout Malaysia, and is widely grown as ornamental plants in tropical and subtropical areas. This plant species has been traditionally used in many countries as food products and for medicinal uses. The bark and leaves of *C. surattensis* are said to be antiblenorrhagic [22]. The decoction of the roots [23] is commonly used to treat snake bites. The leaves are consumed for cough and sore throat and used for both internal and external cooling medicine. *C. surattensis* flowers and leaves have been studied extensively and the therapeutic properties such as antioxidant [24], antimicrobial [25], and antidiabetic [26] have been reported. According to Deepak et al. [27], *C. surattensis* seed showed good antioxidant, antifungal, and antibacterial activities on bacterial and fungal cultures. A finding by El-Sawi and Sleem [28] indicated the efficacy of *C. surattensis* leaf extract as hepatoprotective agent in CCl_4-induced albino rats. Hence, present study is focused on redox control of antioxidant and antihepatotoxic activities of *Cassia surattensis* seed extract against paracetamol intoxication in mice. The outcome from this work may add to the overall therapeutic value of traditional and herbal medicine in hepatic diseases.

2. Materials and Methods

2.1. Plant Sample Collection. The matured pods of *C. surattensis* were collected from Universiti Sains Malaysia (USM), Pulau Pinang, Malaysia. The *C. surattensis* plant (leaves with flowers and pods) was authenticated by a botanist at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, where a sample with voucher number 11464 has been deposited. The seeds were removed from the pods and were washed under running tap water to remove dirt prior to the drying process. The seeds were dried in an oven at 50°C. Then, the dried seeds were ground into powder and stored in airtight bottles.

2.2. Preparation of Plant Seed Extract. The powdered seeds (150 g) were soaked in methanol (500 mL) for 7 days under room temperature, 28°C [29]. The whole extract was filtered and methanol was evaporated from the filtrate by a rotary evaporator (Buchi, Switzerland) at 40–50°C to form a paste. Then, the extract was dried in the oven at 60°C to get a thick paste form. The crude extract was sealed in Petri plate and stored at room temperature, 28°C.

2.3. Total Phenolic Content. The total phenolic content of the extracts was determined using the method described by Li et al. [30]. One mL diluted Folin-Ciocalteau reagent was added to 1 mL of methanolic seed extract. Then, 4 mL sodium carbonate and 10 mL distilled water were added to the mixture. Subsequently, the mixture was allowed to stand 2 hours at room temperature, 28°C. The contents were centrifuged and absorbance was measured at 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated using various concentrations of gallic acid and a standard curve was constructed. Based on the measured absorbance, the concentration of phenolics was read (mg/mL) from the standard curve and the total phenolic contents of the extract were expressed as milligrams of gallic acid equivalents (GAE) per gram dry weight (mg GAE/g dw).

2.4. In Vitro Antioxidant Activity

2.4.1. Inhibition of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay. The DPPH radical scavenging activity of *C. surattensis* seed methanolic extract was carried out by previously described method by Sangetha et al. [24]. Five mL of a 0.004% (w/v) solution of DPPH in 80% methanol was added to 50 μL of methanolic seed extract at different concentrations (0.078, 0.16, 0.31, 0.63, 1.25, 2.50, 5.00, and 10.00 mg/mL resp.). The reaction mixture was shaken vigorously. Butylated hydroxytoluene (BHT, Sigma) was used as a reference standard. The discoloration of DPPH was measured at 517 nm after 30 minutes incubation in the dark. The lower absorbance of the reaction mixture indicated higher free

radical scavenging activity. All the tests were performed in triplicate.

The percentage DPPH radical scavenging was calculated using the following equation:

$$\%\ \text{DPPH radical scavenging} = \frac{A_o - A_1}{A_o} \times 100, \quad (1)$$

where A_o is the absorbance of the control and A_1 is the absorbance in the presence of the extract/standard.

2.4.2. Inhibition of Nitric Oxide Radical Scavenging Assay. The assay was conducted based on the modification method by Chakraborthy [31]. The reaction mixture contained 1.5 mL sodium nitroprusside (10 mM) in phosphate buffer saline pH 7.4 and 0.5 mL of the seed extract at various concentrations (1.95, 3.91, 7.81, 15.63, 31.25, 62.50, 125.00, 250.00, 500.00, and 1000.00 μg/mL) and incubated for 150 minutes at 25°C. Then, 1 mL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid) was added and the mixture was incubated for 30 minutes at room temperature, 28°C. The pink chromophore formed during diazotization of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylenediamine dichloride was measured at 546 nm. The activity of seed extract was compared with ascorbic acid which was used as a reference standard. All tests were performed in triplicate. The nitric oxide radicals scavenging activity were calculated according to the equation:

$$\%\ \text{Inhibition} = \frac{A_o - A_1}{A_o} \times 100\%, \quad (2)$$

where A_o is the absorbance of the control and A_1 is the absorbance in the presence of the extract/standard.

2.4.3. Reducing Power Assay. The reducing power assay was evaluated by the method of Oyaizu (1986) as described by Yildirim et al. [32]. One mL seed extract at various concentrations (0.02, 0.039, 0.078, 0.16, 0.31, 0.63, 1.25, 2.50, 5.00, and 10.00 mg/mL) was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide [K3Fe(CN)6] (1% w/v). The mixture was incubated at 50°C for 20 minutes in a water bath. The reaction was stopped by adding 2.5 mL of trichloroacetic acid (TCA) solution (10% w/v) to the mixture and then centrifuged at 3000 rpm for 10 minutes. Then, 2.5 mL of the upper layer solution was mixed with 2.5 mL distilled water and 0.5 mL Ferric chloride solution (0.1% w/v). The reaction mixture was then incubated for 10 minutes at room temperature. Absorbance of the resultant mixture was measured at 700 nm. The increased absorbance of the reaction mixture indicated enhanced reducing power. Ascorbic acid was used as a reference standard.

2.4.4. Calculation of Inhibition Concentration (IC_{50}). The IC_{50} is defined as the concentration of the sample that provides inhibition of 50% of the initial radical concentration with the unit, mg/mL or μg/mL. The IC_{50} values were calculated from the linear regression plots of various concentrations of methanolic extract of *C. surattensis* seed/reference standard against the mean percentage of % inhibition obtained from three replicate tests.

2.5. In Vivo Antihepatotoxic Activities

2.5.1. Animals. Fifteen adult male Swiss albino mice aged 6 to 8 weeks old and weighed 25 to 30 g were used to study the hepatoprotective activity of *C. surattensis* seed extract. The Animal Ethics Committee, Universiti Sains Malaysia, has approved the animal study for this project (USM/Animal Ethics Approval/2013/(90)(514)). The animals were kept under standard conditions (27 ± 2°C, relative humidity 44–56% and light and dark cycles of 10 hours and 14 hours, resp.) and fed with standard mice diet and purified drinking water ad libitum for 1 week before and during the experiments. The animals were obtained from Animal house of Universiti Sains Malaysia, Penang, and kept in cages under uniform husbandry condition, standard animal diet, and drinking water ad libitum. The food was withdrawn 18–24 hours before starting the experiment. All experiments were performed in the morning according to current guidelines for the care of the laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals [33].

2.5.2. Preparation of Paracetamol Dose Regimen and Treatments. The paracetamol tablets were obtained from a nearby pharmacy. Each tablet contains 500 mg of paracetamol. The mice were administered with paracetamol at a dose of 1 g/kg body weight (b.w.). The paracetamol was made into fine powder using a mortar and pestle. The powdered paracetamol was suspended in distilled water and was administered according to the body weight of mice. An aqueous suspension of seed extract was prepared in distilled water and different doses of *C. surattensis* seed extract (250 mg/kg b.w. and 500 mg/kg b.w.) and silymarin (200 mg/kg b.w.) were administered orally according to the body weight of mice [34].

2.5.3. Mice Groupings and Treatments. Fifteen adult male Swiss albino mice were divided into 5 groups and each group consists of 3 mice each (Table 1). The pretreated normal control group received 10% dimethyl sulfoxide (DMSO) orally. The induced group was pretreated with 10% DMSO orally and given paracetamol once only (dose 1 g/kg b.w.) orally. The treatment Group I received orally both doses of 250 mg/kg b.w. of *C. surattensis* seed extract and 1 g/kg b.w. paracetamol, while the treatment Group II received orally both doses of 500 mg/kg bw of *C. surattensis* seed extract and 1 g/kg bw paracetamol, respectively. The positive control group was given silymarin at the dose of 200 mg/kg b.w. and paracetamol at the dose of 1 g/kg b.w. The mice in treatment and positive control groups were pretreated with the respective dose of seed extract/silymarin orally once daily for 7 consecutive days. Paracetamol dose at 1 g/kg b.w. was given to mice to induce hepatotoxicity. The oral administration of paracetamol was performed 3 hours after the last seed extract/silymarin administration on the 7th day

Table 1: Mice groupings and administrated treatments.

Groups	Treatment
Negative control	10% DMSO
Induced	1.0 g/kg paracetamol per body weight
Treatment Group I	250 mg/kg seed extract per body weight + paracetamol
Treatment Group II	500 mg/kg seed extract per body weigh + paracetamol
Positive control	200 mg/kg silymarin per body weight + paracetamol

15 adult mice were divided into 5 groups ($n = 3$).

except for the normal control group, which received only 10% DMSO. All mice were euthanized after 48 hours after paracetamol-induced hepatotoxicity [34].

2.5.4. Biochemical Analysis. The mice of each group were anaesthetized with ketamine/xylazine and blood was collected directly from the heart. Then centrifuged at 3000 rpm for 15 minutes to separate the serum and kept at 4°C for analysis of various biochemical parameters including ALT, AST, and ALP [35]. All the analyses were performed using Hitachi 902 Automatic Analyzer using the adapted reagents from Roche (Germany) at Gribbles Pathology Laboratory Malaysia (M) Sdn. Bhd., Penang, Malaysia.

2.5.5. Determination Body Weight and Relative Liver Weight. The mice were weighed daily during the study and the body weights of the mice were determined and recorded. After the mice were euthanized, the livers were isolated and washed with saline and weights were determined by using an electronic balance [36]. The liver weight was expressed with respect to relative liver weight. Relative liver weight was calculated using this formula:

$$\text{Relative liver weight (\%)} = \frac{\text{Liver organ weight} \times 100}{\text{Body weight}}. \quad (3)$$

2.5.6. Evaluation of the Antioxidant Status in the Liver Homogenate. Livers were perfused with saline and homogenized in chilled potassium chloride (1.17%) using a homogenizer to determine the in vivo antioxidant level in the liver tissues.

(1) Glutathione (GSH) Activity Assay. The GSH activity was quantified by using commercially available Glutathione Assay Kit (Sigma-Aldrich, USA). Initially, 10 mL of liver homogenate was added and mixed properly with 150 μL of working solution consisting of 1.5 mg/mL DTNB, 6 U/mL glutathione reductase, and 1x assay buffer in a 96 well-plate before being incubated for 5 min. Subsequently, 50 mL of NADPH solution with a concentration of 0.16 mg/mL was added to each well in a 96 well-plate. Finally, the absorbance was measured by using an ELISA Plate Reader (Molecular Devices Inc., USA) at 412 nm wavelength at 1 min intervals for 5 min [37].

(2) The Malonyldialdehyde (MDA) Assay. Each liver homogenate (200 μL) was diluted with 800 μL of PBS and mixed with 25 μL of 8.8 mg/mL butylhydroxytoluene and 500 μL of 50% trichloroacetic acid. The mixture was vortexed, incubated for 2 h on ice, and centrifuged at 2000 ×g for 15 min. The supernatant (1 mL) was transferred into a new tube and mixed with 75 μL of 0.1 M EDTA and 250 μL of 0.05-M 2-thiobarbituric acid. The mixture was boiled for 15 min and allowed to cool to room temperature before the absorbance was measured at 532 and 600 nm in an ELISA Plate Reader (Molecular Devices Inc., USA) [37].

(3) Super Oxide Dismutase (SOD) Assay. Initially, a master mixture comprised 0.1 mol/L phosphate buffer, 0.15 mg/mL sodium cyanide in 0.1 mol/L ethylenediaminetetraacetic acid (EDTA), 1.5 mmol/L nitroblue tetrazolium, and 0.12 mmol/L riboflavin was prepared. Afterward, 200 μL of master mixture was added to 100 μL of serially diluted liver homogenates in a 96 well-plate before mixed thoroughly. Lastly, the absorbance was read by using an ELISA Plate Reader (Molecular Devices Inc., USA) at 560 nm wavelength and the SOD activity in the liver homogenate was expressed as units SOD/mg protein [37].

2.5.7. Histopathological Observations. The liver samples of the mice were fixed in 10% buffered formalin. After fixation, the livers were dehydrated in a graded series of alcohol, cleared in xylene, and embedded in paraffin wax. Multiple 5 μm sections from each block were mounted on slides. After staining with hematoxylin and eosin (H&E), slides were examined under a microscope for histopathological changes [34].

2.6. Statistical Analysis. Data are expressed as mean ± Standard Deviation (SD). Significance was evaluated using t-test and one-way ANOVA test (SPSS 13.0, SPSS Inc., Chicago, III) followed by Tukey post hoc multiple comparisons test for unpaired values. Regression analysis was performed to calculate the dose-response relation. Linear regression analysis was performed to find out the correlation coefficient. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Extract Yield. The extraction was carried out using matured *C. surattensis* seeds. The weight of powdered seeds was 52.22 g. The weight of the seeds extract in paste form was 14.92 g. The extraction process yielded 28.57% of *C. surattensis* seeds extract.

3.2. Total Phenolic Content. The total phenolic content of the *C. surattensis* seed extract was expressed as mg gallic acid equivalent/g dry weight and calculated by using the gallic acid standard curve equation: $y = 0.052x + 0.311$ ($R^2 = 0.992$). The total phenolic content of *C. surattenis* seed extract was 100.99 mg GAE/g dry weight.

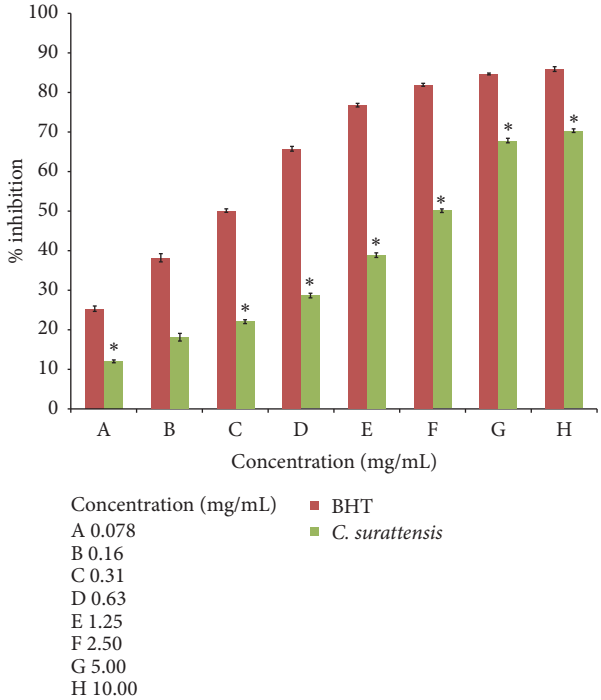

Figure 1: Percentage inhibition of methanolic seed extract of *C. surattensis* on DPPH free radicals compared to butylated hydroxytoluene (BHT). Each value is expressed as mean ± SD ($n = 3$), $^{*}p < 0.05$ compared with BHT.

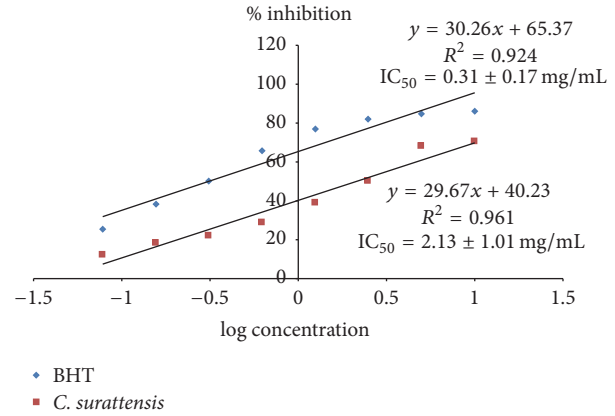

Figure 2: Inhibition effect of *C. surattensis* seed extract on DPPH free radicals compared with butylated hydroxytoluene (BHT).

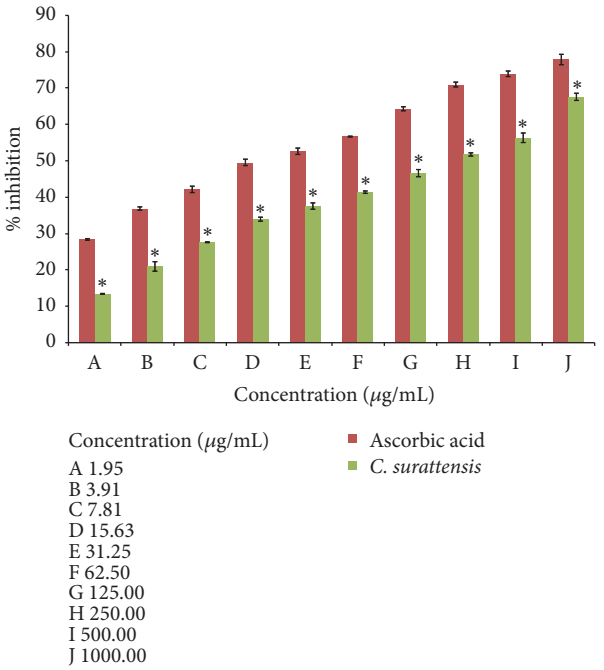

Figure 3: Percentage inhibition of methanolic seed extract of *C. surattensis* on nitric oxide radicals compared to ascorbic acid. Each value is expressed as mean ± SD ($n = 3$), $^{*}p < 0.05$ compared with ascorbic acid.

3.3. Radical Scavenging Activity

3.3.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay. Figures 1 and 2 show that the dose-response activity of DPPH radical scavenging activity of the methanol extract of the *C. surattensis* seed compared to the standard antioxidant BHT. The methanolic seed extract showed the highest scavenging activity, 70.39% ± 0.50 at 10 mg/mL, and lowest, 12.18% ± 0.35 at 0.078 mg/mL. The methanolic seed extract exhibited concentration dependent antioxidant activity by inhibiting DPPH radical with inhibitory concentration 50% (IC_{50}) value of 2.13 ± 1.01 mg/mL and BHT was 0.31 ± 0.17 mg/mL (Figures 1 and 2).

3.3.2. Nitric Oxide (NO) Radical Scavenging Assay. Dose-dependent NO scavenging activity of the methanol extract of the *C. surattensis* seed is shown in Figures 3 and 4. The methanolic seed extract showed the highest scavenging activity (67.60% ± 1.07) at 1000 μg/mL and lowest (13.42% ± 0.13) at 1.95 μg/mL. The methanolic seed extract of *C. surattensis* exhibited concentration dependent antioxidant activity by inhibiting nitric oxide radical with IC50 value of 164.06±1.13 μg/mL and ascorbic acid was 22.39±0.98 μg/mL (Figure 3). The IC50 values of the ascorbic acid were comparatively lower than seed extract which indicates higher antioxidant activity of ascorbic acid compared to the seed extract (Figure 4). Previously, Parul et al. [38] demonstrated that ascorbic acid has a strong antioxidant activity on the NO radical.

3.3.3. Reducing Activity. The dose-response action for the reducing activity of methanol extract of *C. surattensis* seed is shown in Figure 5. The seed extract demonstrated reducing power activity in all the concentration tested, in a concentration-dependent manner. The seed extract reduced ferricyanide complex (Fe3+) to the ferrous form (Fe2+) and this showed that the seed extract has antioxidant properties. The methanolic seed extract showed the highest absorbance, 1.366 ± 0.0036 at 10.00 mg/mL, and lowest, 0.235 ± 0.0021 at 0.02 mg/mL. The higher absorbance of the reaction solution indicates the greater reducing power and greater antioxidant activity [39]. Ascorbic acid showed greater reducing power than that of the *C. surattensis* seed extract.

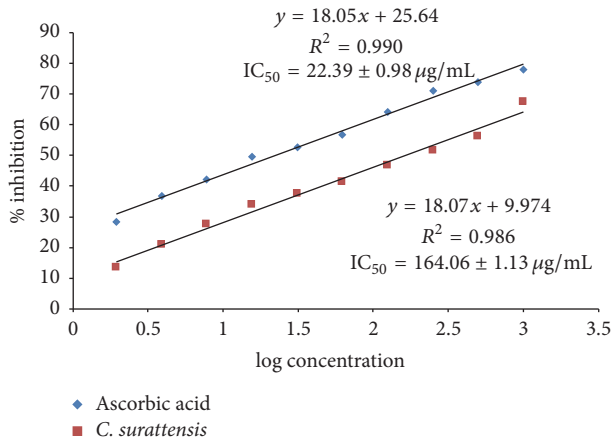

Figure 4: Inhibition effect of *C. surattensis* seed extract on nitric oxide radicals compared with ascorbic acid.

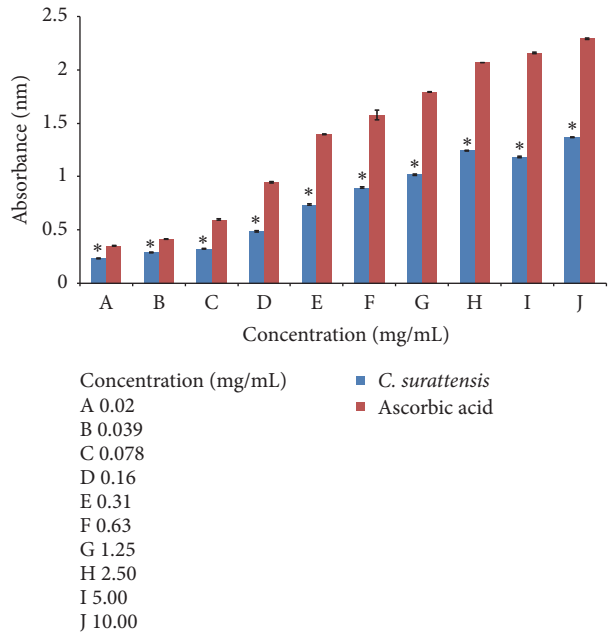

Figure 5: Reducing power of methanolic seed extract of *C. surattensis* compared to ascorbic acid. Each value is expressed as mean ± SD ($n = 3$), $^{*}p < 0.05$ compared with ascorbic acid.

3.4. In Vivo Antihepatotoxic Activities

3.4.1. Determination of Body Weight and Relative Liver Weight. Table 2 shows that the average body weights of the experimental animals were not affected by paracetamol, silymarin, and *C. surattensis* seed extract. The paracetamol administration caused a significant increase in the average liver weight of the paracetamol-induced group compared to the negative control group. The pretreatment with *C. surattensis* seed extract, at doses of 250 and 500 mg/kg b.w., and silymarin at a dose of 200 mg/kg bw significantly reduced the increased liver weight in paracetamol-induced group. A significant elevation of relative liver weight was seen in paracetamol-induced group, 8.15 ± 0.35%, when compared to the negative control group, 5.82 ± 0.31%, indicating the paracetamol-induced hypertrophy of these tissues. By contrast, *C. surattensis* seed extract, at dose of 250 mg/kg bw and 500 mg/kg bw, and silymarin at a dose of 200 mg/kg bw in combination with paracetamol significantly ($p < 0.05$) reduced the value of the relative liver weights to 5.99 ± 0.18%, 6.03 ± 0.47%, and 6.12 ± 0.41%, respectively, suggesting the possibility of *C. surattensis* seed extract to give protection against liver injury upon paracetamol administration.

3.4.2. Biochemical Analysis. The effect of *C. surattensis* seed extract on liver marker enzymes (ALT, AST, and ALP) is displayed in Table 3. The data exhibited that the negative control group demonstrated a normal range of ALT, AST, and ALP levels. However, paracetamol administration caused a significant elevation in the ALT, AST, and ALP levels to 1689 ± 102.14 U/L, 2998 ± 189.22 U/L, and 341.51 ± 38.11 U/L, respectively, compared to the negative control group with pretreated 10% DMSO. A single oral dose of paracetamol at 1 g/kg bw caused a drastic increase in the serum liver marker enzyme activities of ALT, AST, and ALP [40], indicating an acute hepatotoxicity induced by administration of paracetamol. According to the Table 3 data, the biochemical parameters of the *C. surattensis* seed extract pretreated group were greater than those of the negative control group ($p < 0.05$), but it showed much lower levels of ALT, AST, and ALP than the paracetamol-induced group; that is, the extract treatment significantly reduced the previously elevated levels of ALT, AST, and ALP in liver tissue of hepatotoxic mice.

3.4.3. The Antioxidant Status in the Liver Tissues. The in vivo antioxidant level in the liver tissues of paracetamol-intoxicated mice pretreated with *C. surattensis* seed extract was evaluated by various antioxidant assays, namely, GSH assay, SOD assay, and MDA assay (Table 4). The data exhibited that the negative control group revealed a normal range of GSH, SOD, and MDA levels, while the paracetamol-treated group displayed elevated levels of MDA and with decreased level of GSH and SOD, approving that paracetamol triggered liver injury at higher doses. However, the *C. surattensis* seed extract or silymarin pretreatment significantly elevated the previously dropped levels of GSH and SOD which led to depletion of the MDA levels in liver tissue. These findings clearly demonstrating the in vivo antioxidant activity *C. surattensis* seed extract at the dose of 250 mg/kg and 500 mg/kg by significantly reversing ($p < 0.05$) the effect produced by the paracetamol triggered liver injury.

3.4.4. Histopathological Observation. To further explore the biochemical analysis findings, histopathological observation was conducted on liver tissue. Liver sections taken from paracetamol-induced mice (Figure 6) had severe necrosis, vacuolar degeneration, loss of cellular boundaries, and obstruction of sinusoids, and hepatocytes were disrupted and showed hypertrophy compared to the healthy negative control group. The accumulation of neutrophils was also seen in the central vein. The neutrophils act as an indicator of the occurrence of cell damage as they are absent in

Table 2: Effect of *C. surattensis* seed extract on the body and liver weight of mice in paracetamol induced hepatotoxicity.

Groups	Dose (mg/kg)	Body weight, BW (g)	Liver weight, LW (g)	Relative liver weight (%) (LW/BW)
10% DMSO pretreated negative control	—	39.88 ± 3.15	2.32 ± 3.01	5.82 ± 0.31
Paracetamol-induced	—	38.99 ± 5.12**	3.18 ± 5.01**	8.15 ± 0.35**
Silymarin + paracetamol	200.00	41.51 ± 3.12*	2.54 ± 2.82*	6.12 ± 0.41*
Seed extract + paracetamol	250.00	39.85 ± 3.12*	2.39 ± 3.08*	5.99 ± 0.18*
Seed extract + paracetamol	500.00	40.08 ± 3.48*	2.42 ± 3.40*	6.03 ± 0.47*

Results are expressed in means ± SD ($n = 3$).
* $p < 0.05$ compared with paracetamol-induced group.
** $p < 0.05$ compared with 10% DMSO pretreated negative control group.

Table 3: Effect of *C. surattensis* seed extract on ALT, AST, and ALP (U/L) levels of mice in paracetamol-induced hepatotoxicity.

Groups	Dose (mg/kg)	ALT (U/L)	AST (U/L)	ALP (U/L)
10% DMSO pretreated negative control	—	16.72 ± 2.31	89.89 ± 4.12	114.3 ± 4.22
Paracetamol-induced group	—	1689 ± 102.14[a]	2998 ± 189.22[a]	341.51 ± 38.11[a]
Silymarin + paracetamol	200.00	601.1 ± 184.47[ab]	921.3 ± 298.21[ab]	185.47 ± 12.21[ab]
Seed extract + paracetamol	250.00	1105 ± 204.28[ab]	2031 ± 388.12[ab]	271.55 ± 27.32[ab]
Seed extract + paracetamol	500.00	801.43 ± 99.21[ab]	1389 ± 212.33[ab]	231.12 ± 11.17[ab]

Results are expressed in means ± SD ($n = 3$).
[a] $p < 0.05$ compared with 10% DMSO pretreated negative control group.
[ab] $p < 0.05$ compared with paracetamol-induced group.

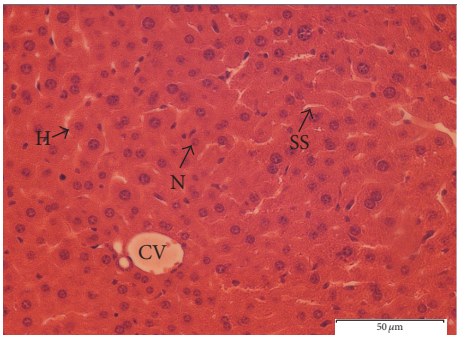

Figure 6: Light microphotograph of negative control liver. (H: hepatocytes; N: nucleus; SS: sinusoid; CV: central vein). Magnification: 40x.

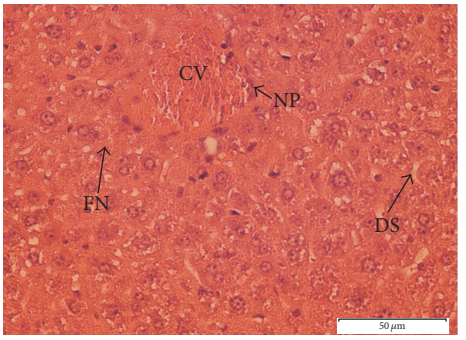

Figure 7: Light microphotograph of paracetamol-induced liver. (NP: neutrophil; DS: dilated sinusoid; FN: focal necrosis; CV: central vein). Magnification: 40x.

normal healthy cells (Figure 7). Histopathological analysis showed that silymarin as well as the seed extract at a dose of 500 mg/kg b.w. significantly improved the degree of hepatocytes degeneration and necrosis in paracetamol-induced mice.

The hepatocyte nucleases are at a recovery stage and there are very minimal numbers of neutrophils surrounding the central vein.

The pretreated group with seed extract at a dose of 500 mg/kg b.w. (Figure 9) and silymarin at a dose of 200 mg/kg b.w. (Figure 10) was very close to the negative control group which showed intact liver cells. Histopathological liver sections of control group showed normal cellular structure and clear central vein, and hepatic cells were distinct and separated by sinusoidal spaces. The seed extract at a dose of 250 mg/kg b.w. did not show significant improvement over the effect of paracetamol on the liver (Figure 8). Though the extent of hepatocytes degeneration was a little lower than in the paracetamol-induced group, liver architecture (necrosis) was slightly improved.

4. Discussion

4.1. Total Phenolic Content. This study showed that *C. surattensis* seed extract contained favourable amount of phenolic compounds. A few studies have reported that *C. surattensis* plant parts possess a significant total phenolic content. For

Table 4: Effect of *C. surattensis* seed extract on oxidative status in paracetamol-induced hepatotoxicity mice liver tissue.

Groups	Dose (mg/kg)	GSH (mg/mg protein)	SOD (U/mg protein)	MDA (nmol/mg protein)
10% DMSO pretreated negative control	—	4.13 ± 0.12	120.82 ± 1.53	1.45 ± 0.11
Paracetamol-induced	—	2.35 ± 0.05	50.07 ± 1.22**	5.04 ± 0.08**
Silymarin + paracetamol	200.00	4.40 ± 0.04	136.69 ± 1.31*	1.64 ± 0.01*
Seed extract + paracetamol	250.00	3.80 ± 0.01	108.56 ± 3.51*	1.87 ± 0.08*
Seed extract + paracetamol	500.00	4.21 ± 0.09	130.34 ± 1.00*	1.68 ± 0.05*

Results are expressed in means ± SD ($n = 3$).
* $p < 0.05$ compared with paracetamol-induced group.
** $p < 0.05$ compared with 10% DMSO pretreated negative control group.

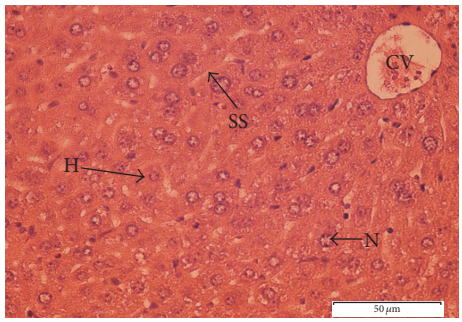

Figure 8: Light microphotograph of liver cells of mice treated with *C. surattensis* (250 mg/kg) (H: hepatocytes; N: nucleus; SS: sinusoid; CV: central vein). Magnification: 40x.

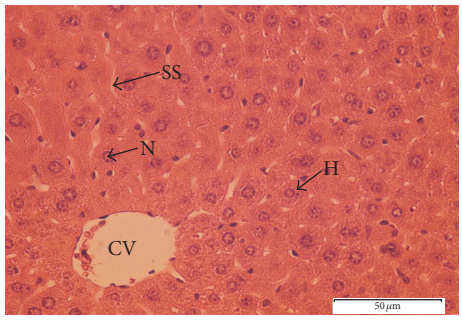

Figure 9: Light microphotograph of liver cells of mice treated with *C. surattensis* (500 mg/kg) (H: hepatocytes; N: nucleus; SS: sinusoid; CV: central vein). Magnification: 40x.

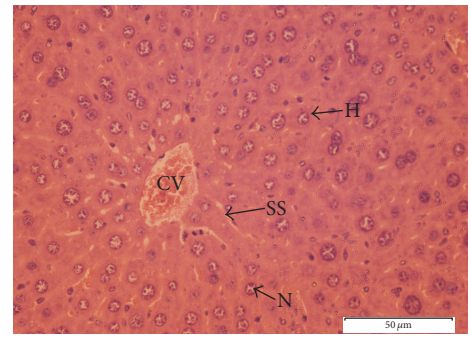

Figure 10: Light microphotograph of liver cells of mice treated with silymarin (H: hepatocytes; N: nucleus; SS: sinusoid; CV: central vein). Magnification: 40x.

example, Sangetha et al. [24] reported that phenolic compounds were found in flower, stem, leaves, and pod of *C. surattensis* extracts and among the plant parts, only the flower, stem, and leaves of *C. surattensis* extracts showed a significant high content of total phenolics. Chew et al. [41] have reported that the flowers of *C. surattensis* extract contain total phenolic content of 3330 ± 309 mg GAE/100 g.

The result of the present study clearly indicated that the methanol extract of *C. surattensis* seed exhibited the presence of phenolic compounds and there was a strong positive correlation with the DPPH radical scavenging activity ($r = 0.968$) and NO radical scavenging activity ($r = 0.979$). The same relationship was also observed between phenolics and antioxidant activity in Sea Buckthorn extracts [42]. Kaneria et al. [43] reported that there are high correlation between phenolic contents and antioxidant activities of some medicinal plant extracts such as *Azadirachta indica, Hemidesmus indicus, Manilkara zapota, Psoralea corylifolia, Rubia cordifolia,* and *Tinospora cordifolia*. These show that the presence of a significant amount of total phenolic content in the seed extract might effectively inhibit radicals and contribute directly to the effective antioxidant activities. The phenolic compound in plant donates their hydrogen atoms from their hydroxyl groups to radicals and form a stable phenoxyl radical and this reaction contributes to their antioxidant activity [44].

4.2. Radical Scavenging Activity

4.2.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay. The DPPH radical is regularly used as a substrate to evaluate the antioxidant activity of medicinal plant; it is a stable free radical that can receive an electron to become a stable molecule. The *C. surattensis* seed extract with higher concentration showed higher bleaching ability of the $DPPH^{\bullet}$ solution with greater hydrogen atom donating activity and higher antioxidant activity. The antioxidant activity of the extract might be attributed to the hydrogen donating ability of the phenolic hydroxyl groups [45, 46]. The DPPH scavenging activities of *C. surattensis* seed extracted with acetone have been reported in previous study by Deepak et al. [27].

According to the study, acetone extract showed maximum antioxidant activity, 76.11% than other extracts (methanol, chloroform) in comparison to standard drug, ascorbic acid.

4.2.2. Nitric Oxide (NO) Radical Scavenging Assay. Nitric oxide (NO) is a key chemical facilitator involved in the regulation of various physiological processes. Higher concentration of NO is related with numerous diseases. Oxygen molecules react with the excess amount of NO to generate nitrite and peroxynitrite anions, which eventually act as free radicals [47]. The results of this study indicate that all the concentrations of *C. surattensis* seed extract and ascorbic acid tested have noticeable inhibition effect on nitric oxide radicals. The seed extract exhibited antioxidant activity through competing with oxygen to scavenge for the nitrite radical that was generated from sodium nitroprusside (SNP) at physiological pH in an aqueous environment. The *C. surattensis* seed extract acts as a potent antioxidant and donate protons to the nitrite radical. This decreased the absorbance and increased the percentage of inhibition. The decrease in absorbance and increase in percentage of inhibition were used to measure the extent of nitrite radical scavenging and antioxidant activity [48]. The NO scavenging activity of phenolic compounds is known [49–51], and this suggests that these compounds in the seed extract might contribute to the NO scavenging activity observed in this study.

4.2.3. Reducing Activity. Reducing power assay is commonly used to assess the capability of a natural antioxidant to donate an electron [52]. This finding of this study affirms the reducing power of *C. surattensis* seed extract. It was hypothesized that the reducing power of *C. surattensis* seed extract might be due to their electron-donating ability and higher amount of reductants contains. Electrons or hydrogen atoms might react with free radicals to stabilize or transform the free radicals into more stable and nonreactive products subsequently blocking the free radical chain reactions [53, 54]. Therefore, *C. surattensis* seed extract could act as electron donors to free radicals and then dismiss the free radical chain reactions.

4.3. In Vivo Antihepatotoxic Activities

4.3.1. Determination of Body Weight and Relative Liver Weight. The body weight of the mice in this study was not affected by the treatment of paracetamol, silymarin, and *C. surattensis* seed extract. This finding was parallel with the findings by Saad et al. [55] on the failure of thioacetamide to cause changes in body weight of rats in acute liver injury study. The increase in the liver weight and relative liver weight of paracetamol-induced liver hepatotoxicity group compared to negative control, silymarin and *C. surattensis* seed extract (doses of 250 mg/kg b.w. and 500 mg/kg b.w.), pretreated groups might be associated with the blocking of hepatic triglyceride secretion into plasma or might be due to the very large increase in liver hemoglobin [56]. On the other hand, the pretreatment with silymarin and seed extract prevented the increase in liver weight and relative liver weight when compared to paracetamol-induced group, which proved the significant hepatoprotective effect.

4.3.2. Biochemical Analysis. Paracetamol induced mice liver damage in this study because of the formation of toxic metabolite, NAPQI, which is initially detoxified by conjugation with glutathione to form mercapturic acid [57]. The overdose of paracetamol used in this study caused the glutathione content of hepatocytes exhausted and the hepatocytes become vulnerable to the noxious effects of NAPQI resulting in liver cell necrosis or liver failure that eventually increased the ALT and AST levels [58, 59] which may explain the findings of this study. It was concluded that a single dose of paracetamol had caused damage to the parenchymal cell in the liver that leads to the leakage of the aminotransferases (ALT and AST) into the blood serum, resulting in increased of its concentrations. The aminotransferases that are released into the blood circulation when there is hepatic necrosis making the enzymes measurable in serum [60–62].

However, pretreatment of high dose of *C. surattensis* seed extract at 500 mg/kg b.w. and silymarin at 200 mg/kg b.w. exhibited an ability to counteract the toxic effect of paracetamol by decreasing the level of these enzymes. The pretreatment of *C. surattensis* seed extract at a dose of 250 mg/kg b.w. caused a slight decrease in the elevated serum liver enzymes. The results of this study supported the generally accepted view that serum levels of aminotransferases return to normal levels with the healing of hepatic parenchyma and regeneration of hepatocytes [63, 64]. The decrease in AST and ALT levels by the seed extract was also an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by paracetamol.

The pretreatment with the 250 mg/kg b.w. dose of seed extract slightly reduced the elevated serum liver enzyme levels but not as effective as the treatment with 500 mg/kg b.w. dose of the seed extract. This proved that *C. surattensis* seed extract at higher dose, 500 mg/kg b.w., showed a better hepatoprotective effect compared to the dose at 250 mg/kg b.w. This might be because the amount of antioxidant polyphenol compound(s) in the extract which render the free radical scavenging activity present at higher dose in 500 mg/kg b.w. compared with 250 mg/kg b.w. dose of *C. surattensis* seed extract. Interestingly, the in vitro antioxidant activity study showed that the *C. surattensis* seed extract exhibited a concentration dependent activity which supports this explanation.

This study also showed that in mice group with paracetamol-induced liver hepatotoxicity, the ALT and AST levels were elevated more compared to the lesser increase in the ALP level. This finding indicated that paracetamol caused cell necrosis at a single dose of 1 g/kg b.w. as cell necrosis involves the initial increase of ALT and AST and modest increase in ALP. The ratio of ALT/AST : ALP plays an important role in the determination of the types of liver damage by hepatotoxins. The ratio is greater than or equal to five for cell necrosis injury compared to if the ratio is less than or equal to two for cholestasis injury.

4.3.3. The Antioxidant Status in the Liver Tissues. The ROS induced oxidative stress inside the cells triggered damage to hepatic parenchymal cells leading to hepatic injury [65]. The raise of ROS levels related with hepatic injury is due to high generation of ROS and reduced scavenging potential of the cells intrinsic antioxidants such as GSH and SOD [65]. Moreover, ROS may cause membrane damage by lipid peroxidation, and MDA is one of the final products of polyunsaturated fatty acids peroxidation in the cells [66]. In this study, paracetamol-induced liver hepatotoxicity significantly reduced the levels of SOD and GSH and elevated the level of MDA, which is a marker of lipid peroxidation, compared to negative control. The overproduction of NAPQI by overdose of paracetamol caused the glutathione content of hepatocytes exhausted and resulting in the reduction of GSH levels in liver tissue [58, 59]. The *C. surattensis* seed extract pretreatment was found increased in the level of intrinsic antioxidants such as GSH and SOD, with reduction in the elevated level of MDA, caused by the paracetamol-induced liver hepatotoxicity. Silymarin also caused significant favourable effects on the levels of GSH, SOD, and MDA relative to the paracetamol-induced group. These results propose that *C. surattensis* seed extract caused hepatoprotective activity linked with the improvement of in vivo antioxidant activity.

4.3.4. Histopathological Observation. The methanolic extract of *C. surattensis* seeds showed significant hepatoprotective activity on paracetamol-induced liver toxicity when administered at a high dose of 500 mg/kg orally. The histopathological liver sections of *C. surattensis* seed extract (dose 500 mg/kg b.w.) pretreated group showed a comparable results to the silymarin pretreated group (dose 200 mg/kg b.w.) in recovering the paracetamol-induced histopathological lesions. These findings suggest the protection ability of 500 mg/kg b.w. dose of *C. surattensis* seed extract on membrane fragility and thus decreased the leakage of the serum liver enzymes into the blood circulation as observed in this study.

The seed extract which exhibited a concentration dependent activity was not effective in recovering histopathological lesions at a dose of 250 mg/kg b.w. as *C. surattensis* seed extract is more effective at higher concentration. The liver histopathological analysis was positively concomitant with the biochemical analysis of this study. The macroscopic appearance of the paracetamol-induced liver showed broad areas of necrosis that helps the leakage of the serum liver enzymes into the blood stream, and this explains the rise in AST, ALT, and ALP in the blood. The hepatotoxic effect of paracetamol is mainly due to generation of free radicals following the depletion of endogenous antioxidants such as glutathione [67]. Therefore, the herbal green *C. surattensis* seed antioxidant activity is effective in treating paracetamol-induced liver hepatotoxicity by scavenging the free radicals generated by the reaction of paracetamol.

In the present study, the *C. surattensis* seed extract was administrated at 250 mg/kg b.w. (low dose) and 500 mg/kg b.w. (high dose) based on the finding on acute oral toxicity of *C. surattensis* flower methanolic extract on Swiss albino mice. No sign of toxicity in mice was reported in the study conducted by Sumathy et al. [68]. According to the OECD guidelines for testing of chemicals 420 for testing of chemicals [69], animals should only be administrated a single dose of 5000 mg/kg. Hence in this study, the high dose of 500 mg/kg bw was selected as 1/10 of the dose that was used in the acute oral toxicity study (5000 mg/kg) and the low dose of 250 mg/kg b.w. was 2 times reduction of the high dose. Moreover, there were several studies that used doses of 250 mg/kg b.w. and 500 mg/kg b.w. to evaluate the hepatoprotective activity of plants [34, 70].

The methanolic extract of *C. surattensis* seeds in this study showed a good scavenging activity of DPPH and nitric oxide radicals. Hence, the in vitro findings further explained the potential of *C. surattensis* seed hepatoprotective activity in paracetamol-induced liver toxicity in mice and proved that the hepatoprotective activity may be due to the observed redox control of antioxidant activity of *C. surattensis* seeds. Similar hepatoprotective activity of *C. surattensis* leaf extract was also determined in the previous study designed to investigate the effect of ethanolic extract of *C. surattensis* leaf on CCl_4-induced liver toxicity in rat. The results of the previous study suggested that *C. surattensis* leaf extract significantly reduced the elevated levels of AST, ALT, and ALP in serum which indicates the efficacy of the leaf extract as a redox control hepatoprotective agent [28]. These current histopathological findings further verified the redox control antihepatotoxic activities of *C. surattensis* seed extract in paracetamol-induced liver toxicity in mice model.

5. Conclusions

In conclusion, the results of the present study strongly suggested that *C. surattensis* seed has redox control of antioxidant and antihepatotoxic activities at lower dose by enhancing antioxidant activity and protecting the liver from the toxic effect of the hepatotoxic agents.

Competing Interests

The authors have no conflict of interests to declare concerning this article.

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Research Article

Colonic and Hepatic Modulation by Lipoic Acid and/or N-Acetylcysteine Supplementation in Mild Ulcerative Colitis Induced by Dextran Sodium Sulfate in Rats

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Lipoic acid (LA) and *N*-acetylcysteine (NAC) are antioxidant and anti-inflammatory agents that have not yet been tested on mild ulcerative colitis (UC). This study aims to evaluate the action of LA and/or NAC, on oxidative stress and inflammation markers in colonic and hepatic rat tissues with mild UC, induced by dextran sodium sulfate (DSS) (2% w/v). LA and/or NAC (100 mg·kg·day^{-1}, each) were given, once a day, in the diet, in a pretreatment phase (7 days) and during UC induction (5 days). Colitis induction was confirmed by histological and biochemical analyses (high performance liquid chromatography, spectrophotometry, and Multiplex®). A redox imbalance occurred before an immunological disruption in the colon. NAC led to a decrease in hydrogen peroxide (H_2O_2), malondialdehyde (MDA) levels, and myeloperoxidase activity. In the liver, DSS did not cause damage but treatments with both antioxidants were potentially harmful, with LA increasing MDA and LA + NAC increasing H_2O_2, tumor necrosis factor alpha, interferon gamma, and transaminases. In summary, NAC exhibited the highest colonic antioxidant and anti-inflammatory activity, while LA + NAC caused hepatic damage.

1. Introduction

The use of antioxidants is considered an important complementary therapy in several conditions such as cardiometabolic, neurological, and gastrointestinal (GI) diseases, for cancer prevention and others [1]. Among the GI disorders, inflammatory bowel diseases (IBD), composed of ulcerative colitis (UC) and Crohn's disease (CD), affect 150–250/100,000 people, especially in the USA and Europe, with substantial health care costs of approximately US$6 billion and €4.6–5.6 billion, per year, respectively [2, 3].

Despite extensive research, the etiology of IBD has yet to be elucidated; however it is considered closely connected to genetic and immunologic factors, microbiota, and oxidative stress, which, in the case of UC, concerns both etiology and symptoms such as increase of intestinal permeability by destruction of tight junctions and increase of infection and inflammation by neutrophil infiltration. Hence, the presence

of colonic ulcers, as a consequence of lipid peroxidation and protein damage and development of cancer, is due to DNA damage [4]. However, the cause of these symptoms and clinical manifestations is heterogeneous, varying according to the UC level: mild, moderate, or severe. In humans, the most common and problematic stage of UC is mild colitis [5]. It is characterized by normal albumin, body temperature, pulse, and hematocrit ratio, associated with an erythrocyte sedimentation rate of <20 mm/h, less than 4 bowel movements per day and no weight loss [6]. Nevertheless, this classification may differ according to authors and adopted criteria [7, 8].

The extraintestinal manifestations of IBD, such as the systemic effects of alternative therapy used for IBD treatment, are poorly explored. Among these manifestations, emphasis is given to hepatobiliary disorders. The close relationship between the liver and intestine is justified by their common embryogenesis until later in adult life (intestine to portal vein) [9]. However, the main focus of the cited studies was microbiota, which has received special attention due to its intimate connection with metabolic syndrome, obesity, and nonalcoholic fatty liver diseases [10].

A pioneer study showed that, after colitis induction (4% of DSS for 7 days), important liver injury occurred, confirmed by higher serum levels of haptoglobin and various histological findings, such as signs of necrosis and ballooning of hepatocytes [11].

The use of antioxidants has emerged as an alternative therapy for IBD. Among them, lipoic acid (LA) and *N*-acetylcysteine (NAC) stand out, which have been tested separately in UC [4], and in combination for other clinical conditions [11, 12], with positive results.

Due to its antioxidant property in both forms, oxidized (LA) and reduced (dihydrolipoic acid, DHLA), LA is called a "universal antioxidant." The LA/DHLA couple is a scavenger of superoxide anion radical ($O_2^{\bullet -}$), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$), and nitric oxide ($^{\bullet}NO$). It is able to restore the exogenous antioxidants tocopherol and ascorbic acid and the endogenous antioxidant system of reduced/oxidized glutathione (GSH/GSSG) [6]. Additionally, *in vitro* studies suggest that LA acts as an inhibitor of IκB kinase-2 (IKK2), with subsequent release of nuclear factor kappa B (NF-kB) [13]. Another anti-inflammatory effect is the elevation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) intracellular levels that occurs through independent mechanisms, by breaking links between Nrf2 and Keap 1 (Kelch ECH associating protein 1). These actions lead to decreased expression of various proinflammatory cytokines and increased expression of enzymatic antioxidants, such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) [14].

NAC is a strong reducing agent [15]. Like LA, NAC is an important scavenger of reactive molecules and plays a role as a metal chelator. However, the most important antioxidant action assigned to NAC is the increase in antioxidant defense activity by providing cysteine, which is required for GSH synthesis. The anti-inflammatory effects of NAC have also been confirmed, through observation of NF-kB inhibition [16].

Taking into consideration that LA and NAC, alone and in combination, were successfully tested in several systems and in regard the intimate bowel-liver association, the aim of this study was to evaluate the action of LA and/or NAC, on oxidative stress and inflammation in mild colitis induced by DSS.

2. Materials and Methods

2.1. Chemicals. DSS MW 36,000–40,000 Da is from MP Biomedicals. LiChrolut®, RP-18 E is from Merck, cytokine kits are from Milliplex MAP Mouse Cytokine/Chemokine Panel (Merck Millipore®), protease inhibitor cocktail tablets are from Roche®, and RIPA buffer are from Cell Signaling®. LA and NAC from Ao Pharmaceutico (Alagoas/Brazil) and all other chemicals and enzymes from Sigma-Aldrich® Chemicals.

2.2. Equipment. HPLC coupled to UV detector (Shimadzu®), freezer VIP Series Sanyo, spectrofluorometer (ThermoScientif® Multiskan), and an optic microscopy Olympus BX51 attached to a DP70 Digital Camera System and a MAGPIX® Multiplex Reader were used.

2.3. Animals. Experimental protocol was approved by the Institutional Animal Ethic Committee/Universidade Federal de Alagoas (IAEC/UFAL) (05/2014) and was performed in accordance with the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) guidelines. Adult male Wistar rats, 185–203 g, were obtained from the Central Animal House of UFAL and kept in individual cages, under controlled conditions, at room temperature ($22 \pm 2^{\circ}$C), humidity ($50 \pm 10\%$), and light (12/12 h light/dark). Commercial feed (Nuvilab®) and water were provided *ad libitum*.

2.4. UC Induction and Experimental Design. Animals were divided into 5 groups. Healthy rats received commercial feed (control group). Mild UC was induced in four groups with 2% w/v DSS, administered in drinking water for 5 days. Antioxidants-treated rats were supplemented 7 days before mild colitis induction with lipoic acid (LA, 100 mg/kg/d), N-acetylcysteine (NAC, 100 mg/kg/d), or LA + NAC (100 mg/kg/d, each). Doses of the two antioxidants used are considered safe to rats/mice (LA up to 1800 mg/d [17] and NAC up to 6000 mg/d) and have been tested successfully in several physiological/pathophysiological conditions in mice/rats [16, 18].

2.5. Euthanasia, Blood Sample Collection, General Biochemical Profile, and Preparation of Tissue Homogenates. At the 13th day, after 12 h of fasting, the animals were anesthetized (ketamine, 100 mg/kg, and xylazine, 15 mg/kg, via i.p.), and blood was collected by cardiac puncture. After perfusion with heparine solution (1 : 50 v/v), aorta was sectioned. The organs were, then, dissected, placed on liquid nitrogen, and immediately stored at -80°C. Tissue homogenates were prepared, within ice cold, with Ripa buffer with protease inhibitor cocktail (one tablet for 50 mL of Ripa buffer), and were

centrifuged at 19.600 ×g/20 min, at 4°C. The supernatants were stored at −80°C.

Biochemical analyses were made by certified laboratory, using well-established methods. Colon and liver tissues were washed in saline and weighed. The left lobe of the liver and distal colon were cut, for use in histological analysis.

2.6. Histological Analysis. After fixation with 10% buffered formalin, the organs were cleaved and the sections were processed by embedding in paraffin and stained with hematoxylin and eosin (HE) to histological evaluation.

Total collagen was evaluated by Masson trichrome staining kit. Briefly, one section of each, liver and bowel, was obtained and stained by using Masson's original trichrome stain, with collagen stained in blue, nuclei in purple-brown, and cytoplasm in pink. Collagen area was defined as the distinct blue color region and was distinguished from muscle, blood, and inflammatory cells. Afterwards, ImageJ® software was used to quantify the blue area (pixel/camp).

2.7. Malondialdehyde (MDA), Hydrogen Peroxide (H_2O_2), and Nitrite Levels. MDA contents were measured by reverse phase ion pair HPLC, with UV detector at 270 nm, according to Tatum et al. [19]. HPLC system conditions were C18, ultrasphere column 150 mm × 4.6 mm and 45 mm × 4.6 mm guard column, the mobile phase comprised acetonitrile HPLC/UV grade, and Trizma buffer (pH 7.4) (1 : 9). The intestinal or hepatic tissue was homogenized with Trizma buffer, BHT, and acetonitrile. Then, the homogenate was centrifuged at 872 ×g/10 min, at 4°C, and the supernatant was filtrated with HPLC filter (0.22 mm). The flow rate was 1.0 mL·min^{-1} and MDA was calculated from the standard curve, generated using 1,1,3,3-tetramethoxypropane and expressed as nmol MDA·mg tissue^{-1}. The retention time was around 2 min 40 s.

H_2O_2 was measured according to Pick and Keisari [20]. Hepatic or colonic tissues were homogenized in 1.0 mL of assay buffer containing 140 mM NaCl, 10 mM PBS, pH 7.0, and 5.5 mM dextrose. After centrifugation (2000 ×g/5 min at 4°C), the supernatant was transferred to microtubes, containing 0.28 mM phenol red, 8.5 U/mL of horseradish peroxidase, and assay buffer. After incubation (37°C for 30 min), NaOH 1 M was added. The samples were read at 610 nm. The concentration was expressed as nmol·mg protein^{-1}.

Nitrite assay was performed based on Griess method adapted for microplates. Supernatant was mixed with 2,3-diaminonaphthalene (50 μg·mL^{-1} in 0.2 M HCl) and incubated at room temperature/10 min. The reaction was interrupted by the addition of NaOH (2.8 M) and monitored at 540 nm. The result was expressed as μmol·mg protein^{-1}.

2.8. Measurement of Enzyme Activity. SOD was measured following S. Marklund and G. Marklund [21]. Liver or colon supernatant was added to 0.2 mM pyrogallol (dissolved in 50 mM potassium phosphate buffer (PBS), pH 6.5) to initiate the reaction, and the decrease of the absorbance related to pyrogallol was monitored at 420 nm. One unit of SOD was defined as the amount required for inhibiting pyrogallol autoxidation by 50% per min. The result was expressed as U·mg protein^{-1} [22].

CAT activity was measured as the rate of decomposition of H_2O_2, as described elsewhere [23], and was monitored at 540 nm. Relative activity was expressed as IU·min·mL·mg protein^{-1}.

Total GPx activity was measured according to Flohe and Gunzler [24], adapted to microplate. The hepatic or colonic tissue was homogenized with assay buffer (PBS 0.1 M, EDTA 5 mM, pH 7.4) and centrifuged (14.000 ×g/20 min, at 4°C). Supernatant was added to wells (in duplicate), followed by the addition of glutathione reductase (GR) (0.048 U) and GSH (10 mM), incubated at 37°C/10 min and afterwards, nicotinamide adenine dinucleotide phosphate (NADPH) (1.5 mmol) and *tert*-butyl hydroperoxide (0.5 mM) were added. The decrease in the absorbance of the system was measured, for 5 min, at 340 nm. One unit of tGPx was defined as the amount of enzyme able to catalyze the oxidation of 1 μmol of NADPH to $NADP^+$ in 1 min. The result was expressed as U·mg protein^{-1}.

MPO activity was measured according to Bradley et al. [25]. Hepatic or colonic tissue was homogenized using assay buffer pH 6.0 (PBS, 50 mM, 0.5% hexadecyltrimethylammonium bromide and EDTA, 5 mM) and centrifuged at 1550 ×g/15 min (4°C). Supernatant was collected and centrifuged at 14.000 ×g/15 min (4°C). The sample was transferred (duplicate) to microplate and *ortho*-dianisidine solution (0.8 mg/mL) was added. After incubation (37°C/15 min), a solution of H_2O_2 (0.3%) was added. A new incubation was performed (37°C/10 min) and the reading was made at 460 nm. One unit of MPO was defined as the quantity that decomposes 1 μmol of H_2O_2. The result was expressed as U·mg protein^{-1}.

2.9. Measurement of Total GSH. GSH and GSSG were measured according to Tripple and Rogers [26], with slight modifications. First, total protein was precipitated, after centrifugation (190 ×g/10 min, at −20°C) with metaphosphoric acid (1 : 1). To obtain total GSH (tGSH), this supernatant was diluted in assay buffer (PBS 0.1 M, EDTA 5 mM, pH 7.4). Then, the homogenates were transferred to microplates, and the final volume (150 μL) was completed with assay buffer. Afterwards, the reaction was started, upon addition of Reaction Mixture 1 (containing 5,5′-dithiobis-(2-nitrobenzoic acid), DTNB, 10 mM, and GR, 4.2 U/mL) and NADPH, 1% w/v.

To measure GSSG, the supernatant was diluted (1 : 50) in assay buffer, containing N-ethylmaleimide (NEM), and centrifuged (10,000 ×g/20 min at 4°C). This solution was incubated for 50 minutes to complete GSH complexation and removal. To exclude NEM, this supernatant with assay buffer were eluted through Sep-pak® Classic C18 cartridges. Afterwards, the eluent was transferred to microplate and Reaction Mixture 1 and NADPH,1% w/v, were added.

In both analyses, the absorbance was measured over 3 min at 412 nm with 30 s intervals. GSH was determined according to the following equation: GSH = tGSG − (GSSG/2). Results were expressed in nM·mg protein^{-1}.

Table 1: Absolute and relative weights of colon and liver for the different groups (mean ± SEM), according to group: control; mild colitis; lipoic acid, LA; N-acetylcysteine, NAC; LA associated with NAC – LA + NAC.

Tissue/Ratio	Control	Mild colitis	LA	NAC	LA + NAC
Colon (g)	2.54 ± 0.10	2.30 ± 0.14	2.21 ± 0.11	2.19 ± 0.15	2.02 ± 0.10
Colon/body weight ratios	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Liver (g)	8.74 ± 0.40	8.76 ± 0.28	8.57 ± 0.31	8.22 ± 0.39	8.40 ± 0.14
Liver/body weight ratios	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00

2.10. TNF-α, IL-10, and Interferon Gamma (INFγ) Levels. Cytokine production was quantified by Milliplex MAP Mouse Cytokine/Chemokine Panel (Merck Millipore), following the manufacturer's instruction. The results were expressed as pg·mg protein^{-1}.

2.11. Statistical Analysis. Normality was assessed by the Kolmogorov-Smirnov test. Parametric variables were evaluated using the paired one-way analysis of variance (ANOVA), followed by Tukey's or Bonferroni's posttest. Student's *t*-test was performed just to confirm the disease in Mild colitis group. The Kruskal-Wallis test was used for assessing the nonparametric variables and corresponding *post hoc* analysis. Results were shown as mean ± standard error (SEM) for those with normal distribution and as median values and interquartile range for the nonparametric ones. Two-sided p value <0.05 was considered statistically significant. GraphPad® Prism version 5.0 for Windows software (San Diego, CA, USA) was used.

3. Results

For the sake of clarity, results are divided into three topics: general, colonic, and hepatic results.

3.1. General Results

3.1.1. Mild Colitis and Supplementation Did Not Alter Body Weight, Food Intake, or Liver and Colon Weights. In both phases (PT and T) of the study, DSS or supplementation by antioxidants did not induce effects on body weight or food intake patterns compared to the control group (Figures 1(a) and 1(b)). Similarly, body weight was unchanged over the evaluation period (Figure 1(c)). Absolute and relative liver and colon weights were unchanged (Table 1). Water ingestion modification was also not observed (data not shown).

3.1.2. LA and NAC, but Not LA + NAC, Decreased Anemia and Leukocytosis Caused by Mild Colitis. Anemia in the mild colitis group was confirmed by a decrease in red blood cells (RBC) and hemoglobin (HB) (Figures 2(a) and 2(b)), since clinical changes such as rectal blood, diarrhea, and weight loss were not observed or did not show differences between the groups. No macroscopic change was observed after euthanasia (data not shown). All treatments increased the parameters (RBC, HB). However, LA and NAC alone ameliorated the typical UC inflammation, represented by a decrease in leukocytes, while the combination of LA + NAC did not show beneficial effects (Figure 2(c)).

3.1.3. LA + NAC Increased Levels of Aminotransferases. The use of DSS and NAC did not cause a change in the available serological biomarkers, unlike LA supplementation, which promoted a decrease of globulin levels versus the mild colitis group ($p < 0.01$). However, this biochemical alteration did not exhibit physiological relevance, since albumin, the most important biomarker of hepatic function, remained statistically unaltered among the groups (Table 2). Moreover, it is important to observe that the combined action of LA and NAC on biomarkers of hepatic injury differed from the control ($p < 0.05$) and NAC ($p < 0.05$) groups. Compared to the NAC group, ALT and AST in the LA + NAC group were 2.2x and 2x higher, respectively. Despite the fact that these enzymes are not exclusive markers of liver damage, their increase in clinical situations such as heart disease and myopathies, when analyzed together with oxidative (Figure 7) and inflammatory (Figure 8) parameters, may be considered as a disruption of the liver metabolism balance. The other systemic biomarkers analyzed were not seen to be statistically significant.

3.2. Colonic Results

3.2.1. LA or NAC Reduced Histological Damage on the Colon Induced by DSS. Samples from DSS-treated animals (mild colitis) showed higher histological damage (Figure 3(a)) than other groups. Although all the treated groups showed these changes, damage to the mucosal architecture was reduced when antioxidants were used, showing a protective effect of the antioxidants, when compared to the mild colitis group. Collagen deposition (Figure 3(b)), marked with a blue color, confirms the presence of fibrotic tissue in mild colitis (Figure 3(b)). LA and/or NAC treatments were able to decrease this deposition but were not sufficient to prevent mild UC lesions and their collagen counts were equal to the mild colitis and control groups ($p > 0.05$) (Figure 3(c)).

3.2.2. Colonic Oxidative Damage Is the First Signal Observed in Mild Colitis. Oxidative damage represented by increased H_2O_2 (Figure 4(a)), nitrite (Figure 4(b)), and MDA (Figure 4(c)) levels, together with a decrease in CAT activity (Figure 4(d)), was already present in the mild colitis group and confirms the involvement of oxidative stress in the pathogenesis of UC. NAC was able to restore both H_2O_2 and MDA to levels equal to the control group and decreased MPO levels versus the mild Colitis group ($p < 0.05$), thereby confirming its higher antioxidant power relative to LA. Interestingly, NAC decreased SOD activity (Figure 4(e)) compared to the LA group. At the same time, it decreased

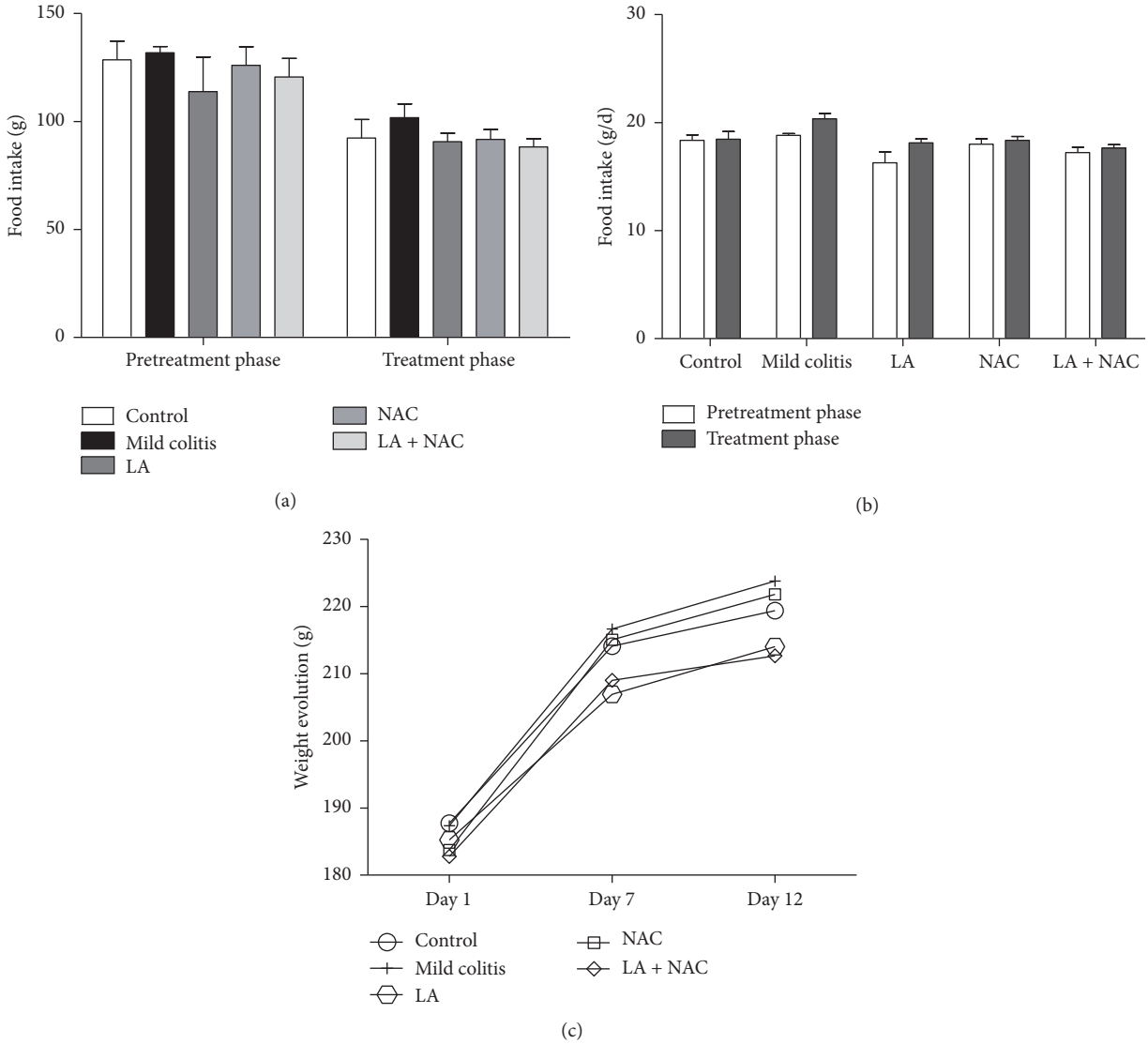

Figure 1: Food intake (g) (a); daily food intake (g/d) (b); weight evolution (g) (c) and daily weight gain (g/d) according to phase (pretreatment, PT; days 1 to 6, and treatment, T; days 7 to 12) and group (control; mild colitis; lipoic acid, LA; *N*-acetylcysteine, NAC; LA associated with NAC – LA + NAC).

colonic oxidative damage. In mild colitis, NAC activity may be maintained due to an increase in GSH (Figure 4(g)) and consequently GSSG (Figure 4(h)), as a response attempt of the body to oxidative damage. There were no alterations in the GSH/GSSG ratio (Figure 4(i)) and GPx levels for all groups (Figure 4(j)).

3.2.3. Changes in Intestinal Cytokines Were Not Observed in Mild UC and LA + NAC Provoked Inflammation. Colonic inflammation represented by proinflammatory cytokines TNF-α and INF-γ, involved in innate immunity, and the anti-inflammatory cytokine IL-10, was not altered in the mild colitis group, compared to the control (Figures 5(a), 5(b), and 5(c)). However, LA + NAC promoted an increase in TNF-α (versus control, LA, and NAC groups) (Figure 5(a)) and IL-10 (versus all groups) (Figure 5(c)). Probably IL-10 increased to minimize the proinflammatory effects caused by TNF-α.

3.3. Hepatic Results

3.3.1. Mild Colitis, LA, and/or NAC Did Not Cause Histological Alterations in the Liver. Biochemical and histological analyses (Table 2) suggest an absence of hepatic injury caused by DSS. However, despite the fact that the association between LA and NAC indicated concerning effects on this tissue, which can be observed by an increase in ALT and AST levels, only one morphological alteration could be identified: a periportal zone with disorganized hepatocyte cords (Figure 6(a)) without a necrosis area or collagen deposition (Figures 6(b) and 6(c)).

3.3.2. LA and/or NAC Present Different Redox Modulations. DSS did not cause hepatic redox imbalance. NAC exhibited an improved SOD effect (increase) versus the LA group (Figure 7(a)), while LA increased CAT (versus

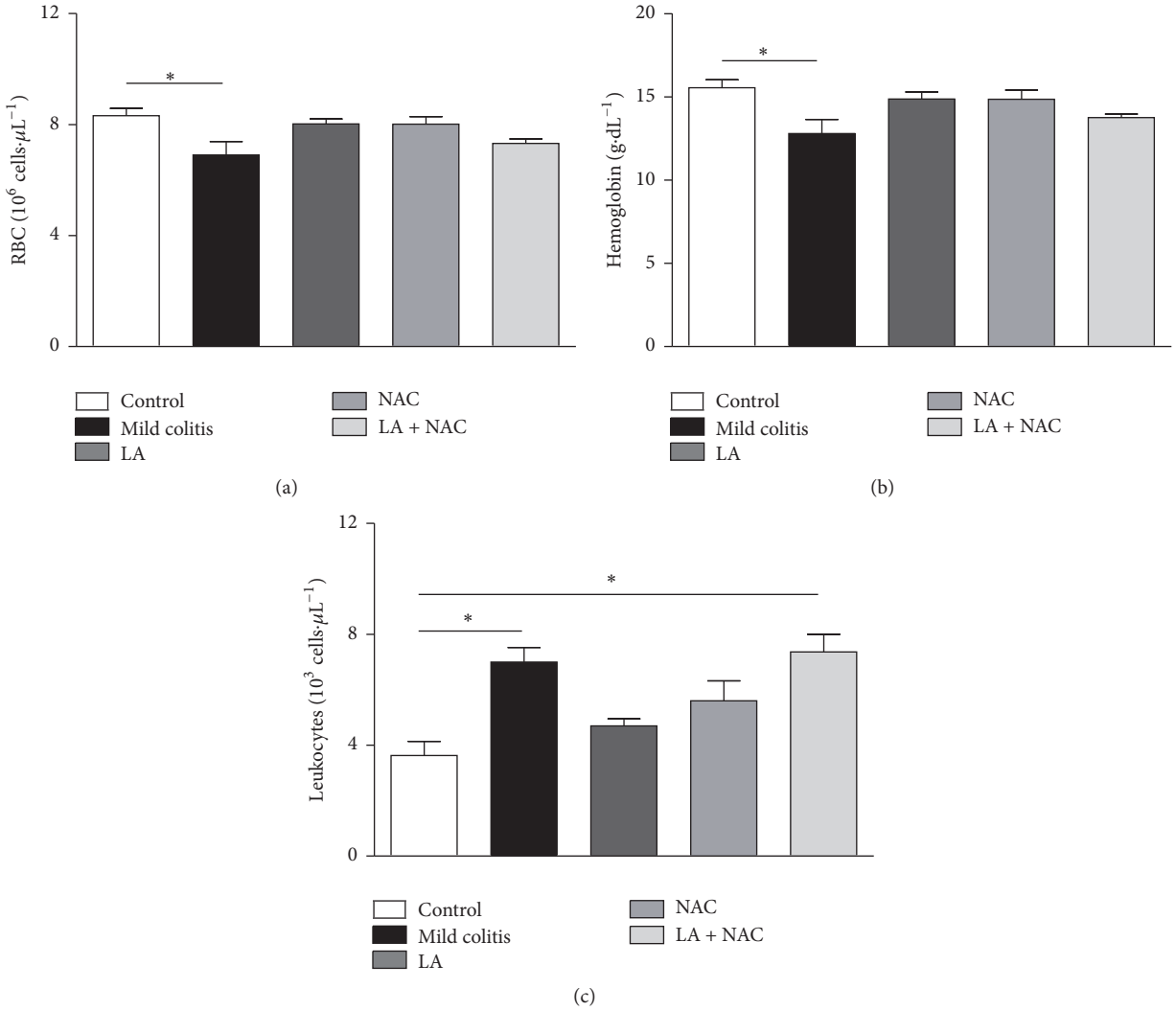

FIGURE 2: Red blood cells (RBC) (10^6 cells·μL^{-1}) (a); hemoglobin (g·dL^{-1}) (b); and leukocytes (10^3 cells·μL^{-1}) (c), according to group (control; mild colitis; lipoic acid, LA; *N*-acetylcysteine, NAC; LA associated with NAC – LA + NAC). $^*p < 0.05$ (Tukey test).

NAC) (Figure 7(f)), GSH (versus mild colitis and NAC) (Figure 7(g)), and consequently GPx (versus control) (Figure 7(j)). These results confirm that different antioxidants act on redox imbalance *via* different pathways. However, as observed by biochemical analysis, LA + NAC acted as a prooxidant supplement, causing an increase in H_2O_2 (Figure 7(b)).

3.3.3. LA + NAC Caused Inflammation in the Liver. In the liver, the levels of cytokines were not modified in the mild colitis group compared to the control group (Figures 8(a), 8(b), and 8(c)). However, it is important to notice, in both tissues (colon and liver), the proinflammatory effects of LA associated with NAC (LA + NAC). In hepatic tissue, this combination provoked the increase of TNF-α and INF-γ levels, when compared to all groups (Figures 8(a) and 8(b)).

4. Discussion

Analyses of histology, oxidative stress, and inflammatory biomarkers were performed on colonic and liver tissues, in order to investigate the role of added antioxidants (NAC and/or LA) in controlling damage caused by DSS. In this context, we observed that redox imbalance was the first alteration in mild colitis; NAC was able to reduce oxidative stress and cell damage, DSS (2% w/w) did not cause hepatic modification, and LA + NAC increased inflammation in the colon and liver.

4.1. Colonic Injury and the Effects of Supplementation. Previous studies have provided compelling evidence for the association between DSS and different degrees of UC [27, 28], from mild colitis up to carcinogenesis, according to its continuous administration, at 2–5% w/v, for a short period of time (4–9 days). Moreover, histological, biochemical, and immunological alterations caused by DSS are similar to UC in humans [29]. The exact mechanism of colitis induction by DSS is not known, but it seems to involve dysfunctional macrophages, luminal bacterial alterations, and direct toxicity to epithelium [30].

The redox profile was the first biochemical parameter to be altered, before immunological changes, particularly in

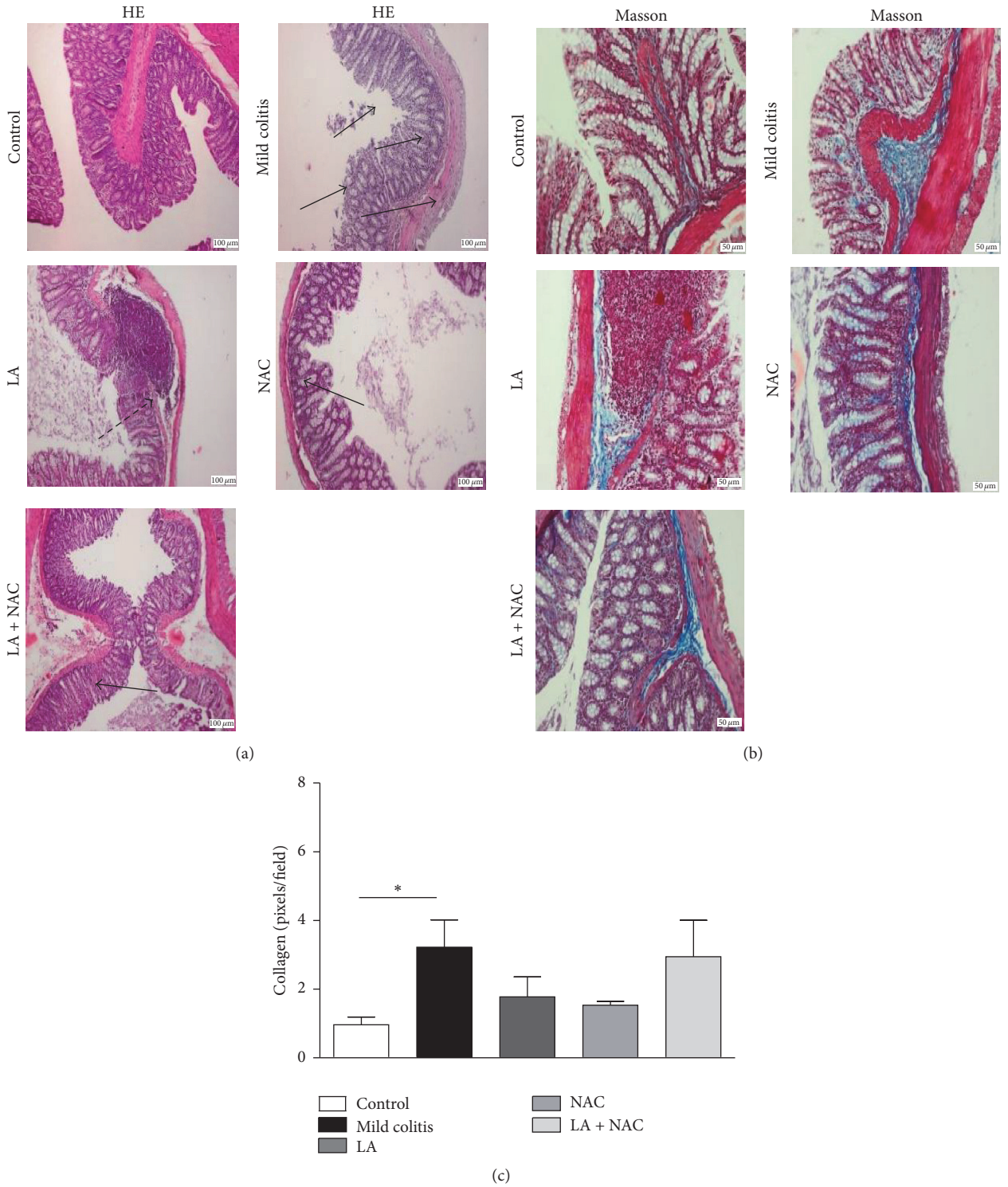

Figure 3: Hematoxylin and eosin staining (a) 50x magnification: arrows show the colonic lesions with neutrophil infiltration in mucosa and crypts' destruction; Masson trichrome staining (b) 100x magnification: the blue color shows areas with accumulation of mucous and collagen, especially on the lamina itself, submucosa, and between crypts. (c) Colonic collagen counts confirm fibrosis in the mild colitis group (control; mild colitis; Lipoic acid, LA; *N*-acetylcysteine, NAC; LA associated with NAC – LA + NAC). $^{*}p < 0.05$ (*Dunn's* test).

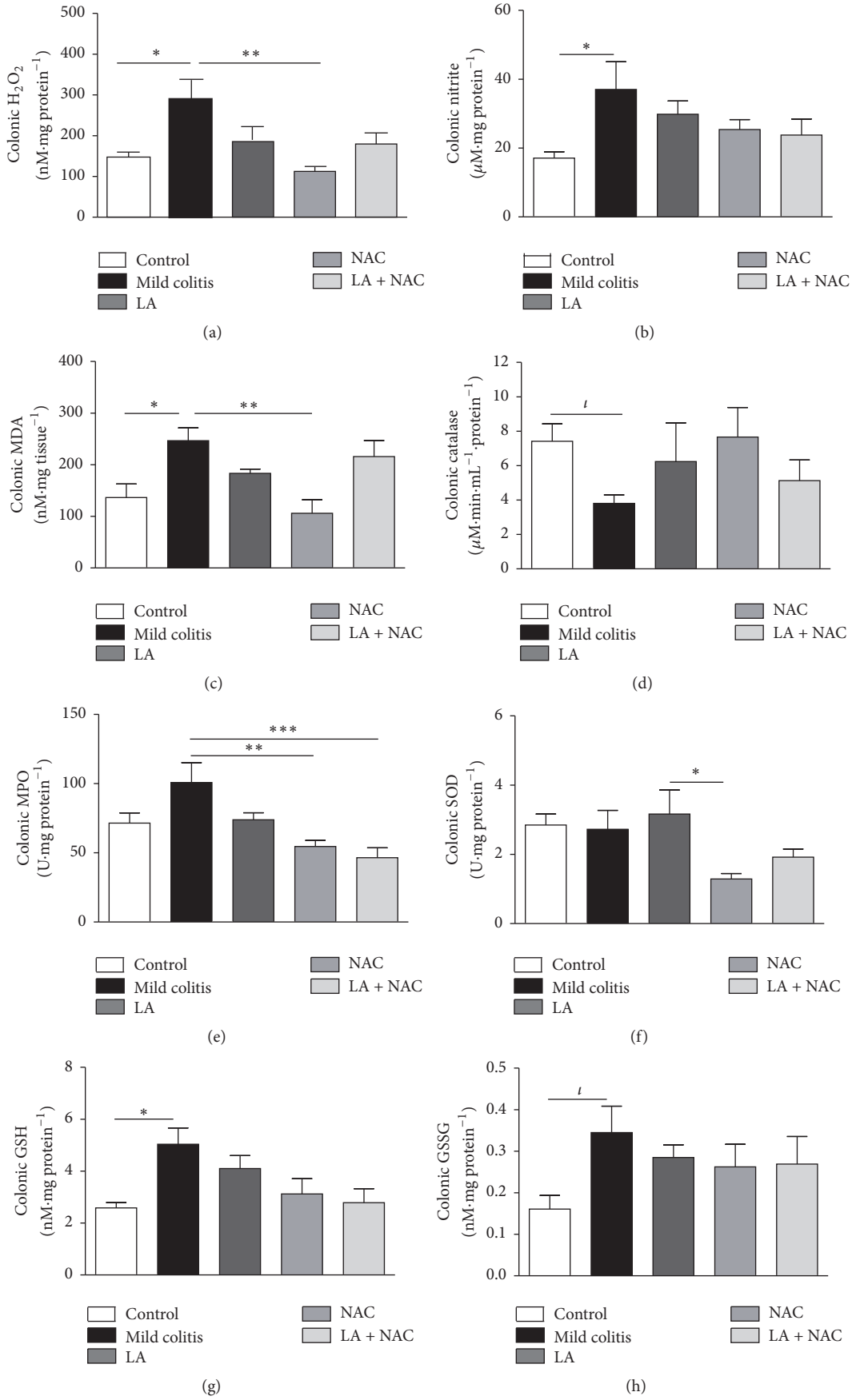

Figure 4: Continued.

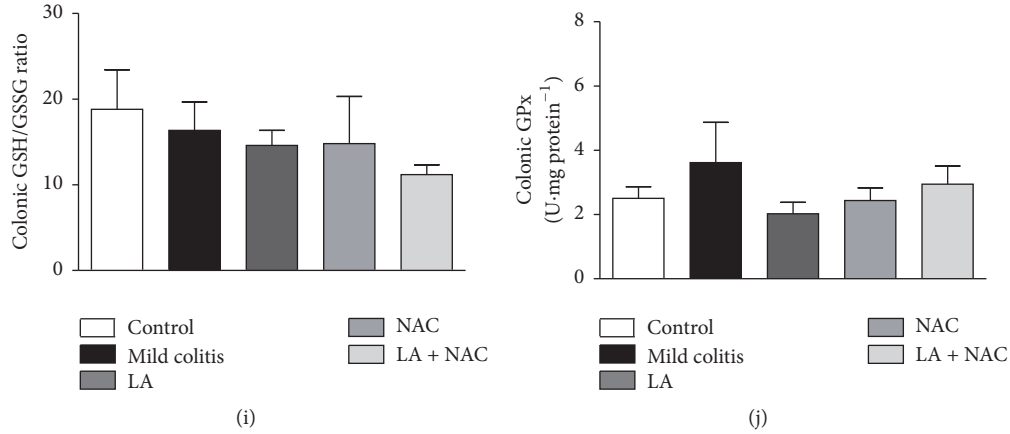

Figure 4: Colonic oxidative markers: hydrogen peroxide (H_2O_2) levels (a); nitrite levels (b); malondialdehyde (MDA) levels (c); catalase activity (d); myeloperoxidase (MPO) activity (e); superoxide dismutase (SOD) activity (f); reduced glutathione (GSH) levels (g); oxidized glutathione (GSSG) levels (h); GSH/GSSG ratio (i); and glutathione peroxidase (GPx) activity (j), according to group: control; mild colitis; lipoic acid, LA; N-acetylcysteine, NAC; LA associated with NAC – LA + NAC. $^{*}p < 0.05$ (Tukey test); $^{**}p < 0.01$ (Tukey test); $^{***}p < 0.001$ (Tukey test); $^{\iota}p < 0.05$ (t-test).

Table 2: Biochemical plasma analysis (mean ± SEM), according to group: control; mild colitis; lipoic acid, LA; *N*-acetylcysteine, NAC; LA associated with NAC – LA + NAC.

	Control	Mild colitis	LA	NAC	LA + NAC
Hb1Ac (%)$^{\Psi}$	7.80 ± 0.15	7.97 ± 0.29	8.50 ± 0.40	8.13 ± 0.33	7.88 ± 0.28
Glucose (mg/dL)	189.00 ± 31.35	207.80 ± 38.77	196.00 ± 61.01	156.2 ± 23.66	176.40 ± 18.76
CRP (mg/dL)	2.22 ± 0.32	2.20 ± 0.16	1.90 ± 0.25	2.15 ± 0.30	2.18 ± 0.29
Calcium (mg/dL)	9.25 ± 0.31	8.90 ± 0.29	8.13 ± 0.90	8.68 ± 0.23	8.64 ± 0.41
TC (mg/dL)	76.00 ± 7.69	65.75 ± 7.20	70.50 ± 5.50	72.25 ± 5.45	64.33 ± 8.67
TG (mg/dL)	55.50 ± 14.56	63.25 ± 14.48	53.50 ± 0.71	54.25 ± 7.01	50.20 ± 19.83
LDL-C (mg/dL)	37.56 ± 14.37	30.32 ± 5.67	31.80 ± 3.60	35.90 ± 5.37	29.96 ± 5.70
HDL-C (mg/dL)	27.33 ± 2.26	24.50 ± 1.76	28.00 ± 2.00	25.50 ± 0.87	23.60 ± 3.17
AST (U/L)	167.50 ± 27.91	194.00 ± 20.72	297.75 ± 59.74	153.40 ± 20.34	$359.75 \pm 76.95^{\alpha\delta}$
ALT (U/L)	82.83 ± 5.83	104.25 ± 6.14	133.00 ± 24.78	85.25 ± 6.02	$168.25 \pm 38.60^{\alpha\delta}$
AP (U/L)	420.50 ± 33.84	345.00 ± 12.45	315.00 ± 66.24	302.40 ± 46.64	305.00 ± 53.97
TB (mg/dL)	0.12 ± 0.00	0.10 ± 0.02	0.10 ± 0.02	0.12 ± 0.01	0.10 ± 0.02
DB (mg/dL)$^{\Psi}$	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
IB (mg/dL)$^{\Psi}$	0.10 ± 0.00	0.07 ± 0.02	0.08 ± 0.00	0.09 ± 0.01	0.08 ± 0.01
TP (g/dL)	5.24 ± 0.19	4.63 ± 0.48	4.20 ± 0.41	4.78 ± 0.17	4.66 ± 0.14
ALB (g/dL)	2.90 ± 0.27	2.28 ± 0.89	2.50 ± 0.46	2.74 ± 0.18	2.68 ± 0.15
GLOB (g/dL)	2.30 ± 0.09	1.95 ± 0.03	$1.70 \pm 0.15^{\alpha\alpha}$	2.04 ± 0.10	1.98 ± 0.10

Hb1Ac = glycosylated hemoglobin; CRP = C reactive protein; TC = total cholesterol; TG = triacylglycerol; LDL-C = low-density lipoprotein; HDL-C = high density lipoprotein; AST = aspartate aminotransferase; ALT = alanine aminotransferase; AP = alkaline phosphatase; TB = total bilirubin; DB = direct bilirubin; IB = indirect bilirubin; TP = total protein; ALB = albumin; GLOB = globulin; $^{\alpha}p < 0.05$ versus control; $^{\alpha\alpha}p < 0.01$ versus control; $^{\delta}p < 0.05$ versus NAC (Tukey test). $^{\Psi}$Kruskal-Wallis and Dunn's test.

virtue of increased H_2O_2 and nitrite levels. Colonic injury typical of UC, represented by destruction of crypts and disorganization of intestinal structure, was observed in all groups that received DSS. Similar results were reported by other authors [31, 32]. However, LA and/or NAC supplementation could not prevent this damage, including collagen deposition (Figures 3(b) and 3(c)).

Even alone, LA has shown to exhibit negative effects in human studies. Wray et al. analyzing cardiovascular risk in elderly people observed that 600 mg/d of LA + Vit C (1000 mg) and Vit E (600 IU), 3x per week for 6 weeks, nullified positive effects on blood pressure, caused by exercise [33]. Additionally, McNeilly et al. [34], studying cardiovascular risk in obese patients with glucose intolerance, tested 1 g/d of LA for 12 weeks, with or without exercise, and detected no improvement on serum lipid profile and increased *oxLDL*. Showkat et al. [35] tested LA (600 mg), 30 minutes prior to iron administration in patients with chronic renal failure on hemodialysis, and observed an increase in F2 isoprostanes and lipid hydroperoxide,

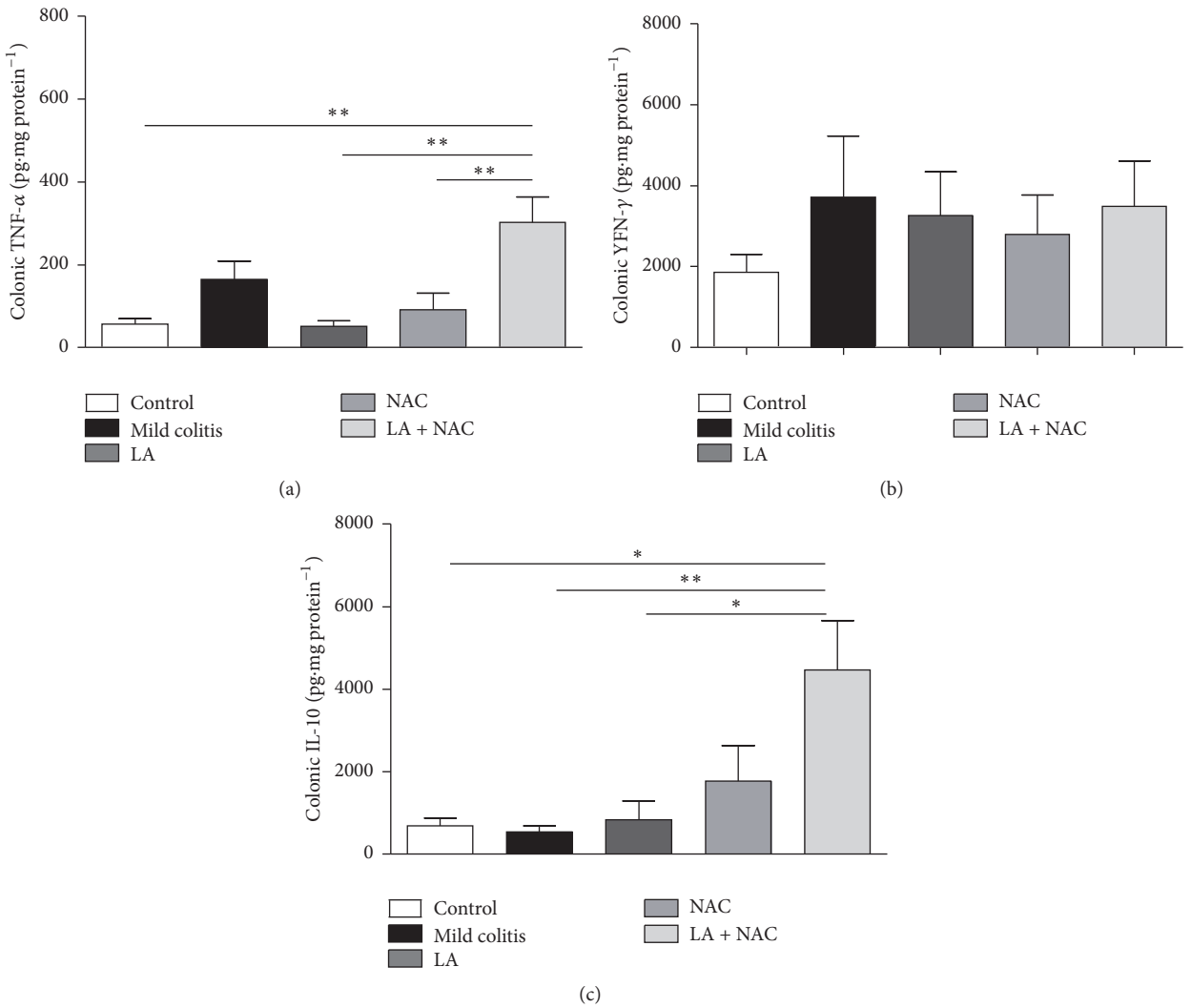

Figure 5: Colonic inflammatory markers: tumor necrosis factor alpha (TNF-α) (a); interferon gamma (INF-γ) (b); and interleukin 10 (IL-10) (c) levels according to group: control; mild colitis; lipoic acid, LA; *N*-acetylcysteine, NAC; LA associated with NAC – LA + NAC. $^{*}p < 0.05$, $^{**}p < 0.01$ (Tukey test).

biomarkers of lipid peroxidation, thereby confirming cell disruption.

LA was able to scavenge H_2O_2 and decrease LP (MDA levels), but NAC was more effective, completely preventing the increase of these markers. NAC was also shown to be effective in chronic UC, induced by DSS (5% w/v for 5 days), as observed by Amrouche-Mekkioui and Djerdjouri [28]. According to these authors, NAC (150 mg·kg·d^{-1} for 45 days) decreased colitis symptoms, inflammation, cell apoptosis, and MPO and $^{\bullet}$NO levels. Collectively, these results indicate the antioxidant and anti-inflammatory effects of NAC at different stages of UC.

$^{\bullet}$NO is a reactive molecule associated with UC progression, especially regarding toxic megacolon. In addition, $^{\bullet}$NO reacts with $O_2^{\bullet-}$, forming peroxynitrite, which causes LP and consequent ulcers in the colon mucosa. Both lesions are common in IBD [4]. $^{\bullet}$NO production is catalyzed by the enzyme nitric oxide synthase (NOS). In inflamed tissue, such as the colon in UC, the inducible isoform of the enzyme (iNOS) is highly expressed in DSS-inflamed colons and the colon of UC patients [36]. This enzyme, present in its own colonocyte, may be responsible for the increase of nitrite levels in the mild colitis group.

F2-isoprostane is the best general indicator of nonenzymatic lipid peroxidation in complex biological systems [37]. However, in a recent systematic review on antioxidant therapy published by our group, we observed that the majority of the studies used MDA (identified directly by HPLC, or indirectly by thiobarbituric acid reactive substances, TBARS) to measure LP. In our study, a MDA assay was chosen.

In the present study, alterations in colonic SOD and GPx (Figures 4(f) and 4(j)) in the mild colitis group were not observed, which is similar to data reported by Akman et al. [38], who studied patients with active intestinal inflammation. On the other hand, the lower SOD activity observed in the NAC versus LA group, without evidence of increased

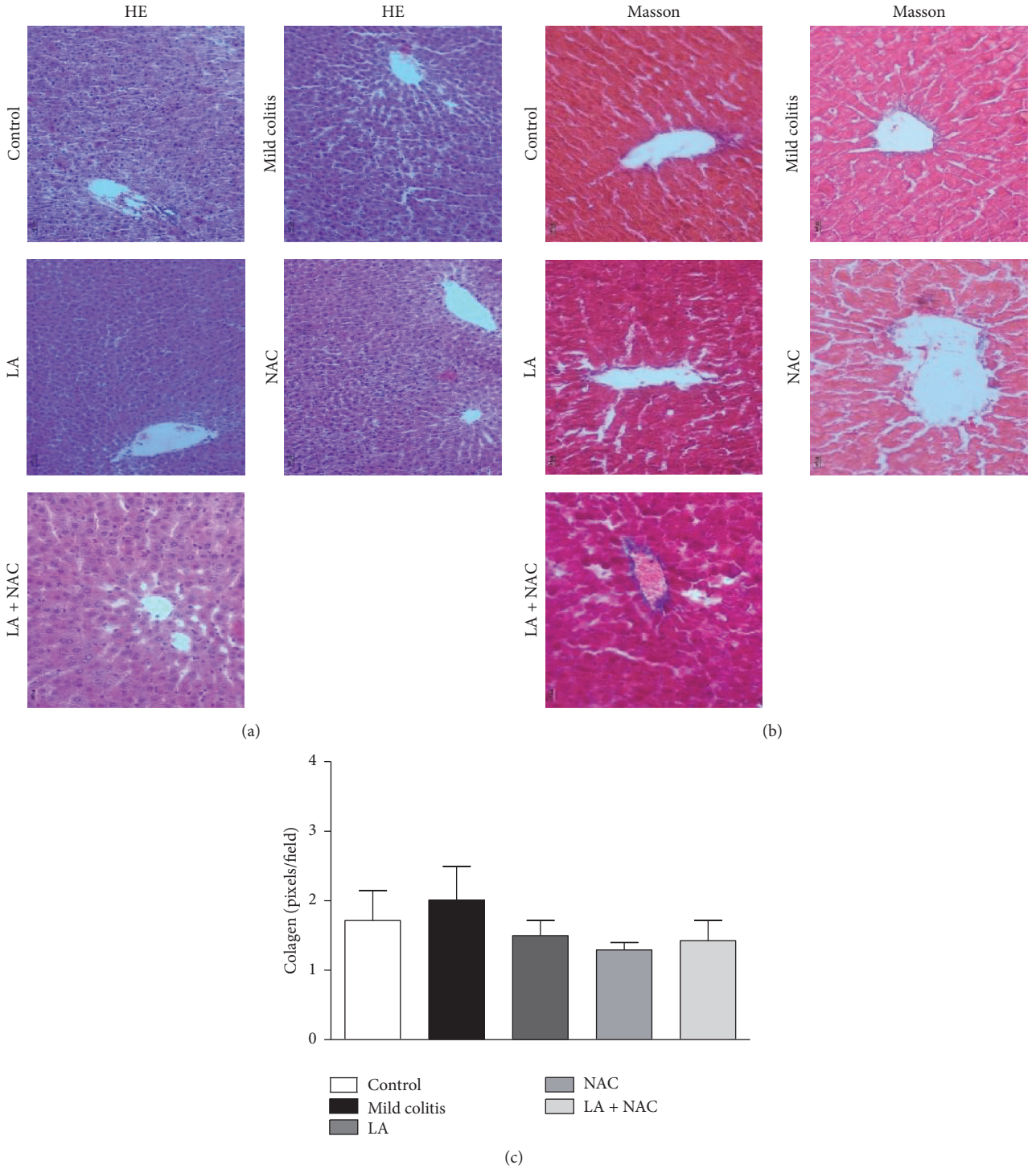

Figure 6: Hematoxylin and eosin staining (a) 50x magnification: Masson trichrome staining (b) 200x magnification. Hepatic collagen deposition (Figures 3(b) and 3(c)) confirms the absence of fibrotic tissue (control; mild colitis; lipoic acid, LA; *N*-acetylcysteine, NAC; LA associated with NAC – LA + NAC).

H_2O_2 levels, confirms the major antioxidant power of NAC in our model.

Recently, antioxidant therapy for IBD has gained increased recognition [4]. However, SOD activity has been poorly investigated regarding LA and NAC administration, probably because SOD is an enzyme whose activity is modestly reduced, during tissue inflammation, unlike GPx2, a gastrointestinal-specific form of GPx [36], which is closely associated with H_2O_2 metabolism, and whose gene expression is downregulated in several experimental models of UC [39].

Our findings on elevated GSH and GSSG levels in the mild colitis group are different from the findings of Amrouche-Mekkioui and Djerdjouri [28]. This increase may

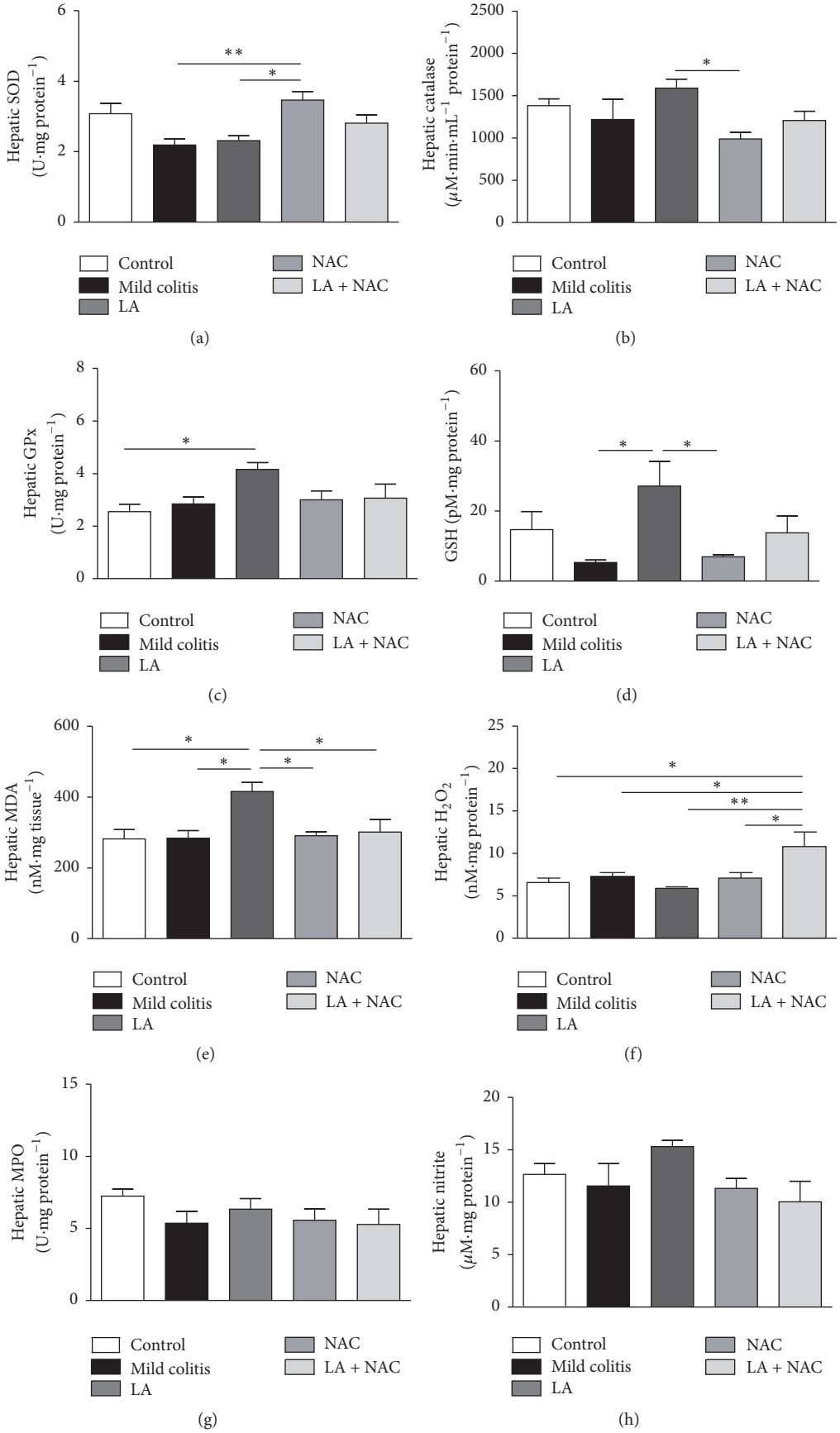

Figure 7: Continued.

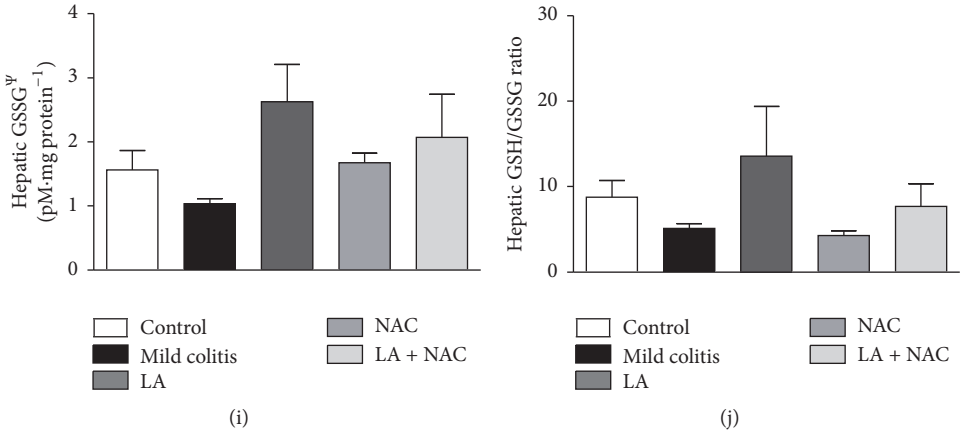

Figure 7: Hepatic redox markers: superoxide dismutase (SOD) activity (a); catalase activity (b); glutathione peroxidase (GPx) activity (c); reduced glutathione (GSH) levels (d); malondialdehyde (MDA) levels (e); hydrogen peroxide (H_2O_2) levels (f); myeloperoxidase (MPO) activity (g); nitrite levels (h); glutathione oxidized (GSSG) levels (i); and GSH/GSSG ratio (j), according to group: control; mild colitis; lipoic acid, LA; N-acetylcysteine, NAC; LA associated with NAC – LA + NAC. $^{*}p < 0.05$ (Tukey test). $^{**}p < 0.01$ (Tukey test). Ps.: GSSG (Kruskal-Wallis test).

be explained by the role played by GSH in inhibiting apoptosis signaling not only by scavenging intracellular ROS but also by inhibiting cytochrome c release from mitochondria and regulating the activity of redox-sensitive caspases [40]. In our results, the influence of oxidative stress on GSH cycling was confirmed by an increase of GSSG. However, the concomitant elevation of GSH levels did not cause a change in the GSH/GSSG ratio, the most important biomarker.

The GI tract is a major site for generation of prooxidants, whose production is primarily due to the presence of a plethora of microbes, food ingredients, and interactions between immune cells [15]. The enhanced production of reactive species is associated with chronic intestinal inflammation in the early stages of IBD. Their destructive effects on DNA, proteins, and lipids may contribute to initiation and progression of UC, causing several symptoms, such as loss of blood and anemia, carcinogenesis, hepatotoxicity, nephrotoxicity, and hypersensitivity [41]. Besides that, oxidative stress increases inflammation and stimulates activation of NF-kB, with consequent production of proinflammatory cytokines, chemokines, growth factors, and adhesion molecules, which cause inflammation and fibrosis, identified in our study by the increase of leukocyte levels and collagen deposition.

TNF-α has been shown to play a critical role in the pathogenesis of IBD and biological therapy with TNF-α-blockers has been used as a mainstream treatment for downregulating aberrant immune responses and inflammatory cascades [42]. Additionally, INF-γ is involved with overexpression of several chemokines, such as IFN-γ-inducible protein 10 and IFN-inducible T-cell α chemoattractant, in the intestinal mucosa for colitis induced in mice, and in UC patients [43]. On the other hand, IL-10, an anti-inflammatory cytokine, is required for regulating immune functions by promoting the widespread suppression of immune responses through its pleiotropic effects [44]. Imbalance in the production of these cytokines, such as TNF-α, plays a pivotal role in the signaling cascade of inflammatory pathways.

Guijarro et al. [45] in studying the effect of NAC plus mesalamine in UC patients also observed no changes in TNF-α plasma levels. It is possible that alterations were not identified because the model used in this study is for mild UC and upregulation of proinflammatory cytokines, such as IFN-γ and TNF-α, was observed more consistently in severe inflammation, such as colitis associated with carcinogenesis.

Unexpectedly, LA + NAC did not promote a beneficial action, even in increasing colonic IL-10 levels, which may be explained by the increase of leukocytes and dendritic cells, the latter responsible for its secretion and that are increased in colonic infiltrates [46]. IL-10 elevation has an anti-inflammatory response, especially in Th2 (T helper 2 lymphocytes) [47], related to autoimmune disorders such as UC [48]. In contrast to other studies [49, 50], a colonic increase of proinflammatory cytokines (Figures 5(a), 5(b), and 5(c)), despite the increase of leukocytes (Figure 2(c)), was not observed in the present study.

4.2. Hepatic Injury and the Effects of Supplementation. The extraintestinal manifestations of IBD are poorly explored by the scientific community. However, recent results have associated these manifestations to IBD activity [51] and the use of TNF-α inhibitors [52], exemplified by hepatobiliary manifestations, in terms of frequency and severity [53–55]. At the same time, these IBD complications remain underdiagnosed [56].

Despite the intimate connection between liver and colon, the oxidative and inflammatory alterations found in our model of mild colitis (2% of DSS, v/v, for 5 days) could have been insufficient to cause liver damage, unlike the results of Trivedi and Jena [57] and Farombi et al. [58]. However, when histological (Figure 6(a)), serological (Table 2), oxidative (Figure 7), and inflammatory (Figure 8) parameters were

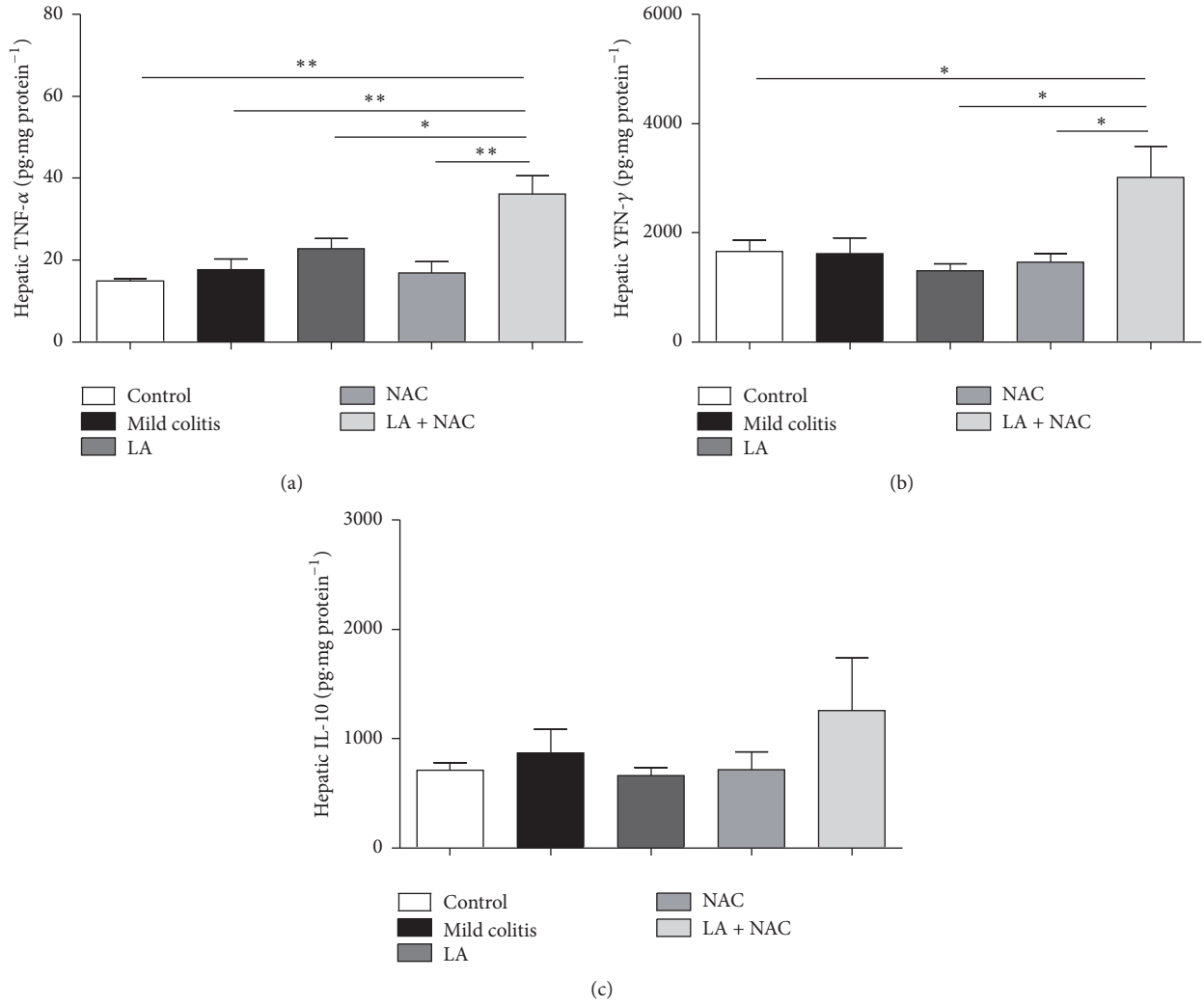

Figure 8: Hepatic inflammatory markers: tumor necrosis factor alpha (TNF-α) (a); interferon gamma (INF-γ) (b); and interleukin 10 (IL-10) (c) levels according to group: control; mild colitis; lipoic acid, LA; *N*-acetylcysteine, NAC; LA associated with NAC – LA + NAC. $^{*}p < 0.05$, $^{**}p < 0.01$ (Tukey test).

evaluated, hepatic damage in the LA + NAC group was identified.

Unlike hepatic $^{\bullet}$NO (Figure 7(d)), H_2O_2 production was seen to be stimulated by the combination of LA + NAC (Figure 7(f)), while no modification in histology and collagen depositions was observed in this group.

Mitochondria and redox-active enzymes can generate $O_2^{\bullet-}$ and H_2O_2 as byproducts in liver cells and these reactive molecules are increased under different conditions of chronic liver injury, caused by alcohol, xenobiotics, viral infections, nonalcoholic fatty liver disease, and others. Additionally, high concentrations of oxidative species, such as H_2O_2 and $^{\bullet}$OH, induce hepatic stellate cell death and cause reduction of collagen deposition [59], which would explain the absence of changes in collagen deposition despite the higher levels of ALT and AST found in the serum of the LA + NAC group. Hepatic injury, whether acute or chronic, eventually results in an increase of serum concentrations of aminotransferases [60], suggesting stronger harmful action on the liver tissue, caused by the combination of these two antioxidants.

NAC supplementation led to an increase in hepatic SOD but without increasing H_2O_2 levels (versus mild colitis and LA), that is, improved redox status by activation of the antioxidant defense system. On the contrary, LA significantly increased CAT, GSH, and GPx, possibly due to increased oxidative stress observed in this group and confirmed by an elevation of MDA. Oxidative stress may be observed by GSSG levels, and although without statistical significance, these levels were approximately 61% higher than in the NAC group (Figure 7(i)).

Relative to lower CAT and GSH levels observed in the NAC group, these levels could be justified by the shorter period of supplementation. NAC protection against oxidative stress occurs by directing cysteine into the GSH synthesis pathway, with a consequent increase on the intracellular GSH content [16].

In a recent review, the authors showed that LA is generally administered associated with other substances and that this multiple therapy impairs the identification of the specific role of each component, raising difficulties in attributing

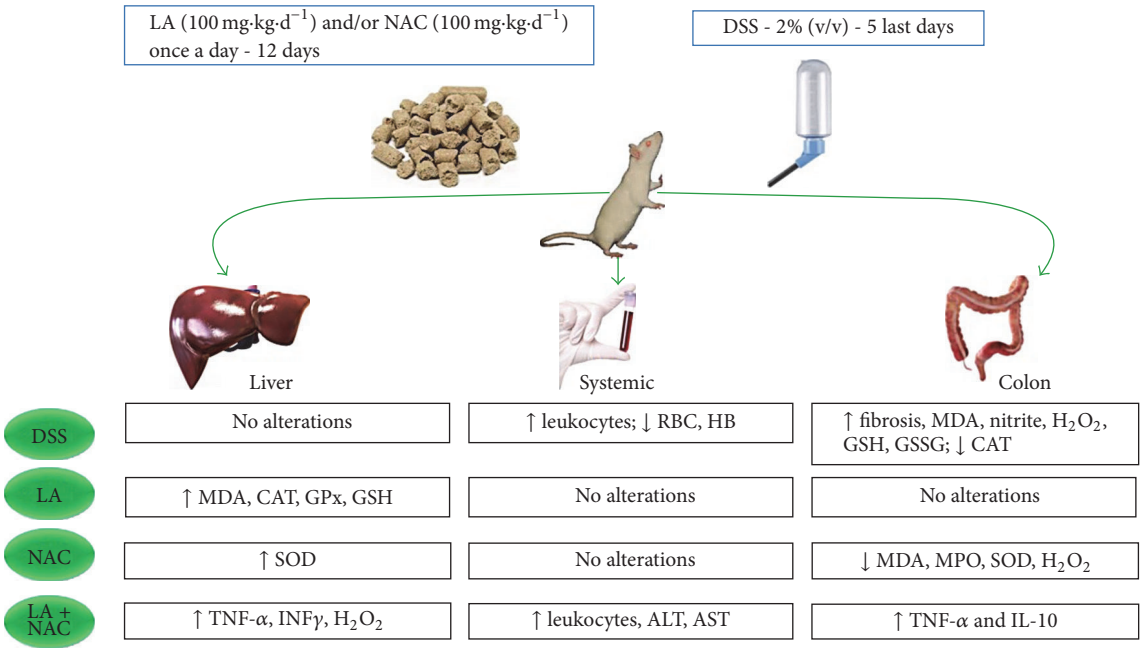

Figure 9: ↑ = increased; ↓ = decreased; ALT = alanine aminotransferase; AST = aspartate aminotransferase; CAT = catalase; d = day; DSS = dextran sodium sulfate; GSH = glutathione reduced; GSSG = glutathione oxidized; H_2O_2 = hydrogen peroxide; HB = hemoglobin; IL = Interleukin; INFγ = interferon gamma; LA = lipoic acid; MDA = malondialdehyde; MPO = myeloperoxidase; NAC = N-acetylcysteine; RBC = red blood cells; SOD = superoxide dismutase; TNF-α = tumor necrosis factor alpha.

beneficial, synergistic, or antagonistic effects [18]. In this context, El-Gowelli et al., also using an animal model, observed that LA plus cyclosporine, an immunosuppressant used routinely in UC treatment, aggravates colon damage. Pop-Busui et al. studying patients with type 1 diabetes observed similar noxious effects in diabetic patients, using LA plus allopurinol (xanthine oxidase inhibitor) and nicotinamide whose combination did not prevent the progression of cardiovascular autonomic neuropathy.

It is important to emphasize that the LA + NAC group received 200 mg·kg·d^{-1} (100 mg·kg·d^{-1} of each antioxidant), an amount much lower than the maximum limit established for safety in the oral delivery of LA (10x less) [61] and NAC (30x less) [62]. Taken together, these results cast doubt on the concept of the "universal antioxidant" given to LA.

5. Conclusions

In our study, oxidative stress was the first biochemical manifestation of mild UC and happened before an increase in TNF-α and INF-γ. Additionally, NAC exhibits better antioxidant effects, especially regarding MDA and H_2O_2 levels. LA, administered daily, as a single dose increased hepatic MDA. LA + NAC increased oxidative and inflammatory profiles in the colon and liver (Figure 9).

In summary, our work provides evidence that the antioxidant and anti-inflammatory power of NAC involves not only the colon but also the liver. This fact confirms, for UC, the necessity to broaden the investigation to the liver, which is intimately connected to the colon.

The management of UC with alternative therapies is a large field of investigation and experimental colitis must mimic the human disease spectra. As presently shown, investigation on several tissues and organs is necessary, before a definite choice of a treatment can be made.

Abbreviation List

ALB: Albumin
ALT: Alanine aminotransferase
ANOVA: One-way analysis of variance
AP: Alkaline phosphatase
AST: Aspartate aminotransferase
BHT: Butylated hydroxytoluene
CAT: Catalase
CD: Crohn's disease
CRP: C reactive protein
d: Day
DB: Direct bilirubin
DHLA: Dihydrolipoic acid
DNA: Deoxyribonucleic acid
DSS: Dextran sodium sulfate
DTNB: 5,5′-Dithiobis-2-nitrobenzoic acid
GI: Gastrointestinal
GLOB: Globulin
GPx: Glutathione peroxidase
GR: Glutathione reductase GR
GSH: Glutathione reduced
GSSG: Dlutathione oxidized
H_2O_2: Hydrogen peroxide
HB: Hemoglobin
Hb1Ac: Glycosylated hemoglobin
HDL-C: High-density lipoprotein
HE: Hematoxylin and eosin

HPLC: High performance liquid chromatography
IBD: Inflammatory bowel diseases
IB: Indirect bilirubin
IKK2: IκB kinase-2
IL: Interleukin
INFγ: Interferon gamma
i.p.: Intraperitoneal
Keap 1: Kelch ECH associating protein 1
LA: Lipoic acid
LDL-C: Low-density lipoprotein
LP: Lipid peroxidation
MDA: Malondialdehyde
MPO: Myeloperoxidase
NAC: *N*-Acetylcysteine
$NADP^+$: Nicotinamide adenine dinucleotide phosphate oxidized
NADPH: Nicotinamide adenine dinucleotide phosphate reduced
NEM: N-Ethylmaleimide
NF-kB: Nuclear factor kappa B
Nrf2: Nuclear factor (erythroid-derived 2)-like 2
PBS: Potassium phosphate buffer
PT: Pretreatment
RBC: Red blood cells
SEM: Mean ± standard error
SOD: Superoxide dismutase
T: Treatment
TB: Total bilirubin
TC: Total cholesterol
TG: Triacylglycerol
tGPx: Total GPx
Th2: T helper 2 lymphocytes
TNF-α: Tumor necrosis factor alpha
TP: Total protein
UC: Ulcerative colitis
Vit: Vitamin.

Additional Points

Highlights. Oxidative stress occurs before inflammatory alterations in mild colitis. *N*-Acetylcysteine is a promising antioxidant toward alleviating ulcerative colitis. Lipoic acid and *N*-acetylcysteine improved oxidative biomarkers for colonic disease. The combination of lipoic acid and *N*-acetylcysteine causes important hepatic injury and colonic inflammation. Antagonist effects of antioxidants have been proven, when administered together in mild colitis.

Competing Interests

The authors confirm that this article's content has no conflict of interests.

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Review Article

Oxidative Stress and Liver Cancer: Etiology and Therapeutic Targets

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Accumulating evidence has indicated that oxidative stress (OS) is associated with the development of hepatocellular carcinoma (HCC). However, the mechanisms remain largely unknown. Normally, OS occurs when the body receives any danger signal—from either an internal or external source—and further induces DNA oxidative damage and abnormal protein expression, placing the body into a state of vulnerability to the development of various diseases such as cancer. There are many factors involved in liver carcinogenesis, including hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, alcohol abuse, and nonalcoholic fatty liver disease (NAFLD). The relationship between OS and HCC has recently been attracting increasing attention. Therefore, elucidation of the impact of OS on the development of liver carcinogenesis is very important for the prevention and treatment of liver cancer. This review focuses mainly on the relationship between OS and the development of HCC from the perspective of cellular and molecular mechanisms and the etiology and therapeutic targets of HCC.

1. Introduction

Oxidative stress (OS) is a process whereby the body receives stimulation from harmful endogenous or exogenous factors. Free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are common metabolic products of several oxidation-reduction (redox) reactions in the cells, are increased when OS occurs. OS also induces DNA oxidative damage and abnormal protein expression, placing the body into a state of vulnerability. This is closely related to the occurrence and development of various diseases such as diabetes, cancer, and cardiovascular and nervous system diseases [1, 2]. A better understanding of the mechanisms of OS on human illnesses is very important for disease prevention and treatment.

Hepatocellular carcinoma (HCC) is the most common type of hepatic malignant tumor worldwide. Liver cirrhosis is acknowledged as a main risk factor for HCC, and the association rate is high, at 80–90% [3]. Many factors are involved in liver carcinogenesis, including HBV and HCV infection, alcohol abuse, nonalcoholic fatty liver disease (NAFLD), aflatoxin B1, obesity, diabetes, dietary habits, and iron accumulation [4]. Few studies have been conducted on the role of OS in the development of HCC; however, the relationship between OS and the pathogenesis of liver cancer has been attracting increasing attention. This report will provide a review of OS and the development of liver cancer from the perspective of cellular and molecular mechanisms and the etiology and therapeutic targets of HCC.

2. Mechanisms of OS-Related Liver Cell Injury and Carcinogenesis

In general, OS can be triggered by any dangerous or inflammatory signal and affects multiple cells in the liver. The mechanisms of OS on the development of liver cancer are summarized in Figure 1 and are described below.

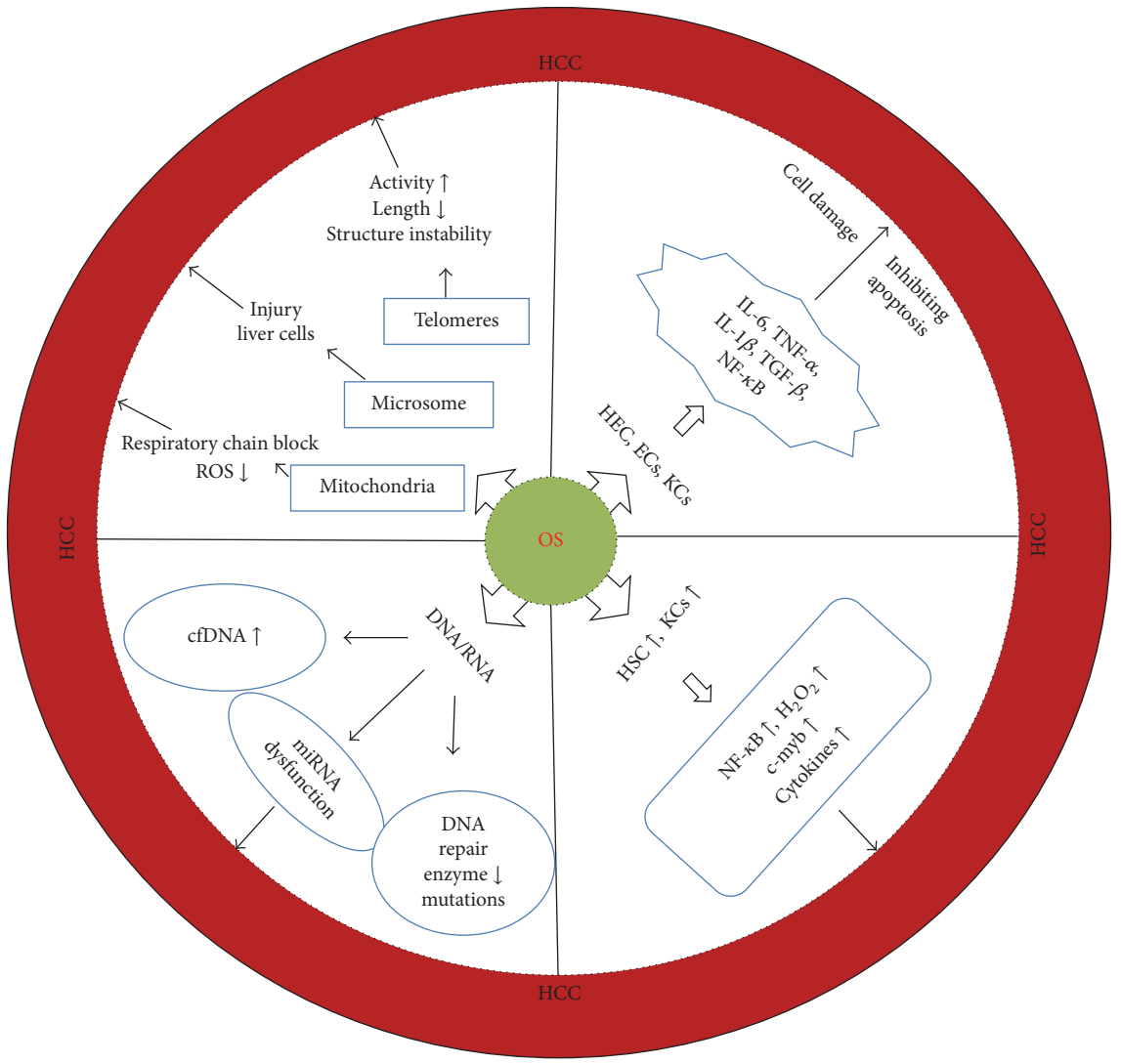

Figure 1: Mechanisms of oxidative stress on the regulation of liver cells.

2.1. Effects of OS on Cytokine Production and Cellular Apoptosis. Liver injury can be either an acute or a chronic inflammatory process. In the environment of local inflammation, many types of liver cells, such as liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), dendritic cells (DCs), and Kupffer cells (KCs), are activated. These cells produce many kinds of immune mediators, cytokines, and chemokines. For example, interleukin- (IL-) 6 is an important proinflammatory cytokine that can inhibit tissue inflammation and cellular apoptosis [5]. Tumor necrosis factor alpha (TNF-α) is a proinflammatory immune mediator that induces tissue damage, produces other cytokines, replenishes inflammatory cells, promotes the occurrence of fibrosis, and further activates the OS reaction [6]. One of the important functions of TNF-α is to activate cellular apoptotic and/or antiapoptotic pathways. The role of TNF-α in the development of HCC remains controversial [7]. OS-associated injury in chronic hepatitis patients is often associated with an increase in fibrosis factor TNF-α and transforming growth factor beta (TGF-β). TGF-β elevation is directly related to the severity of tissue injury and liver fibrosis [8].

Cytokines have been reported to affect liver inflammation, fibrosis, and apoptosis, regulate the process of alcoholic steatohepatitis (ASH)/nonalcoholic steatohepatitis (NASH), and participate in many metabolic changes of ASH/NASH, such as insulin resistance, lipid metabolism, appetite disorders, fever, and increased neutrophils [9].

2.2. OS and Mitochondria, Microsomes, and Telomeres. Mitochondrial dysfunction can impact many important cellular functions, leading to a variety of diseases [10]. New evidence shows that mitochondria play an important role in the process of carcinogenesis. During OS, mitochondrial transcription and replication are increased. The electron transport chain is blocked in the damaged mitochondria, resulting in accumulation of ROS. Further, TNF-α released by liver parenchymal cells and KCs directly damages the mitochondrial respiratory chain, consequently damaging

Table 1: Summary of HCC patients with oxidative DNA damage and inflammation markers.

Ref number	Damage factors	Inflammation markers
[5]	HBV, HCV, NASH	IL-6
[6, 7]	HBV, HCV, NASH	TNF-α
[8]	HBV, HCV, NASH	TGF-β, H_2O_2, NO
[13]	Mitochondrial dysfunction	p53
[14]	NASH	CYP2E1
[17, 18]	HBV, HCV	RASSF1A, GSTP1, CHRNA3, DOK1
[27]	HBV, HCV, NASH	cfDNA
[28, 29]	HBV, HCV, NASH	miRNA
[30]	HBV, HCV, NASH	miRNA-199a, miR-199b, miR-122a, miR-92, miR-222
[31]	HBV, HCV, NASH	NF-kB, OxLDL
[32–35]	NASH	IL-17
[36–47]	NASH	Adiponectin
[48]	NASH	Sulfatase 2
[49–51]	NASH	Adiponectin
[52]	HBV	IL-1β, IL-6, CXCL-8, TNF-α
[53–55]	HBV	HBx
[56]	HCV	PD-L1
[57]	HBV, HCV	8-OHdG
[58, 59]	HCV	Fe^{2+}

mitochondrial cytochrome oxidase. On the other hand, the production of ROS is increased due to the blockade of any part of the respiratory chain; accumulation of ROS increases oxidative lipid deposition, which induces more lipid peroxidation, inhibiting the respiratory electron transport chain, creating a vicious circle [11]. Another vicious circle is the consumption of antioxidants. Fatty degeneration causes lipid peroxidation, and ROS can consume antioxidant enzymes, glutathione (GSH), and vitamin E; the loss of such protective material can enhance the effect of ROS on mitochondria [11]. Mass accumulation of ROS can change the mitochondrial metabolic process, increase the permeability of the mitochondrial membrane, promote the release of apoptotic factors, and further damage mitochondrial DNA and its additive effects of deletion and mutation [12]. The specific mechanism of signaling pathways becomes clear by illustrating how ROS and cancer-related proteins (p53, oncogenes) regulate mitochondrial functions [13].

Cytochrome P4502E1 (CYP2E1) is a microsomal oxygenase of fatty acid oxidation that can reduce the content of molecular oxygen and generate prooxidants. This process can lead to OS if it is not effectively blocked by an antioxidant. Administration of anti-CYP2E1 serum and a CYP2E1 inhibitor can block the process of OS and protect the cells from damage. In a human experimental liver NASH model, CYP2E1 surrounded the venules and was consistent with the most seriously damaged liver cells. All of these factors have proved that OS in microsomes can induce cell injury [14].

Telomeres play a very important role in cell proliferation, aging, immortalization, and carcinogenesis [15]. Telomere shortening may lead to an end-to-end fusion; consequently, somatic cells stop proliferating and enter into the stage of aging and apoptosis [16]. OS can accelerate the process of telomere shortening and speed up the accumulation of oxidative damage. In comparison with liver tissue from patients who have HCC with or without cirrhosis, HBV or HCV can induce changes in specific genes in the process of DNA repair, cell cycle control, and signal transduction of apoptosis (RASSF1A, GSTP1, CHRNA3, and DOK1 are specific genes that exist in HCC tumors) [17, 18]. According to recent reports, a chronic state of OS may cause migration of reverse transcriptase subunits of telomerase in the cytoplasm, thus reducing the activity of the enzyme. Reduction of the apoptosis signal in cells/tissue is a significant factor in carcinogenesis [19–21].

2.3. OS and Genetic Material. OS can cause DNA damage. One study showed that increased liver oxidative damage of DNA, combined with histological fibrosis, is a recognized risk factor for HCC [22]. Chronic viral infections cause liver cell necrosis and inflammation and liver regeneration, all of which are associated with infiltration of immune cells that produce reactive oxygen and nitrogen [23]. DNA damage induced by oxygen free radicals and DNA repair of the adaptation disorder leads to the accumulation of cancer-related gene mutations. There is much evidence that chronic inflammation is one of the causes of human cancer [24, 25]. Oxidative stress and accumulation of DNA damage play an important role in the process of virus-induced cancer [26]. The summary of HCC patients with oxidative DNA damage and inflammation markers was indicated in Table 1.

Circulating free DNA (cfDNA), which mainly comes from the oxide that DNA releases from dead cells, is a kind of DNA with double or single chain strands outside the cells. Circulating free DNA can be released by necrotic cells and apoptotic tumor cells. Low levels of cfDNA can also

be detected in healthy people, but a higher level of cfDNA indicates the possibility of the presence of various diseases including cancer [27]. This phenomenon provides the basis for further research on the relationship between HCC and cfDNA.

Recently, microRNA (miRNA), a somewhat small noncoding RNA family (containing 21–23 nucleotides), has been found to play an important role in different phases of the process of HCC development [28]. In fact, miRNA inhibits the translation process by combining specific complementary sequences, or combining with specific complementary sequences on 3′UTR of mRNA to induce the degradation of mRNA [29]. miRNA is considered to be an important mediator in the immune system. Dysfunction of miRNA in inflammatory reactions and oncogenesis is the central event in the development of various cancers. When OS occurs, the expression of a variety of miRNA, as in HCC, is changed. The expression of miRNA-199a, miRNA-199b, and miRNA-122a in most (50%–70%) HCC is strongly downregulated, and the expression of miRNA-92 is indistinctively downregulated. On the contrary, the expression of miRNA-222 is upregulated [30].

2.4. OS and HSCs and KCs. In recent years, studies on the relationship between OS and HSCs have been increasing. HSCs have been proven to play a central part in the process of liver fibrosis [4]. HSCs can induce collagen production after activation in the body by free radicals, which are produced by ROS and superoxide anions, and further induce damage to liver cells [60, 61]. OS can further activate HSCs and stimulate the activity of nuclear factor kappa B (NF-kB). The NF-kB transcription factor is sensitive to redox. Activation of the NF-kB transcription factor can increase the production of nitric oxide (NO) and ROS, which participate in the formation of oxidized low-density lipoprotein (OxLDL) and further activate NF-kB. This creates a vicious circle and results in OS and cell injury [31].

KCs are liver macrophages that serve the functions of phagocytosis, antigen presentation, and immune regulation. KCs can be activated in response to danger of liver infection and produce various cytokines and inflammatory mediators, resulting in aggravation of liver cell injury [8]. Activated KCs produce a large amount of ROS and induce extracellular OS, which can directly cause liver cell necrosis. Other products of KCs, such as H_2O_2, NO, and various cytokines, may also have toxic effects on liver cells.

3. OS Potentiates Hepatitis Virus Infection and Liver Cell Carcinogenesis

It is known that over 80% of cases of HCC are associated with chronic HBV or HCV infection. Recently, the numbers of patients with obesity, as well as the related conditions of metabolic syndrome and NAFLD, are increasing, and these conditions are becoming an important cause of chronic liver disease in the developed countries, such as European nations and the United States. NAFLD includes simple fatty liver (SFL), nonalcoholic steatohepatitis (NASH), and related cirrhosis. NASH is also considered as one of the causes of liver cancer, and the mechanisms are under investigation. The mechanisms of OS in HBV-, HCV-, and NASH-related HCC are summarized in Figure 2 and are described below.

3.1. NASH-Related HCC and OS. The pathophysiological basis of NASH is a "two-hit" hypothesis. The first hit refers to the fatty degeneration of liver cells, characterized by the accumulation of triglyceride in the liver cells. The second hit includes a variety of cellular stress responses, such as apoptosis, OS, endoplasmic reticulum (ER) stress, and intestinal circumstances [62]. Other studies have demonstrated that the inflammatory response can induce fatty deposition in the liver cells, leading to the "multiple-hit" theory [63]. Fatty toxicity can cause multiple hits to the body, such as OS, ER stress, and immune responses [64]. Cellular stress is also involved in the process of carcinogenesis. The obesity-related diseases such as high blood triglycerides and high blood pressure are definite risk factors of NAFLD. OS is one of the important processes mediated by IL-17, while the IL-17 receptor is widely distributed on the surface of liver cells [32, 33]. The regulation of IL-17-related pathways has been shown to effectively prevent the development of NASH in a mouse model [34]. Patients with increased expression of serum IL-17 have a higher risk of early recurrence of liver cancer after surgery [35]. Thus, OS may be involved in IL-17-mediated NASH-related HCC.

Adiponectin is a protein from fat cells that regulates fat and carbohydrate metabolism [36]. In obese and diabetic patients, the level of adiponectin is usually decreased, and in patients with liver fibrosis, the level of adiponectin usually increases [37]. In HCC, the relationship between the adiponectin level and clinical features of the disease is very complex [38].

Most studies show that adiponectin is a "good" fat factor. Adiponectin has anti-inflammatory, antidiabetic, and anti-fat-accumulation properties; and it participates in energy metabolism, regulation of cell proliferation, and tissue remodeling [39, 40]. Adiponectin also inhibits the growth of cancer cells [41] and induces apoptosis [42], which is directly related to the occurrence and development of cancer [43]. Adiponectin inhibits angiogenesis and thus inhibits the growth and metastasis of liver tumors in mice [44]. Similarly, in a study of human HCC, a lower level of adiponectin has been found to be related to a higher malignant degree of HCC [45]. High adiponectin levels have been found to reduce the risk of prostate cancer, breast cancer, endometrial cancer, colorectal cancer [46], and pancreatic cancer [47]. Adiponectin blocks the protein expression of sulfatase 2 (SULF2), which is oncogenic, and high expression of SULF2 is related to HCC [48]. In addition, the expression levels of adiponectin in primary human liver cancer specimens are lower than in paracancerous tissues [49]. However, other studies have shown that adiponectin increases the risk of liver cancer. Aleksandrova et al. pointed out that non-high-molecular-weight (HMW) adiponectin, not high-molecular-weight adiponectin, was significantly associated with the risk of HCC [50]. Low-molecular forms of adiponectin are more

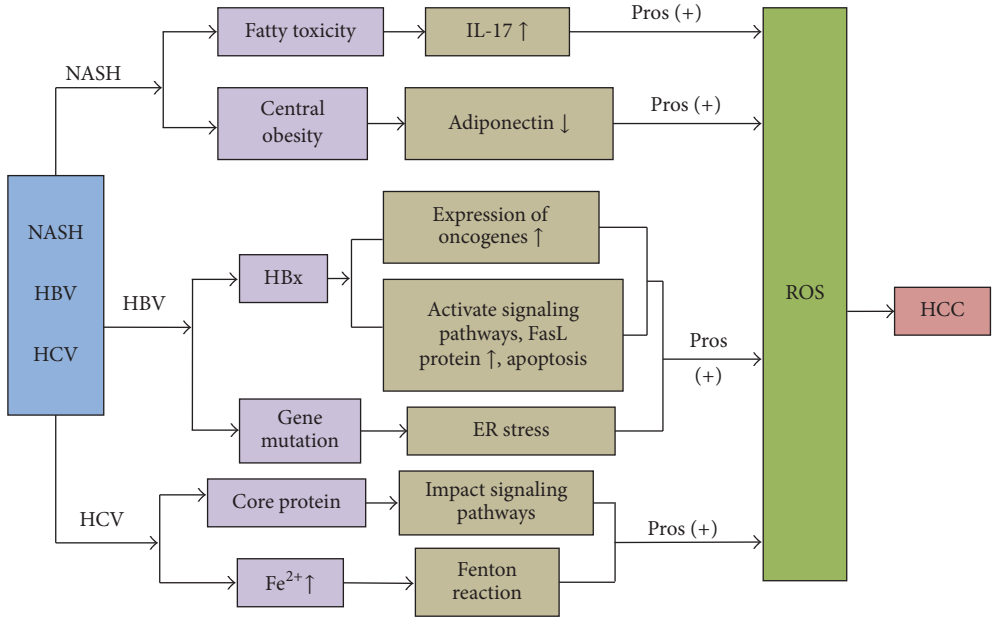

Figure 2: The mechanisms of oxidative stress on HBV-, HCV-, and NASH-related HCC.

closely associated with inflammation compared to high-molecular forms [51].

3.2. HBV/HCV-Related HCC and OS. HBV- and HCV-related chronic inflammation and fibrosis of the liver are usually induced by OS, which contributes to the pathogenesis of hepatocarcinogenesis. HBV infection results in activation of macrophages to produce a variety of proinflammatory cytokines, such as IL-1β, IL-6, CXCL-8, and TNF-α [52]. Such persistent abnormal production of cytokines and the resulting production of ROS have an influence on hepatocarcinogenesis.

The HBV genome can code a variety of gene products, including DNA polymerase (Pol), the capsid protein (core), envelope proteins L, M, and S, and the multifunctional protein HBx. Many studies have indicated that the HBx protein has carcinogenic potential. Transactivated HBx protein stimulates virus replication and expression and protects the virus-infected cells from damage [53]. The HBx protein is concentrated in the cytoplasm, and the c-terminal region from HBx's truncation is the producing region of ROS [54]. This phenomenon can be found in 46% of HCC tissue, but not in nonneoplastic tissue [55]. It is an important process in the development of liver cancer that HBV genes integrate into the host genome. Several cancer-related genes, such as TERT, MLL4, and CCNE1, can also be integrated by HBV [65]. HBx is the most common of these genes that are integrated into the human genome.

Studies on analyzing genetic mutations in HBV patients have found that these gene mutations were associated with the occurrence of liver cancer; this emphasizes the importance of HBx on the development of HCC, and OS is involved in this process. A considerable amount of experimental data has proved that the products from the mutant genes in the pre-s area, which accumulate in the ER, have the potential for promoting carcinogenesis through ER stress and the role of ROS [66].

All in all, OS, at least in part, participates in the process of HBV-related liver cancer development through HBx and the pre-s region.

In the state of HCV infection, liver antigen-presenting cells, KCs and DCs, are activated and modulate the immune functions [82]. The most direct impact of HCV on inflammatory signaling pathways is upregulating immunomodulatory molecules such as PD-L1 in KCs [56]. Persistent inflammation causes the liver cells to go into the circulation between the time of apoptosis and regeneration, produces a spontaneous mutation or damage to DNA, and further results in development of HCC [83]. HCV antigens, in particular the core protein, play a key role in the pathogenesis of chronic HCV and hepatocarcinogenesis through the TNFR, PKR, and STAT3 pathways [84]. There are more OS markers (8-OHdG) or reactive oxygen metabolites in the serum of HCV-related HCC patients than in HBV-related HCC patients, suggesting that there is more OS in HCV infection [57]. OS is also associated with senility, which is also one of the driving factors of hepatocarcinogenesis [85].

In addition, during chronic HCV infection, serological markers and iron accumulation in liver cells (especially in the lysosomes) usually are elevated. An excess of bivalent iron is strongly toxic due to induction of Fenton's reaction, ROS, and hydroxyl free radicals. Iron toxicity is considered to be one of the influencing factors of liver cancer. Some reports have shown that a diet low in iron can reduce the risk of hepatocarcinogenesis in patients with chronic HCV infection [58]. Additional research on iron metabolism and its correlation with liver cancer and OS is underway [59].

Table 2: Summary of antioxidant treatment targets in HCC therapy.

Ref number	Antioxidant treatment	Targets	Pros/cons HCC
[67]	Curcuminoids	Glutathione (GSH)↑, P450↓	Cons
[68, 69]	Ascorbic acid, lipoic acid quercetin, mitoquinone, ebselen	GSH	Cons
[68, 69]	Resveratrol	GSH↑, ROS	Cons
[70–72]	Vitamin E	HBV↓, TGF-β↓	Cons
[73]	Phlebotomy	Fe^{2+}	Cons
[74–76]	Metformin	AMPK↑, Nrf2↑, IL-6↑, hemeoxygenase-1 (HO-1)↑	Cons
[77, 78]	5-Aminoimidazole-4-carboxamide-1-b-ribofuranoside (AICAR)	Nrf2↑	Cons
[79–81]	L-Carnitine	Mitochondria	Cons

4. OS-Related Potential Therapeutic Targets

OS is associated with the development of HBV-, HCV-, and NASH-related HCC. Therefore, antioxidant treatment to control the causes of HCC is significant. In fact, there are many kinds of antioxidant drugs and foods in our everyday life. However, it is difficult to elucidate their specific effects in vivo and in vitro. Studies on the role of antioxidant effects on liver cancer development are still being conducted. The potential therapeutic targets on HCC of antioxidant treatment are summarized in Table 2.

It has been reported that curcuminoids could protect DNA from ROS damage, supporting the liver cells during the course of injury and cirrhosis [67]. Studies on chronic HCV infection have also shown that liver function was improved after antiviral treatment. Ascorbic acid, lipoic acid, and quercetin (types of flavonoid antioxidants) and mitoquinone (antioxidant agent target on mitochondria) are also beneficial to patients with chronic HCV infection. The antioxidant properties of resveratrol can reduce liver lipid peroxidation, increase the content of GSH in the liver, and scavenge ROS. The role of resveratrol is mainly in dealing with external liver damage factors such as alcohol intake. In addition, antioxidant drugs composed of ebselen (glutathione peroxidase analogue) have been used in early liver damage caused by alcohol. Studies have shown that vitamin E inhibits HBV replication and TGF-β gene expression in a rat model of NASH [68, 69].

Phlebotomy is considered an effective method against iron overload in patients with hepatitis and NASH [73]. Currently, alternative antioxidant treatment for liver cancer includes, but is not limited to, antioxidant gene therapy, induction of transcription factors AMPK or Nrf2 activator, and activation of oxygen scavengers and drugs that increase the capacity of mitochondrial oxygen intake. As antioxidant, the AMPK agonist metformin and mitochondrial support drug (L-carnitine) is more effective than vitamin E.

Metformin increases the level of AMP in the cells through the activation of AMPK and causes cell cycle arrest, apoptosis, and STAT3-induced IL-6 [74] and antioxidant enzyme of heme oxygenase-1 (HO-1) production [75] through the Nrf2 signaling pathway. A meta-analysis on the drugs of diabetes treatment found that the application of metformin can result in a 50% reduction in the incidence of HCC [76]. The use of 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR) as an AMPK activator can induce an increase in the Nrf2 protein and expression of antioxidant enzymes in endothelial cells, whereas AICAR activates Nrf2 in hepatoma cell lines resulting in antioxidant enzyme expression [77]. The combination of metformin and AICAR activates AMPK and Nrf2 for the purpose of controlling liver cancer. OS to normal cells can lead to a cancerous cell phenotype, in turn developing high resistance to further oxidative stress. At present, several clinical trials have found that the OS induced under these conditions can treat liver cancer [78]. The American Association for the Study of Liver Diseases (AASLD) recommends that the dose of vitamin E for the treatment of NASH should be 800 IU/d; in actual application, the dose administered is generally higher than the recommended dose [70]. The selection of the recommended dose was based on a two-year randomized study of NASH, which demonstrated that the dose can improve the level of alanine aminotransferase and histologic activity [71]. However, experimental studies found no beneficial effect on liver fibrosis [72]. Animal studies have shown that an L-carnitine dietary supplement can prevent chemically induced hepatitis and subsequent HCC and NASH-related HCC [79, 80]. Supplementation with L-carnitine was observed to significantly improve plasma glucose levels, lipid profiles, and histological manifestations of NASH patients [81].

5. Conclusion

Accumulating evidence has shown that OS plays an important role in the development of liver cell carcinogenesis through disrupting either normal cell function or genetic materials and interfering with the pathways of signal transduction. Application of antioxidant drugs can control OS damage in vitro. However, so far there has been found no effective antioxidant drug that can be used in vivo. In order to design more effective methods for the prevention and

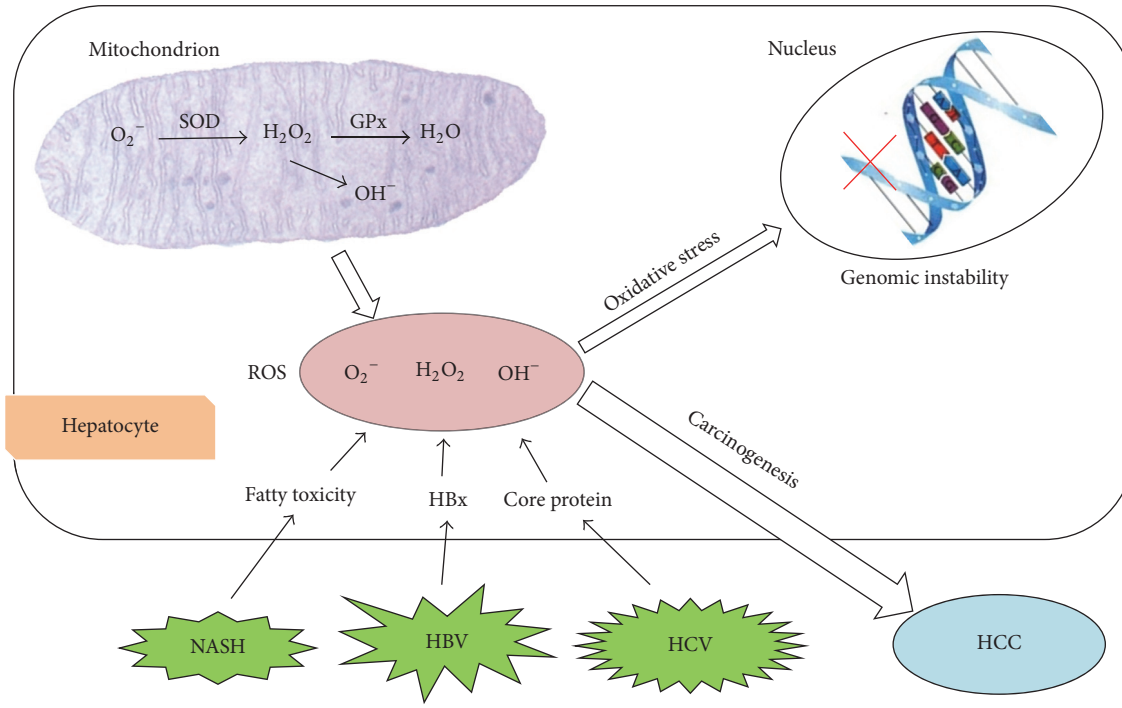

Figure 3: The mechanisms of OS-related HCC.

treatment of HCC, investigations into better understanding the mechanisms of liver cancer development, OS damage, and antioxidants are urgently needed (Figure 3).

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Renalase as a Novel Biomarker for Evaluating the Severity of Hepatic Ischemia-Reperfusion Injury

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Hepatic ischemia-reperfusion (I/R) injury is a serious complication in clinical practice. However, no efficient biomarkers are available for the evaluation of the severity of I/R injury. Recently, renalase has been reported to be implicated in the I/R injury of various organs. This protein is secreted into the blood in response to increased oxidative stress. To investigate the responsiveness of renalase to oxidative stress, we examined the changes of renalase in cell and mouse models. We observed a significant increase of renalase expression in HepG2 cells in a time- and dose-dependent manner when treated with H_2O_2. Renalase expression also increased significantly in liver tissues that underwent the hepatic I/R process. The increased renalase levels could be efficiently suppressed by antioxidants *in vitro* and *in vivo*. Furthermore, serum renalase levels were significantly increased in the mouse models and also efficiently suppressed by antioxidants treatment. The variation trends are consistent between renalase and liver enzymes in the mouse models. In conclusion, renalase is highly sensitive and responsive to oxidative stress *in vitro* and *in vivo*. Moreover, renalase can be detected in the blood. These properties make renalase a highly promising biomarker for the evaluation of the severity of hepatic I/R injury.

1. Introduction

Ischemia is ceasing of blood supply, causing a shortage of oxygen; reperfusion is restoring of blood supply after ischemia. The ischemia-reperfusion (I/R) process occurs in many clinically important events, including hepatic resectional surgery, transplantation, trauma, and hemorrhagic shock [1–3]. Hepatic I/R injury is an inevitable complication causing severe cellular death, tissues damage, and liver dysfunction, which increases the mortality [4–6]. Reactive oxygen species (ROS), which are the main toxicants in oxidative stress, play critical roles in hepatic I/R injury [7–10]. Vascular endothelial cells, hepatic sinusoidal endothelial cells, Kupffer cells, or polymorphonuclear leukocytes are activated by the I/R process and produce a large amount of ROS in oxidative stress process, including superoxide, hydrogen peroxide (H_2O_2), hydroxyl radicals, and nitric oxide. This ROS production is the main factor responsible for damaging liver parenchymal cells, increasing vascular permeability, and inducing inflammatory cell infiltration [2, 11–15]. H_2O_2 is the most abundant and stable ROS. It leads to oxidative stress and is implicated in a variety of inflammatory diseases [16, 17]. H_2O_2 can be converted into hydroxyl radicals, which are extremely reactive and more toxic than other ROS [8, 9].

In this context, H_2O_2 is the perfect agent to establish an oxidative stress model in cells and also to simulate I/R injury *in vitro* to some extent.

It has been proposed that I/R injury is an intricate process in which the oxidant/antioxidant balance is changed in favor of the oxidants [18]. Treatment with superoxide dismutase (SOD) and catalase (CAT) reduces the ROS levels, as SOD rapidly dismutases the superoxide (O_2^-) to H_2O_2, and CAT scavenges H_2O_2 to produce water, which reduces the severity of oxidative stress [5]. Beside SOD and CAT, a variety of compounds and biomaterials has been developed as potential therapeutic agents for I/R injury [6, 17]. However, it still lacks efficient, specific, and sensitive biomarkers for the accurate evaluation of the severity of oxidative stress in hepatic I/R injury [19].

Renalase, a ubiquitous flavin adenine dinucleotide-containing amino oxidase, has been implicated in the process of I/R injury [20]. Since first being identified in 2005, renalase has been reported to be synthesized in various organs, including kidney, heart, liver, and adipose tissues [21]. Renalase is secreted into the blood in response to increased oxidative stress [22–24]. The elevated renalase level under stress conditions makes renalase a potential biomarker for the evaluation of the severity of organ I/R injury.

In the present study, we demonstrated that renalase is a sensitive ROS-responsive gene in hepatocytes. In hepatic I/R injury mouse models, renalase was augmented in liver and blood. Moreover, the augmentation of renalase can be ameliorated by antioxidants pretreating, which can reduce the severity of oxidative stress, *in vitro* and *in vivo*. These findings provide evidence that renalase can serve as an efficient and sensitive biomarker for the early warning or evaluation of the severity of hepatic I/R injury.

2. Material and Methods

2.1. Ethics Statement. All animal experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th edition, 2011) and approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology, China.

2.2. Reagents and Cell Culture. H_2O_2, pentobarbital sodium, SOD, and CAT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human hepatocellular carcinoma cell line HepG2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in DMEM (high glucose; Invitrogen, Madison, WI, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA) and 100 U/mL penicillin-100 μg/mL streptomycin (Gibco, Carlsbad, CA, USA) and kept in a humidified atmosphere at 37°C with 5% CO_2 in an incubator (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3. In Vivo Hepatic I/R Model. The *in vivo* hepatic I/R model was performed as previously described [3, 16, 17, 25]. Male C57BL/6 mice, aged 8–12 weeks, were purchased from Beijing University (Beijing, China) and maintained on a chow diet in a 12 h light/12 h dark environment at 25°C in the Animal Care Facility of Tongji Medical College. Surgical procedures on mice were performed under sterile conditions by administration of pentobarbital sodium (50 mg/kg) by an intraperitoneal injection. One hour before the pentobarbital sodium anesthesia, the I/R+SOD+CAT group was intraperitoneally injected with 300 KU/kg SOD and 60 mg/kg CAT, whereas the sham and I/R mice groups were given physiological saline as the solvent by the same method. Laparotomy was performed by vertically opening 2.5–3 cm in the anterior part of the abdomen of the anesthetized mice. After identifying the portal triad and biliary tree, the main trunk of the hepatic artery and portal vein, except for the vasculatures to the right lower lobe, was clamped with a vascular clip to achieve ischemic injury to approximately 70% of the liver. After 1 h of ischemia, reperfusion was achieved by releasing the vascular clip. No vascular clamp was done for the sham group of mice. Then, the incision was closed with silk suture. Six hours after reperfusion, hepatic lobes underwent I/R and the corresponding hepatic lobes from the mice of the sham group were removed and used for further assays. Histological evaluations (H&E staining and IHC of cleaved caspase-3) were performed to quantify the degree of liver injury. Confocal immunofluorescence imaging of frozen sections was performed to detect the renalase levels. Western blotting and real-time qPCR were performed to detect the protein levels and mRNA expression of renalase in liver tissue. Blood was taken by eyeball extirpating and then centrifuged for serum separation, and the serum was used for detection of levels of the renalase and liver enzymes.

2.4. Western Blot Analysis. As previously described [26–28], total cells and tissues were lysed using RIPA lysis buffer, and the protein concentration was determined with a BCA protein assay kit (Pierce Company, Rockford, IL, USA). Protein extracts were used for SDS-PAGE (Invitrogen, Carlsbad, CA, USA), and the proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA), which was blocked with 5% nonfat milk in TBS for 3 h and incubated with various primary antibodies overnight at 4°C. After incubation with HRP-conjugated secondary antibodies (diluted 1 : 5000) for 1 h at room temperature, the membranes were treated with ECL reagents (170–5061, Bio-Rad, Hercules, CA, USA) prior to visualization using a ChemiDoc MP imaging analysis system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The specific protein expression levels were normalized to β-tubulin on the same nitrocellulose membrane. The following primary antibodies and dilutions were used: anti-renalase (GTX89570, diluted 1 : 1000) was purchased from GeneTex (Irvine, CA, USA); anti-β-tubulin (sc-9104, diluted 1 : 2000) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.5. Real-Time RT-RCR. As previously described [29, 30], total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 2 μg of total RNA was reversely transcripted using an

Table 1: The sequences of primers for real-time qPCR.

Name	Use	Orientation	Sequence
Renalase	Real-time qPCR	F	5′-AGTGAACGCCAGAGGGAGCAA-3′
		R	5′-TAGCGGCAGGACCAAGGGAC-3′
β-Tubulin	Real-time qPCR	F	5′-GCTCATCGCTTATCACCTCC-3′
		R	5′-GAGCGCTCTGTCCACGTACT-3′

RNA PCR Kit (Takara Biotechnology, Otsu, Japan), and the resulting cDNA was used as a PCR template. The mRNA levels were determined by real-time qPCR with an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and β-tubulin was used as endogenous control. The experiment was performed in triplicate. The relative gene expression levels were calculated using the comparative C_T method applying the formula $2^{-\Delta\Delta C_T}$. The primer sequences for real-time qPCR are listed in Table 1.

2.6. Confocal Immunofluorescence. Fresh ischemia-reperfusion hepatic lobes were collected and rinsed in saline to remove remaining blood. Tissues were cut into 6-μm thick sections with a freezing slicing microtome. The sections were then immersed and fixed in 4% paraformaldehyde at room temperature for one-half hour. Afterwards, the sections were incubated with 5% bovine serum albumin (BSA) and immunolabeled with the indicated antibody (renalase GTX89570, GeneTex) at 4°C overnight. After washing, the sections were incubated with Alexa Fluor 594 goat anti-rabbit IgG (R37117, Invitrogen) for 1 h. After washing, 4′,6-diamidino-2-phenylindole (DAPI) was added to stain the cell nuclei on ice. Tissue fluorescence was imaged on a confocal microscope (Alsi, Nikon). For the quantitative expression of renalase, the density of fluorescence was analyzed by the ImageJ 1.44p software.

2.7. Liver Histology and IHC. Formalin-fixed liver specimens were embedded in paraffin blocks and cut into 5 μm sections. The sections were then stained with hematoxylin and eosin for histology. For IHC, the sections were deparaffinized and rehydrated with ethanol and xylene and were heated to 95–98°C for 20 min in 10 mM citrate buffer, pH 6.0. After blocking with PBS containing 10% goat serum for 1 h at room temperature, the sections were incubated with a primary antibody 1 : 200 cleaved caspase-3 overnight at 4°C. After incubation with secondary antibodies, the sections were mounted and evaluated with an Olympus microscope.

2.8. Detection of Liver Enzymes in Serum. Alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) in serum were determined using automatic biochemical analyzer (TBA-40FR, Toshiba, Tokyo, Japan). Related detection kits were purchased from MedicalSystem Biotechnology Co., Ningbo, China.

2.9. Detection of Serum Renalase Protein Levels. The protein levels of renalase in the serum were determined by using a Mouse Renalase (RNLS) ELISA Kit (MU30925, Bio-Swamp, Wuhan, China) according to the manufacturer's instructions. Briefly, dilute standard first. Add serum 40 μL to testing well, then add biotinylated anti-RNLS-antibody 10 μL, and gently mix. Incubate for 30 min at 37°C. Dry and wash every well. Add HRP-Conjugate Reagent 50 μL to each well, except the blank well. Incubate and wash. Then add chromogen solutions and incubate for 15 min at 37°C. Measure the optical density (OD) at 450 nm after adding Stop Solution within 15 min. Calculate according to the manufacturer's instructions.

2.10. Statistical Analysis. As described in previous studies [31, 32], the statistical analysis was performed with the Statistical Package for Social Sciences (SPSS version 13.0; IBM Analytics, Chicago, IL, USA). All the data were expressed as mean ± SD (standard deviation, SD) and the difference was analyzed by a one-way ANOVA test. Statistical analysis was performed using Student's t-test for paired data. The difference was considered statistically significant for $p < 0.05$.

3. Results

3.1. Increase of the Renalase Expression in HepG2 Cells Induced by H_2O_2. To investigate the impact of oxidative stress on the renalase expression in HepG2 cells, the cells were incubated with H_2O_2 in different concentrations and for different times. First, HepG2 cells were incubated with H_2O_2 in increasing concentrations (0 μM, 100 μM, 200 μM, 500 μM, and 1000 μM) for 6 h, respectively. With increasing H_2O_2 concentration, the mRNA expression of renalase increased proportionally (Figure 1(a)). The maximal expression of renalase was detected for a concentration of 500 μM. Renalase expression decreased slightly when incubated with a concentration of 1000 μM compared with 500 μM (Figure 1(a)). This suggests that the concentration of 1000 μM may be beyond the cell's affordable range and causes cell death. We used relatively higher concentrations (1000 μM, 2000 μM, 3000 μM, and 5000 μM) of H_2O_2 to treat cells and found that cell death increased with the increasing concentrations of H_2O_2. Treating with 2000 μM of H_2O_2 for 6 h could even cause more than half of the cell death. No cells could survive in the concentration of 5000 μM H_2O_2 (data not shown). Then, HepG2 cells were incubated with 500 μM H_2O_2 for different times (15 min, 30 min, 1 h, 3 h, 6 h, 12 h, and 24 h). Increasing the incubation time induced a proportional increase of the

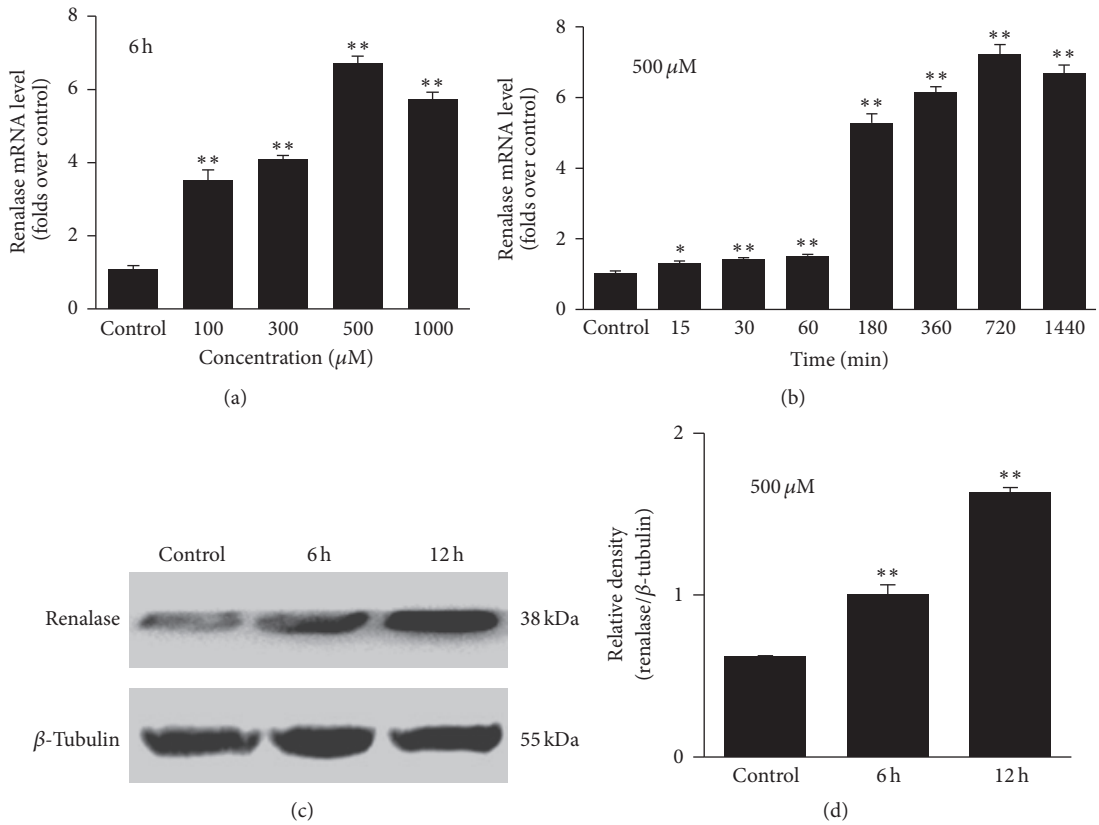

Figure 1: Increase of renalase expression in hepatic cells upon H_2O_2 treatment. (a) Relative expression of renalase mRNA evaluated by real-time qPCR in HepG2 cells treated with increasing concentrations of H_2O_2. (b) Relative expression of renalase mRNA evaluated by real-time qPCR in HepG2 cells treated with H_2O_2 for different times. (c) Western blot analysis of renalase protein levels in HepG2 cells treated with 500 μM H_2O_2 for 6 h or 12 h. (d) Densitometric analysis of Western blot of renalase in HepG2 cells treated with 500 μM H_2O_2 for 6 h or 12 h. $^*p < 0.05$; $^{**}p < 0.01$, compared with control group. Data are plotted as the mean ± SD from five independent experiments. Bars indicate the standard deviation of the mean.

mRNA expression of renalase (Figure 1(b)). The maximal expression of renalase was measured for an incubation time of 12 h. Renalase expression decreased slightly when incubated for 24 h (Figure 1(b)). This indicates that the cells may not tolerate the long-time incubation of H_2O_2. Further, the protein levels of renalase in HepG2 cells that were incubated with 500 μM H_2O_2 for either 6 h or 12 h were measured and found to be larger for the longer incubation ($p < 0.01$, Figures 1(c) and 1(d)).

3.2. Suppression of H_2O_2-Induced Augmentation of the Renalase Expression in HepG2 Cells Mediated by Antioxidant Preincubation. Antioxidants can reduce the severity of oxidative stress. To investigate whether the H_2O_2-induced augmentation of the renalase expression in HepG2 cells can be decreased by antioxidants, cells were preincubated with 300 KU/L SOD and 60 mg/L CAT for 2 h. Before the H_2O_2-treatment, the SOD and CAT-containing medium was replaced by FBS-free medium. The obtained results showed that the SOD/CAT preincubation significantly decreased the H_2O_2-induced augmentation of the renalase mRNA expression in HepG2 cells ($p < 0.01$, Figure 2(a)). Furthermore, the SOD/CAT preincubation significantly decreased the H_2O_2-induced increase of the renalase protein levels in HepG2 cells ($p < 0.01$, Figures 2(b) and 2(c)).

3.3. Increased Renalase Expression and Histologic Damage in Hepatic I/R Injury Mouse Models and the Suppression to the Increased Renalase Expression by Antioxidants. In hepatic I/R injury mouse models, the renalase mRNA expression in livers was significantly augmented ($p < 0.01$, Figure 3(a)), and the intraperitoneal preinjection of SOD and CAT decreased this augmentation significantly ($p < 0.01$, Figure 3(a)). The renalase protein levels in livers of the mouse models were significantly increased ($p < 0.01$, Figure 3(c)) and could also be suppressed by the intraperitoneal preinjection of SOD and CAT ($p < 0.01$, Figure 3(c)). This result was confirmed by Western blot (Figures 3(b) and 3(c)) and confocal immunofluorescence imaging (Figure 4(b)). The histological evaluation revealed that I/R caused hydropic degeneration and necrosis of hepatic tissue and cells. A significant improvement was observed in I/R+SOD+CAT group (Figure 4(a)). Consistent with the observed hydropic degeneration and necrosis during I/R, hepatic apoptosis was increased as

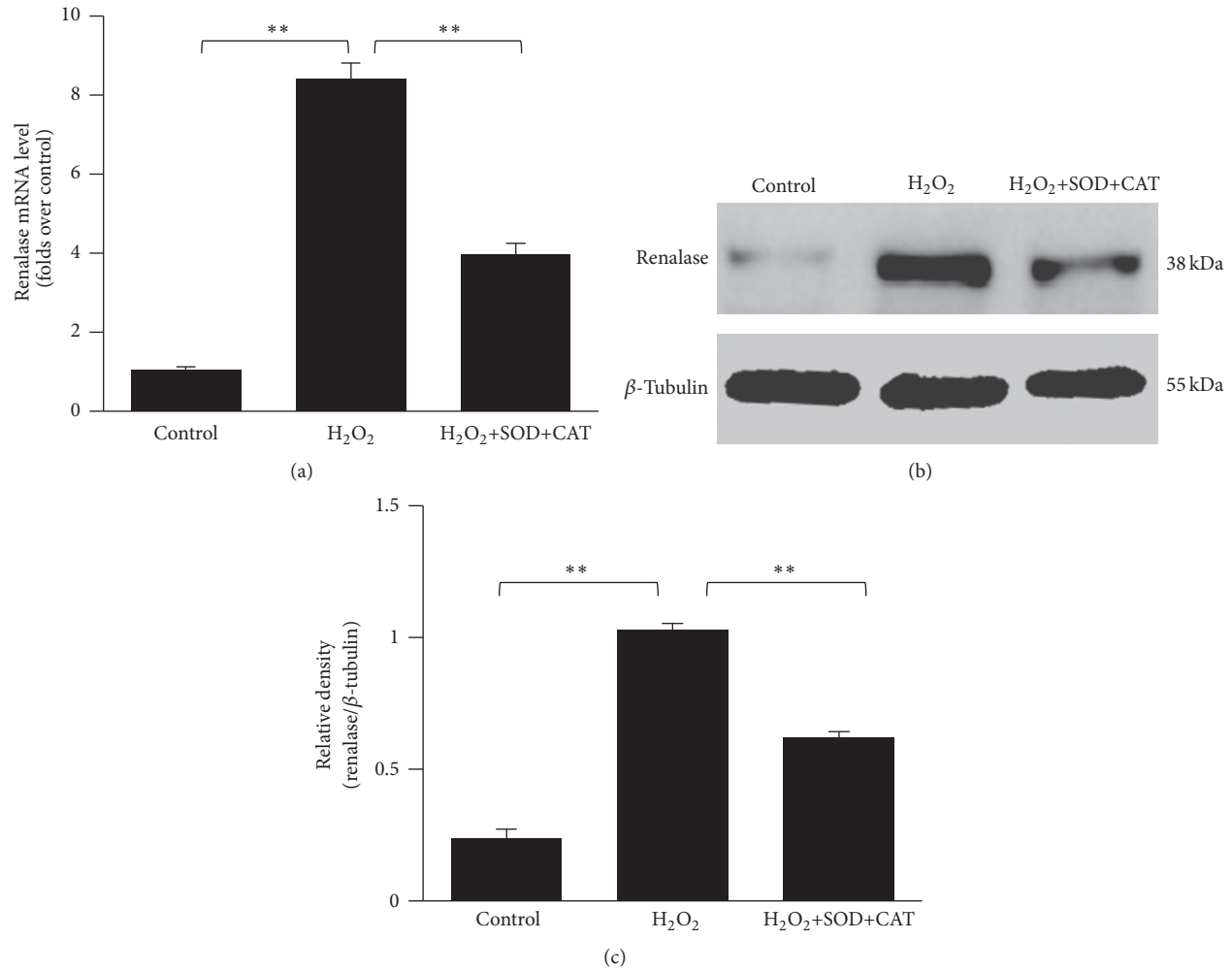

Figure 2: Antioxidant-induced decrease of H_2O_2-induced augmentation of the renalase expression in hepatic cells. (a) Relative expression of renalase mRNA evaluated by real-time qPCR in H_2O_2-incubated (12 h) HepG2 cells with or without pretreatment with antioxidants. (b) Western blot analysis of renalase protein levels in H_2O_2-incubated (12 h) HepG2 cells with or without preincubation with antioxidants. (c) Densitometric analysis of Western blot of renalase in H_2O_2-incubated HepG2 cells with or without preincubation with antioxidants. $^{**}p < 0.01$. Data are plotted as the mean ± SD from five independent experiments. Bars indicate the standard deviation of the mean.

seen by the brown intracellular staining of cleaved caspase-3 in most hepatocytes. A significant reduction of cleaved caspase-3 staining was observed in the I/R+SOD+CAT group (Figure 4(a)).

3.4. Increase of the Serum Levels of Renalase and Liver Enzymes in Hepatic I/R Injury Mouse Models and the Suppression to the Increased Renalase and Liver Enzymes by Antioxidants. The liver enzymes (ALT, AST, GGT, ALP, and LDH) and renalase levels were measured in the serum of the hepatic I/R injury mouse models. The renalase levels were found to be significantly augmented in the hepatic I/R process ($p < 0.01$). The levels of ALT ($p < 0.01$), AST ($p < 0.01$), ALP ($p < 0.05$), and LDH ($p < 0.01$) elevated significantly in the hepatic I/R group compared to the sham group, whereas GGT increased slightly in the hepatic I/R group and with no statistical significance to the sham group ($p > 0.05$). The increase of ALT, AST, LDH, and renalase levels could be effectively suppressed (renalase, ALT, and LDH: $p < 0.01$; AST: $p < 0.05$) by the intraperitoneal preinjection of SOD and CAT. ALP and GGT could also be suppressed by antioxidants, but with no statistical significance ($p > 0.05$) to the I/R group (Figure 5).

4. Discussion

Hepatic I/R injury may occur in hepatic resections, liver transplantations, traumas, and vascular surgeries [33–35]. It is a serious complication in clinical practice. The redox balance in the process of hepatic I/R injury leads to accumulation of ROS, which presents the disease mechanism most commonly invoked in hepatic I/R injury [33]. Suppressing ROS-burst in oxidative stress is an efficient way to alleviate hepatic I/R injury. However, early warning, evaluation of the severity, or therapeutic effect of oxidative stress, which is even more important in clinical practice of hepatic I/R injury

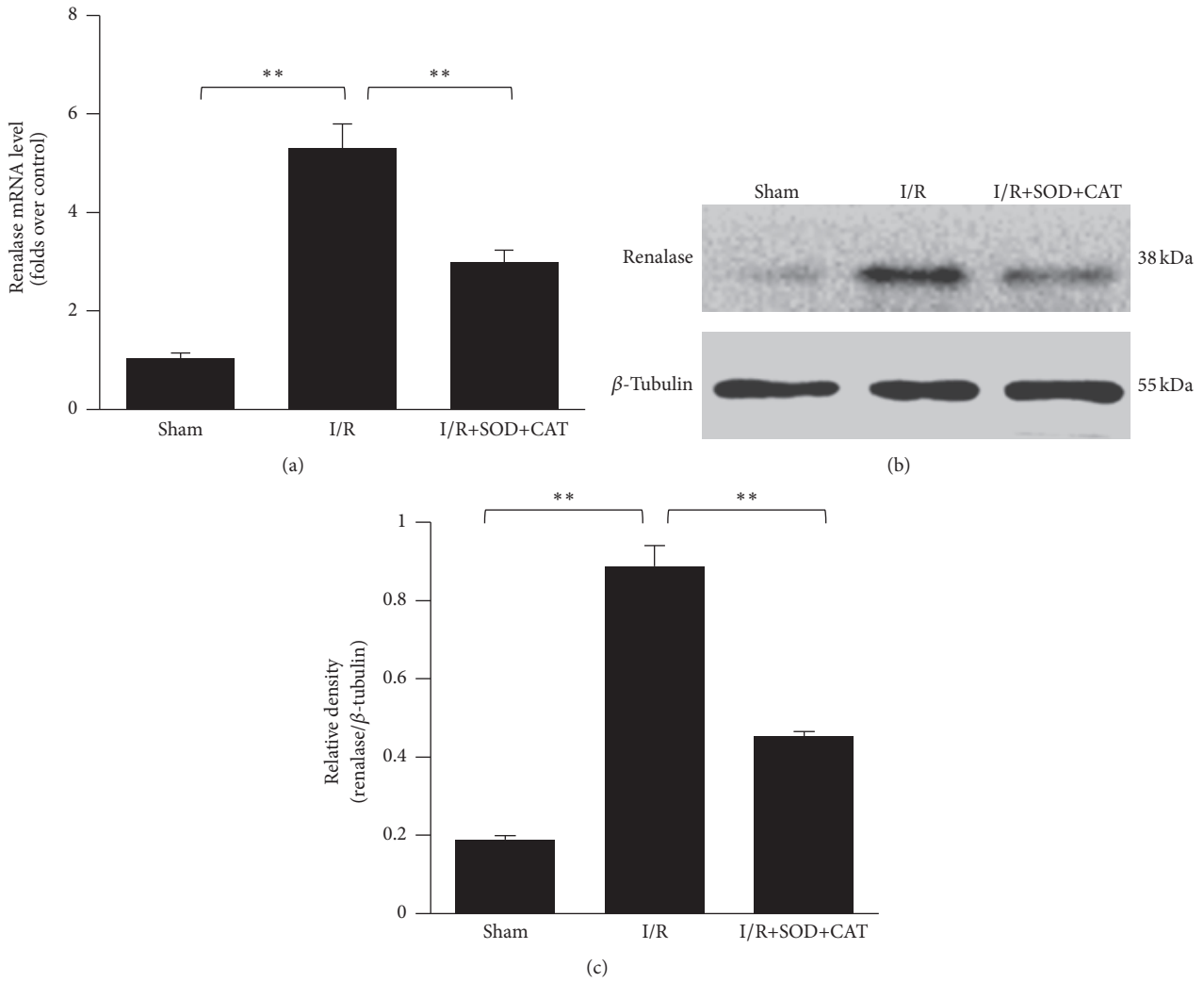

FIGURE 3: Augmentation of renalase expression in hepatic I/R injury models and the subsequent decrease induced by antioxidant preinjection. (a) Relative expression of renalase mRNA evaluated by real-time qPCR in livers of mouse models (sham, I/R injury, and I/R injury with antioxidant preinjection, 6 h after reperfusion). (b) Western blot analysis of renalase protein levels in livers of mouse models (sham, I/R injury, and I/R injury with antioxidant preinjection, 6 h after reperfusion). (c) Densitometric analysis of Western blot of renalase in livers of mouse models (sham, I/R injury, and I/R injury with antioxidant preinjection, 6 h after reperfusion). $^{**}p < 0.01$. Data are plotted as the mean ± SD from five independent experiments. Bars indicate the standard deviation of the mean.

management, is a remaining challenge. This is due to the lack of efficient, specific, and sensitive biomarkers for the accurate evaluation of the severity of oxidative stress [19]. Biochemical markers used to evaluate the severity of oxidative stress include malondialdehyde (MDA) [36, 37], ascorbic acid (AA)/dehydroascorbic acid (DHA) [38, 39], and a series of inflammatory, proinflammatory, or anti-inflammatory biomarkers [40–42]. However, these markers face various limitations. MDA is a marker for lipid peroxidation, which usually proceeds in the I/R injury of the extremities [43]. A close relationship between MDA and cardiac necrosis markers has been reported [36], but whether the MDA level can reflect the severity of oxidative stress or I/R injury of other organs remains unclear. The determination of AA and DHA levels is challenging because of the unstable nature of these compounds [39]. Inflammatory, proinflammatory, and anti-inflammatory markers cover a wide range of cytokines. However, these cytokines are not specific for evaluating oxidative stress. Therefore, an efficient, specific, and sensitive biomarker for evaluating oxidative stress is still urgently needed.

The human renalase gene is located on chromosome 10 at q23.33 and encodes the conserved protein renalase consisting of 342 amino acids. Renalase was reported to efficiently degrade dopamine and epinephrine in the blood [21], and it has been verified that it is the only enzyme involved in the catecholamine metabolism that can be secreted into the blood cycle hitherto [44]. Previous studies indicated that renalase is multifunctional and closely related to oxidative stress conditions such as stroke, heart transplantation, or acute

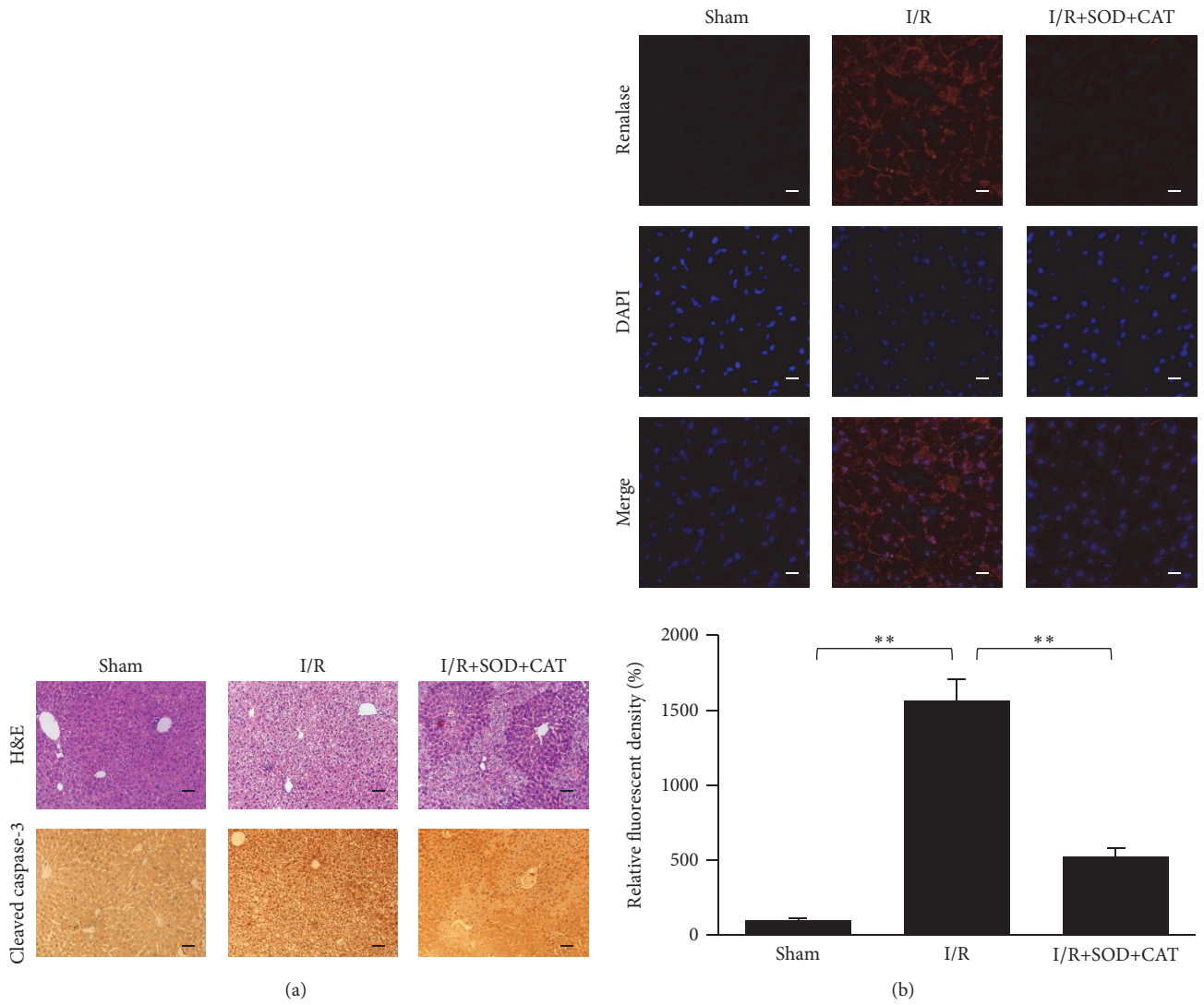

FIGURE 4: Increase of renalase levels in hepatic I/R injury models and its suppression induced by antioxidant preinjection and the corresponding histological changes in liver. (a) The livers of the sham, I/R, and IR+SOD+CAT groups (6 h after reperfusion) were subjected to histological evaluation by H&E staining and IHC of cleaved caspase-3. Scale bar (black) represents 50 μm. (b) Confocal immunofluorescence imaging of renalase in livers of mouse models (sham, I/R injury, and I/R injury with antioxidant preinjection, 6 h after reperfusion). Scale bar (white) represents 30 μm. The relative fluorescent density of each group was analyzed. $^{**}p < 0.01$. Data are plotted as the mean ± SD from five independent experiments. Bars indicate the standard deviation of the mean.

kidney injury [45–48]. Considering its tight relationship with oxidative stress, renalase is thought to play a role, or at least responsive, in the process of hepatic I/R injury.

In particular, H_2O_2 is considered as the most abundant and stable ROS. H_2O_2 is a mild oxidant but is converted into hydroxyl radicals, which are extremely reactive and more toxic than other ROS [49, 50]. Therefore, to investigate the responsiveness of renalase in hepatic cells under the condition of oxidative stress, H_2O_2 was used to mimic the ROS-burst in oxidative stress. For the cell model *in vitro*, the common hepatocellular carcinoma cell line HepG2 was chosen. Renalase expression in HepG2 cells showed a positive correlation with H_2O_2 concentration and incubation time. These results suggest that renalase is responsive to the ROS-burst or oxidative stress in hepatic cells, and it is sensitive to the degree or severity and duration of oxidative stress.

Disturbance of the redox balance is characteristic for oxidative stress in the process of the hepatic I/R injury, which leads to ROS-burst and antioxidant consumption [33]. The most efficient way to ameliorate oxidative stress is replenishing ROS-scavenging antioxidants [17]. SOD is a common antioxidant, which is wildly distributed within mammalian organisms [51]. It is an important free radical scavenger, which has been clinically used in several countries. CAT is also an endogenous antioxidant, which can catalytically decompose H_2O_2 [52, 53], but has not been used in clinical practice. In this study, SOD and CAT were used for the preincubation of the cells. We observed that

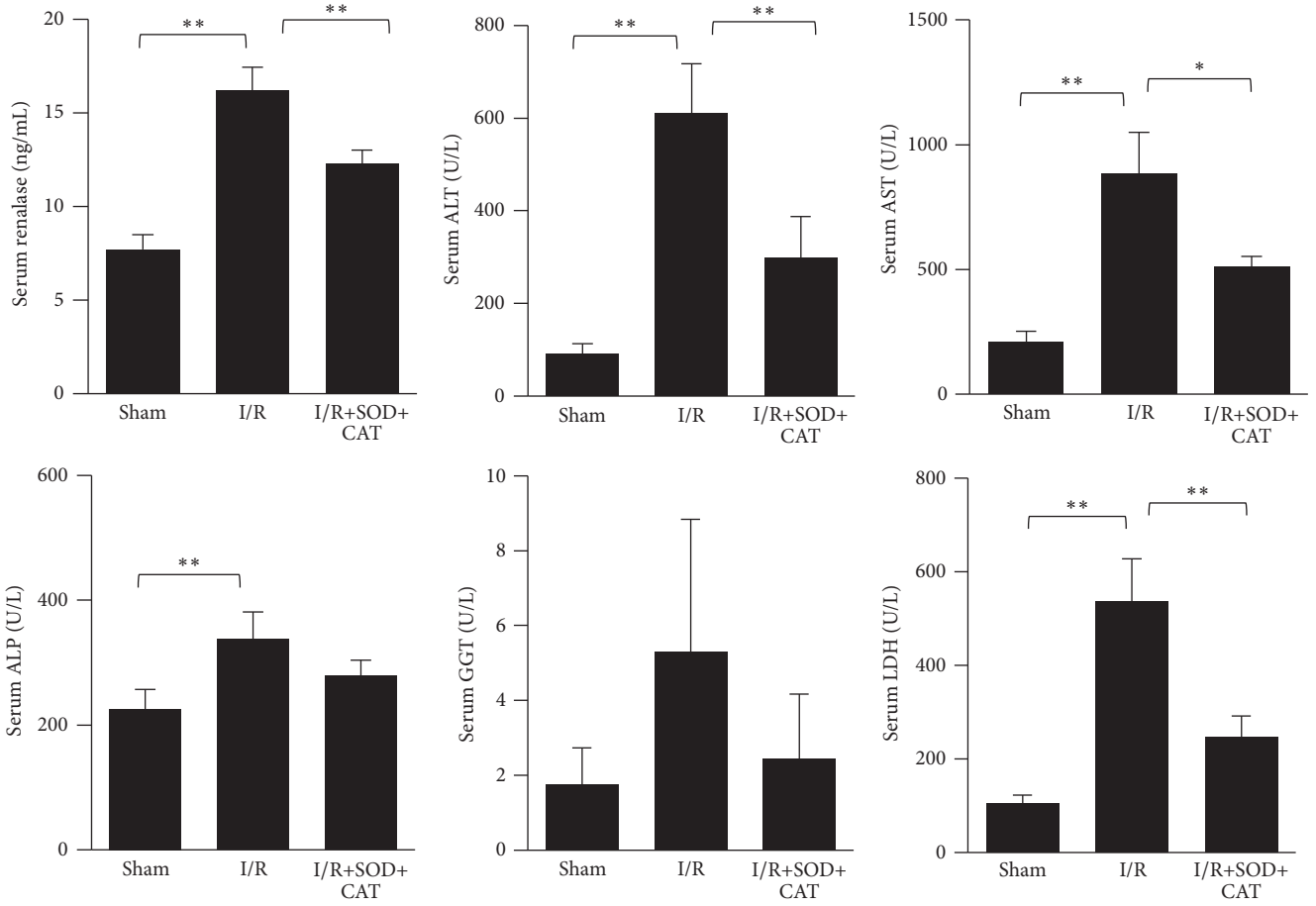

Figure 5: Increase of serum renalase and liver enzymes levels in hepatic I/R injury models and its suppression induced by antioxidant preinjection. Serum renalase and liver enzymes: ALT, AST, GGT, ALP, and LDH levels in mouse models (sham, I/R injury, and I/R injury with antioxidant preinjection, 6 h after reperfusion). $^{*}p < 0.05$; $^{**}p < 0.01$. Data are plotted as the mean ± SD from five independent experiments. Bars indicate the standard deviation of the mean.

the SOD/CAT preincubation significantly ameliorated the elevation of the renalase expression in HepG2 cells. The application of antioxidants reduced the severity of oxidative stress, and the responsive elevation of the renalase expression was ameliorated accordingly. This result suggests that renalase is responsive to the therapeutic means for oxidative stress in hepatic cells. And the expression of renalase is closely related to the degree or severity of the oxidative stress.

The hepatic I/R injury mouse model is the most commonly used animal model in various studies for hepatic I/R injury and oxidative stress *in vivo*. We used this model to investigate whether renalase is responsive to the hepatic I/R injury *in vivo*. Renalase expression was significantly increased in the livers of hepatic I/R injury mouse models. Intraperitoneal preinjection of SOD and CAT ameliorated the elevation of renalase in the livers significantly. Furthermore, a consistent high-and-low variation between the renalase expression in liver and the degree of liver damage, including tissue necrosis and cell apoptosis, has been observed. Liver enzymes are the most important evaluation indexes in the standard liver function test (LFT) panel. Serum levels of liver enzymes, including ALT, AST, GGT, ALP, and LDH, were increased by different degrees in the hepatic I/R injury mouse models. Consistently, the serum renalase levels of the hepatic I/R injury mouse models were significantly elevated. The elevations of serum levels of either the renalase or the liver enzymes could be significantly reduced by the intraperitoneal preinjection of SOD and CAT. These results suggest that renalase is responsive to hepatic I/R injury and therapeutic means. Its expression in liver tissue closely related to the severity of oxidative stress and the subsequent liver damage. Moreover, the serum renalase level can sensitively reflect the severity and therapeutic effect of oxidative stress in the process of hepatic I/R injury. Combining with the standard LFT panel, more accurate and specific evaluations of the hepatic I/R injury can be made.

In conclusion, to the best of our knowledge, this study firstly demonstrates the value and applicability of renalase as a promising biological marker for the evaluation of the severity of oxidative stress in hepatic I/R injury. We investigated the responsiveness of renalase to conditions related to oxidative stress *in vitro* and *in vivo*. Our results revealed that oxidative stress efficiently induces the elevation of renalase expression *in vitro* and *in vivo*. Furthermore, this elevated

renalase expression can be decreased by antioxidants. Its sensitive responsiveness to the severity of oxidative stress and convenient detection in the blood make renalase an ideal biomarker for the evaluation of the severity and therapeutic effect of hepatic I/R injury. In the future, clinical treatment decisions based on the detection of renalase activities in the blood may help to improve the clinical outcomes of hepatic resections, liver transplantations, traumas, or vascular surgeries. Nevertheless, the variation trend of serum renalase level in the hepatic I/R process under different conditions and time points has not been demonstrated in this study, which needs further investigations. Beside the I/R injury, there are various pathophysiological processes which are related to oxidative stress. Renalase may play roles in these processes, and the variation trend of renalase expression in these processes may be different from each other. Related researches may make renalase serve as a novel inspection item, which can assist the existing inspection items to make the evaluations of the diseases more accurate. Other potential applications of renalase in clinical practice require further explorations and investigations.

Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests

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

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Review Article

Schisandrin B: A Double-Edged Sword in Nonalcoholic Fatty Liver Disease

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Nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver lesions ranging from hepatic steatosis, nonalcoholic steatohepatitis, hepatic cirrhosis, and hepatocellular carcinoma. The high global prevalence of NAFLD has underlined the important public health implications of this disease. The pathogenesis of NAFLD involves the abnormal accumulation of free fatty acids, oxidative stress, endoplasmic reticulum (ER) stress, and a proinflammatory state in the liver. Schisandrin B (Sch B), an active dibenzooctadiene lignan isolated from the fruit of *Schisandra chinensis* (a traditional Chinese herb), was found to possess antihyperlipidemic, antioxidant, anti-ER stress, and anti-inflammatory activities in cultured hepatocytes *in vitro* and in rodent livers *in vivo*. Whereas a long-term, low dose regimen of Sch B induces an antihyperlipidemic response in obese mice fed a high fat diet, a single bolus high dose of Sch B increases serum/hepatic lipid levels in mice. This differential action of Sch B is likely related to a dose/time-dependent biphasic response on lipid metabolism in mice. The hepatoprotection afforded by Sch B against oxidative stress, ER stress, and inflammation has been widely reported. The ensemble of results suggests that Sch B may offer potential as a therapeutic agent for NAFLD. The optimal dose and duration of Sch B treatment need to be established in order to ensure maximal efficacy and safety when used in humans.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) constitutes a spectrum of alcohol consumption-independent liver lesions ranging from hepatic steatosis, nonalcoholic steatohepatitis (NASH), hepatic cirrhosis, and hepatocellular carcinoma (HCC) [1]. Recently, an epidemiological meta-analysis study has shown that the global prevalence of NAFLD is 25% [2], indicative of the magnitude of the clinical as well as the economic burden globally. To cope with this situation, preventative interventions are urgently needed. Traditional Chinese medicinal herbs, which have a long history of use in safeguarding health, may offer a promising approach for the prevention and/or treatment of NAFLD. In this review, we will consider the pathogenesis of NAFLD, followed by a discussion of the hepatoprotective action of schisandrin B (Sch B), an active dibenzooctadiene lignan isolated from the fruit of *Schisandra chinensis* (FSC, a traditional Chinese herb), in relation to the pathogenesis of NAFLD.

2. A Brief Introduction to the Pathogenesis of NAFLD

Obesity, as well as the associated insulin resistance, is a predisposing factor in the pathogenesis of NAFLD. About 30–90% of obese individuals will eventually develop hepatic steatosis, which is defined as an abnormal accumulation of lipid at ≥5% of the organ weight [3]. However, 10–20% of patients with hepatic steatosis may go on to develop NASH in which inflammation and hepatic tissue damage occur [4]. Patients (3–5%), with NASH, in whom livers exhibit a repeated damage-and-repair cycle due to chronic inflammation, may go on to develop cirrhosis [1]. Cirrhosis, which is referred to as the dysfunction of fibrotic liver, is one of the risk factors for hepatocellular carcinoma [5] (Figure 1).

2.1. Hepatic Steatosis. The liver plays a pivotal role in the metabolic homeostasis of carbohydrates, lipids, and proteins. Hepatic lipid content is governed by the uptake of free fatty

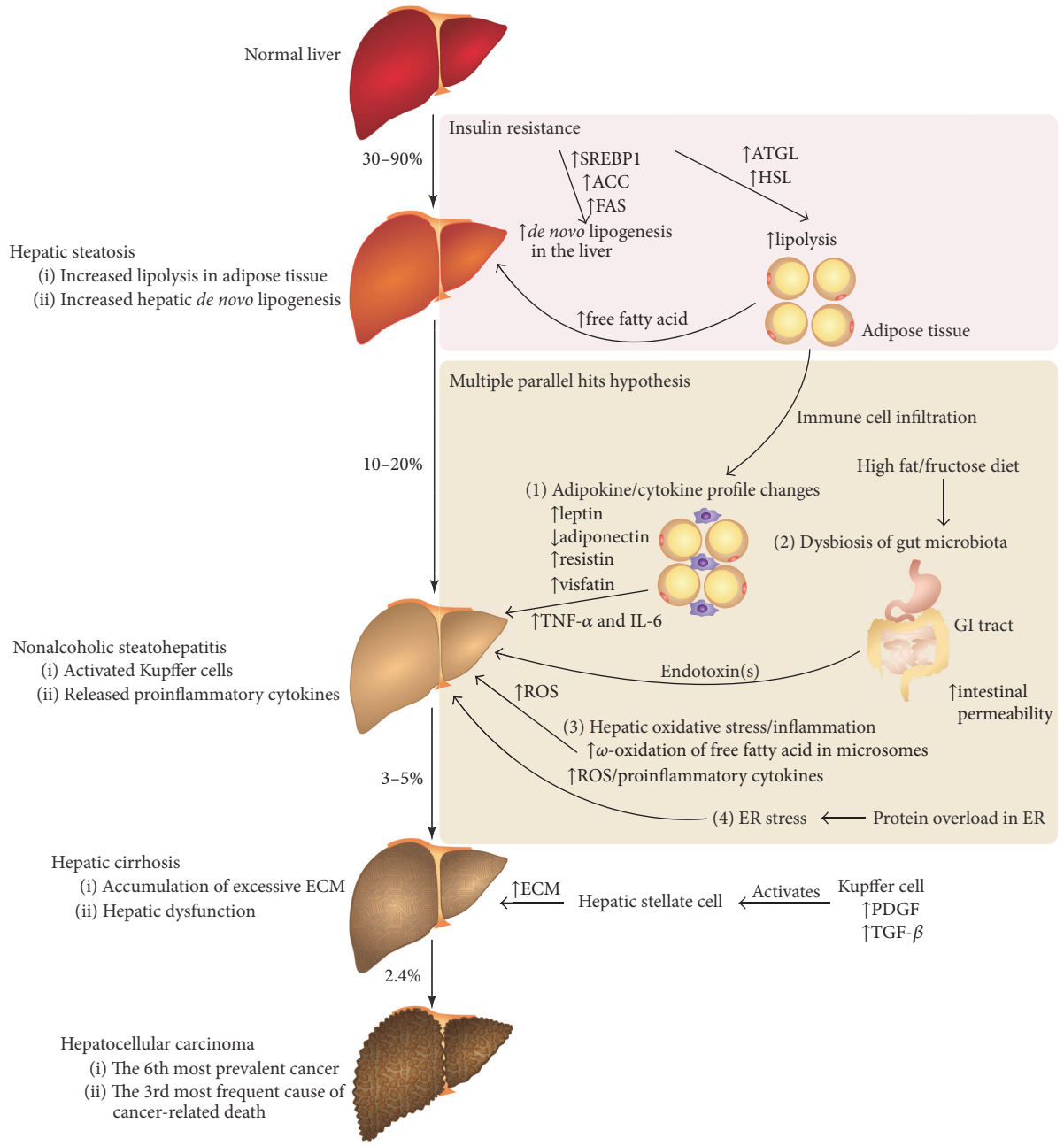

Figure 1: The pathogenesis of nonalcoholic fatty liver disease (NAFLD). The percentage shown along the arrow indicates the prevalence of the pathogenesis leading to the next stage of NAFLD. Hallmarks and important features of each stage of NAFLD are indicated. Insulin resistance is an important pathological factor for the development of hepatic steatosis (a benign stage of NAFLD), presumably due to the induction of hepatic *de novo* lipogenesis as well as lipolysis of adipose tissue. Key enzymes involved in these processes are indicated (in pink box). Changes in the profile of adipokines and cytokine, dysbiosis of gut microbiota, hepatic oxidative stress/inflammation, and endoplasmic reticulum ER stress are regarded as the "multiple parallel hits" of the pathogenesis of NASH from hepatic steatosis. Key factors involved in the pathogenesis are indicated (in orange box). SREBP1: sterol regulatory element-binding protein-1; ACC: acetyl-CoA carboxylase; FAS: fatty acid synthase; ATGL: adipose triglyceride lipase; HSL: hormone-sensitive lipase; GI tract: gastrointestinal tract; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; ROS: reactive oxygen species; ER: endoplasmic reticulum; PDGF: platelet-derived growth factor; TGF-β: transforming growth factor-β; ECM: extracellular matrix.

acids as well as the export of processed lipids [such as very low density lipoprotein (VLDL)] [6]. Free fatty acids in the liver can arise from the lipolysis of triacylglycerol (TAG) which is stored in white adipose tissue under fasting conditions. Another source of fatty acids is the dietary intake of lipid, which is processed into chylomicrons. The liver can also undergo *de novo* lipogenesis, in which excess blood glucose can be converted into TAG under postprandial

conditions [6, 7]. In *de novo* lipogenesis, the major transcription factors, namely, sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP), can induce the expression of an array of enzymes involved in lipogenesis (such as acetyl-CoA carboxylase (AAC) and fatty acid synthase (FAS)) in the presence of high levels of insulin and glucose [6, 7]. In this regard, insulin plays a critical role in the regulation of lipogenesis via the induction of SREBP-1c activity. On the other hand, lipid mitochondrial β-oxidation in the liver can also reduce TAG levels [6, 7]. In the process of mitochondrial β-oxidation, long-chain-fatty-acid-CoA ligase, carnitine palmitoyltransferase I (CPT1), and carnitine palmitoyltransferase 2 (CPT2) play critical roles in the transfer of free fatty acids into the mitochondrial matrix, with resultant production of fatty acyl-CoA which is the initial substrate for β-oxidation [6, 7]. The fatty acyl-CoA is metabolized into acetyl-CoA and water with the generation of ATP. In addition, hepatic lipids can also serve as an endogenous supply of lipid in the body. Hepatic triglycerides (TG), cholesterol, and apolipoproteins can be assembled into VLDLs which circulate in the bloodstream for the delivery of lipids to peripheral tissues [6].

The dysregulation of intermediary metabolism in the liver, which is usually observed in insulin-resistant/obese individuals, can lead to an abnormal accumulation of lipid in the liver [8]. This excessive accumulation predominantly arises from the overflow of free fatty acids following lipolysis in white adipose tissue as well as hepatic *de novo* lipogenesis. However, pathological changes in hepatic mitochondrial β-oxidation and the export of VLDLs are less likely to be involved [9]. Given that insulin regulates lipolysis in adipose tissue as well as *de novo* lipogenesis in the liver, insulin resistance is associated with hepatic steatosis [8]. In healthy individuals, insulin can exert a powerful antilipolytic action by the inactivation of hormone-sensitive lipase (HSL) via the phosphatidylinositol 3-kinase (PI3K)/Akt/phosphodiesterase 3B pathway [10]. In insulin-resistant individuals, the reduced sensitivity of white adipocytes to insulin can therefore lead to increased lipolysis [10]. In healthy individuals, insulin can suppress gluconeogenesis and induce lipogenesis in the liver. However, in the liver of rodents, the induction of insulin resistance is associated with overproduction of glucose (leading to hyperglycemia) as well as overinduction of SREBP-1c (which leads to increased hepatic *de novo* lipogenesis) [11, 12]. This paradoxical observation suggests differential regulation of hepatic gluconeogenesis and lipogenesis by insulin in insulin-resistant rodents, presumably due to diversity in insulin receptor signal transduction pathways [13]. The insulin receptor substrates IRS1 and IRS2 bind to the activated insulin receptor and serve as adaptor molecules for the further propagation of signal transduction. Shimomura et al. have hypothesized that insulin selectively suppresses IRS2 (which regulates gluconeogenic genes) but activates IRS1 (which induces lipogenic genes) [11], with resultant suppression of gluconeogenesis and enhancement of lipogenesis. An alternative hypothesis is that endoplasmic reticulum (ER) stress, during which excessive unfolded/misfolded proteins accumulate in the ER, can induce the expression of lipogenic genes in insulin-resistant liver, leading to the suppression of gluconeogenesis and enhancement of lipogenesis [14].

2.2. Nonalcoholic Steatohepatitis (NASH). Given that only 10–20% of patients with HS will develop NASH, the pathogenesis of NASH was traditionally hypothesized to be a "two-hit" process [15], in which the "first hit" involves the excessive accumulation of lipid in the liver (i.e., hepatic steatosis) and the "second hit" involves risk factors (such as bacteria-derived endotoxin) that can induce liver inflammation [15]. Recently, accumulating experimental evidence has demonstrated the insufficiency of the "two-hit" hypothesis in explaining the complicated pathogenesis of NAFLD. The relatively low incidence rate (10–20%) of NASH in patients with hepatic steatosis suggests that hepatic steatosis is a benign state in the majority of NAFLD patients, while NASH is likely to be an optional diagnosis after fulfilling certain pathological conditions [16]. Tilg and Moschen have proposed a new model of the evolution of inflammation in NAFLD, namely, the "multiple parallel hits" hypothesis [16], in which the development of NASH from hepatic steatosis may involve many "hits," including dysbiosis of the gut microbiota, the release of proinflammatory cytokines, hepatic oxidative stress/inflammation, pathological changes in the plasma level of adipokines, and genetic/epigenetic factors, acting in parallel, with resultant pathogenesis of NASH. In this section, we will mainly focus on the effects of gut microbiota, hepatic oxidative stress/inflammation, ER stress, and adipokines on the pathogenesis of NAFLD. Genetic and epigenetic factors in relation to NAFLD have been reviewed by Dongiovanni and Valenti [17].

2.2.1. Dysbiosis of Gut Microbiota. The involvement of gut microbiota in the development of high fat diet-induced obesity in mice was first demonstrated by Turnbaugh et al., who demonstrated that the relative abundance of two gut bacterial populations, namely, Bacteroidetes and Firmicutes, is associated with the phenotype of obese and lean mice (and this was also seen in human volunteers) [18, 19]. This postulation was also supported by the experimental finding that the inoculation of germ-free mice with "obese microbiota" (isolated from high fat diet-fed mice) can lead to significant increases in body weight, total body fat disposition, hepatic lipogenesis, and insulin resistance relative to those animals transplanted with "lean microbiota" (isolated from control mice) [19, 20]. The ability of a high fat diet to decrease the gut bacterial ratio of Bacteroidetes to Firmicutes (i.e., the dysbiosis of gut microbiota) further confirms the interrelationships among diet, gut microbiota, and NAFLD phenotype [21, 22]. In addition to the feeding of a high fat diet, the effect of a high carbohydrate (particularly fructose) diet on NAFLD in relation to gut microbiota has also recently been described. Given that fructose is primarily metabolized in the liver, a diet with excessive fructose was found to increase the incidence of hepatic steatosis [23–25], presumably due to the dysbiosis of gut microbiota and the increase in the permeability of intestine to microbiome as well as increased levels of endotoxins in blood. The increased permeability of

intestine to microbiome likely increases the risk of microbiome infection, with resultant endotoxemia [26, 27]. In response to endotoxemia, the residential macrophages in the liver (Kupffer cells) are activated *via* the toll-like receptor and subsequently release proinflammatory cytokines, which is a predisposing factor in the pathogenesis of NASH [28–30]. Under normal physiological conditions, mammalian nondigestible carbohydrates (such as dietary fibers) can be metabolized by gut microbiota, with resultant production of short-chain fatty acids (SCFA), which can maintain a favorable environment for microbial flora in the GI tract [31]. In this regard, the replacement of nondigestible carbohydrates with simple carbohydrates (such as fructose) was found to change the microbial composition of the gut, particularly the ratio between Bacteroidetes and Firmicutes [32]. The ensemble of experimental findings strongly suggests the interrelationships among diet, gut microbiota, and NAFLD. However, the direct linkages among diet, gut microbiota, and NAFLD remain to be investigated.

2.2.2. Hepatic Inflammation and Oxidative Stress. Recent studies have indicated that saturated fatty acids can activate the proinflammatory toll-like receptor 4/transforming growth factor β-activated kinase-binding protein/c-Jun N-terminal kinase/nuclear factor-κB (NF-κB) signaling cascade, with the resultant release of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and transforming growth factor-β (TGF-β) [33]. The cytokines further induce the infiltration of inflammatory cells, leading to a vicious cycle that leads to the development of liver damage. The involvement of toll-like receptor 4 in the development of NASH was supported by a study showing that the feeding of a high fat diet did not induce obesity, insulin resistance, or inflammation in toll-like receptor 4 knockout mice [34]. In patients with hepatic steatosis, the increased availability of free fatty acids in the liver likely increases mitochondrial β-oxidation [35], with a resultant increase in the production of acetyl-CoA. The overproduction of acetyl-CoA can overwhelm the capacity of the tricarboxylic acid (TCA) cycle and the electron transfer chain (ETC) as well as the ATP synthase-catalyzed reaction, leading to increased leakage of electrons from ETC and hence the generation of reactive oxygen species (ROS) [36]. In addition, mitochondrial β-oxidation is the major mechanism for the hepatic disposition of free fatty acids under physiological conditions [35]. To cope with the hyperlipidemia in hepatic steatosis, peroxisomal and microsomal ω-oxidation of fatty acids are activated in a complementary fashion. Peroxisomal and microsomal oxidation of fatty acids involve cytochrome P_{450} 2E1 and cytochrome P_{450} reductase, both of which generate ROS as byproducts of the catalytic reaction [37, 38]. ROS can lead to the peroxidation of lipid molecules, which in turn can activate residential macrophages (Kupffer cells) in the liver and thereby increase the extent of inflammation.

2.2.3. Endoplasmic Reticulum Stress. With a high metabolic rate, hepatocytes have a high capacity for protein synthesis and are enriched with ER for protein folding. Any pathological factors that perturb ER folding capacity (e.g., ER stress) can trigger an unfolding protein response, during which both the expression of chaperones and the ER-associated protein degradation are enhanced, with the amount of protein molecules entering the ER being reduced [39]. Recently, it has been demonstrated that the induction of biochemical factors involving a protein unfolding response is present in patients with NASH, suggesting the possible association of an ER stress/unfolding protein response in the pathogenesis of NASH [40]. Consistent with this postulation, ER stress was found to be associated with the aforementioned risk factors involving the "multiple parallel hits" hypothesis in the development of NASH. As such, the increase of protein load in the ER would increase the generation of ROS, presumably due to the activation of ER oxidoreductases, the enhanced electron flow in mitochondrial ETC, and the induction of NADPH oxidase [41]. In addition, ER stress can activate JNK/NF-κB via the IRE1α-TRAF2 complex, leading to the release of proinflammatory cytokines [42]. The interaction of inflammation, oxidative stress, and ER stress in the pathogenesis of NASH remains to be elucidated.

2.2.4. Adipokine/Cytokine Release from Adipose Tissue. In the last decade, the adipose tissue has been considered to be an endocrine tissue by the virtue of its ability to secrete adipokines, which can induce autocrine, paracrine, and endocrine functions relating to energy metabolism. As such, patients with obesity or other metabolic disorders were found to exhibit an abnormality in the secretion profile of adipokines [43]. In addition, the immune cell-infiltrating adipose tissue (particularly as found in obese individuals) as well as endothelial cells of adipose tissue was shown to secrete classical proinflammatory cytokines (such as TNF-α and IL-6). Given the association between obesity/insulin resistance and NAFLD [8], the role of adipokines/cytokine in relation to the pathogenesis of NAFLD has been emphasized recently [43]. The effects of adipokines (such as leptin [44–50], adiponectin [51–54], resistin [55–61], and visfatin [56, 62–64]) as well as proinflammatory cytokines (such as TNF-α and IL-6 [65–70]) arising from adipocytes and/or immune cell-infiltrating adipose tissue on the pathogenesis of NAFLD are summarized in Table 1. While adiponectin can serve as a protective adipokine in the pathogenesis of NAFLD, the high levels of leptin, resistin, visfatin, TNF-α, and IL-6 seem to be involved in the pathogenesis of NAFLD (Table 1).

2.3. Hepatic Cirrhosis. Hepatic steatosis and NASH are associated with the pathogenesis of hepatic fibrosis/cirrhosis. The development of hepatic fibrosis, during which hepatic parenchymal cells undergo regeneration for the replacement of necrotic/apoptotic cells, is a natural wound-healing process in response to liver injury [71]. This wound-healing process is accompanied by an inflammatory response as well as accumulation of newly synthesized extracellular matrix (ECM). Under conditions of repeated and persistent hepatic

Table 1: Physiological/pathological effects of adipokines/cytokines in nonalcoholic fatty liver disease (NAFLD).

Adipokines/cytokines	Site of secretion	Normal physiological functions	Pathological effects in NAFLD	References
Leptin	White adipose tissue	Preserves the insulin sensitivity in the liver [48] (via the cross talk with the insulin signaling by activating the suppressor of cytokine signaling 3 [49])	Proinflammatory and fibrogenic [50] (via the activation of HSCs)	[48–50]
Adiponectin	Adipose tissue Injured hepatocytes	Improves fatty acid metabolism by the induction of free fatty acid oxidation as well as inhibition of gluconeogenesis, free fatty acid uptake, and *de novo* lipogenesis in cultured hepatocytes [53] Suppresses the release of proinflammatory cytokines (TNF-α and IL-6) and induces the release of the anti-inflammatory cytokine (IL-10) in Kupffer cells [54] Elicits an antifibrotic response by the inhibition of the release of TGF-β [54]	The level of adiponectin is reduced in NAFLD [52]	[52–54]
Resistin	Macrophage-infiltrating adipose tissue [56]		Induces glucose intolerance and insulin resistance [55] Proinflammatory (the induction of release of TNF-α, IL-1β, IL-6, and IL-2 in resistin-incubated macrophages) [58–60] Induces hepatic fibrosis (via the activation of HSCs and Kupffer cells [61] which release TGF-β and form collagen type 1)	[55–61]
Visfatin	Macrophage-infiltrating adipose tissue [56]		Possesses nicotinamide phosphoribosyl-transferase activity, which is critical for the glucose-induced release of insulin in pancreatic beta cells *in vitro* and *in vivo* [63] Proinflammatory (the release of TNF-α, IL-6, and IL-1β from macrophages [64])	[56, 63, 64]
TNF-α and IL-6	Adipocytes in individuals with insulin resistance or obesity [65]		Associated with the extent of obesity/adiposity in patients [66, 67] Distally influence the metabolic functions of liver via the JNK1-mediated release of IL-6 [68] Facilitate the development of NASH and HCC (via the induction of oncogenic factor STAT3 and release of TNF-α and IL-6) [70]	[65–70]

HSCs: hepatic satellite cells; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; IL-10: interleukin-10; TGF-β: transforming growth factor-β; IL-2: interleukin-2; IL-1β: interleukin-1β; JNK1: c-Jun N-terminal kinase-1; NASH: nonalcoholic steatohepatitis; HCC: hepatocellular carcinoma; STAT3: signal transducer and activator of transcription-3.

damage, Kupffer cells are activated and release platelet-derived growth factor (PDGF) that stimulates the proliferation of hepatic stellate cells (HSC), which are the major ECM producing cells in the liver [72]. The activated HSC may differentiate into myofibroblast-like cells that are proinflammatory and fibrogenic. The release of proinflammatory cytokines from HSC potentiates the inflammatory response, leading to a vicious cycle of tissue injury [72]. Hepatic cirrhosis is a pathological condition in which metabolic functions of the liver are suppressed by the excessive disposition of ECM arising from activated HSC. Despite the fact that the precise mechanism underlying NAFLD-mediated hepatic cirrhosis has yet to be elucidated, redox-regulated hepatic fibrosis is likely involved in its pathogenesis [73]. ROS, presumably arising during the development of NASH, are hypothesized to induce the release of TGF-β, which is one of the principal cytokines in the pathogenesis of human hepatic fibrosis. In this regard, TGF-β can stimulate the differentiation of HSC into myofibroblast-like cells as well as the associated production of ECM and the inhibition of ECM degradation [72]. Angiotensin II, a vasoactive cytokine, was found to promote fibrogenesis in activated HSC [74].

2.4. Hepatocellular Carcinoma (HCC). Recently, a number of meta-analyses regarding the prevalence of HCC have indicated that the cumulative mortality from HCC is 0–3% in patients who are not in the cirrhotic stage, which includes hepatic steatosis as well as NASH (for a study period of up to 20 years), whereas the cumulative incidence of mortality in patients with cirrhotic NASH ranges from approximately 2% over 7 years to approximately 13% over 3 years [83]. This observation suggests that the presence of cirrhosis may predispose to the development of HCC. Inflammation, diabetes, and obesity are hypothesized to be systemic risk factors for the pathogenesis of HCC. Epigenetic changes as well as mutations in genes that regulate hepatic growth and regeneration can induce "replicative immortality" (which refers to the ability of a cell population to proliferate continuously) in hepatocytes [5]. In addition to the altered receptor signaling arising from accumulated mutations in "immortal" hepatocytes, hyperinsulinemia as well as proinflammatory signaling can further amplify the signal for cell growth and proliferation, with a resultant increased risk of HCC [5].

3. Hepatoprotective Action of Sch B against NAFLD

The use of nutraceuticals, particularly with antioxidant, anti-ER stress, and anti-inflammatory activities, has been utilized as one of the approaches for the treatment of NAFLD [84]. Sch B is the most abundant dibenzocyclooctadiene lignan isolated from the FSC. In the realm of traditional Chinese medicine theory, the FSC is prescribed for the treatment of viral/chemical-induced hepatitis [85]. This suggests the possible therapeutic application of Sch B in NAFLD. In this regard, accumulating experimental evidence has revealed that Sch B, the principal active ingredient found in the FSC, possesses antihyperlipidemic, antioxidant, anti-inflammatory, and anticarcinogenic activities demonstrable in cultured hepatocytes *in vitro* and rodent livers *in vivo* [75, 86–88]. As mentioned earlier, the progression from hepatic steatosis to NASH involves "multiple parallel hits" in the pathological process. In addition, NASH has been recognized as a pathological condition that can favor the development of the end-stage liver disease [89]. In this section, the hepatoprotective action afforded by Sch B in the pathogenesis of NAFLD will be discussed.

3.1. The Differential Effect of Sch B on Lipid Content in Blood and Liver in Relation to the "Benign State" of NAFLD Pathogenesis. As already mentioned, the excessive accumulation of free fatty acids in the liver is the primary event in the pathogenesis of NAFLD (i.e., the "benign state" of NAFLD). In this regard, the effect of Sch B on hepatic/plasma lipid contents has been extensively investigated. A recent study has demonstrated that Sch B dose-dependently suppresses free fatty acid-induced steatosis in cultured L02 hepatocytes, in part *via* the inhibition of adipose differentiation-related protein (ADRP) and SREBP-1 [75]. Paralleling the results obtained in the cell-based study, Pan et al. have shown that the administration of Sch B in mice (50–200 mg/kg/d) with cholesterol/bile salts (2/0.5 g/kg/d) for a period of 4 to 6 days reduces hepatic total cholesterol and TG in hypercholesterolemic mice [76]. In a recent study by Pan et al., a single bolus dose of Sch B (0.2 to 1.6 g/kg, given 24 h prior to sacrifice) was unexpectedly found to increase hepatic cholesterol and TG levels in control mice [77]. Recently, Pan et al. have developed a novel mouse model of combined hyperlipidemia associated with steatosis and liver injury involving the oral administration of a Sch B and cholesterol/bile salt mixture given as a single dose [78]. In this experimental model, the combination of Sch B/cholesterol/bile salt (1/2/0.5 g/kg) was found to increase serum TG and total cholesterol (TC) levels, hepatic TG and TC levels, and serum alanine/aspartate aminotransferase activities (the latter used as indicators of hepatic damage). The results obtained from studies by Pan et al. suggest a complicated mechanism underlying the differential effect of Sch B on hepatic lipid content in control *versus* hypercholesterolemic mice. To reconcile the differential observations obtained from a number of cell-based and animal-based studies, a recent study by Kwan et al. may provide a possible explanation for these seemingly conflicting findings [79]. Kwan et al. have investigated the effect of a single bolus dose of Sch B (0.8 g/kg) in nonfasting and fasting mice as well as the effect of long-term low dose Sch B (50 mg/kg/d $\times$ 14 days) in control and high fat diet-(HFD-) fed mice. Firstly, the single bolus dose of Sch B was found to increase plasma TG and total cholesterol as well as serum free fatty acid levels in fasting but not in nonfasting mice. Secondly, the long-term low dose treatment with Sch B was shown to reduce hepatic TG levels, FAS activity, levels of SREBP1 and TNF-α, and the extent of hepatic fibrosis in HFD-fed mice. The beneficial effect of the long-term low dose treatment with Sch B was also associated with increases

in the levels of adipose triglyceride lipase and hormone-sensitive lipase in the adipose tissue of HFD-fed mice, all of which are indicative of an increase in lipolysis. In support of this, recent studies conducted by Pan et al. have also demonstrated that an aqueous extract of the pulp of FSC, an ethanol extract of the pulp of FSC, and the seed of FSC (all of which presumably contain lower concentrations of Sch B) were found to ameliorate serum/hepatic lipid profiles in control and hypercholesterolemic mice [90, 91].

In an effort to explore the feasibility of Sch B for use in patients with NAFLD, the human equivalent doses of Sch B in the aforementioned studies were estimated, based on a dose conversion equation from animals to humans [92]. We further estimated the equivalent amount of Sch B as well as air-dried FSC for human consumption. While the optimal daily dose of Sch B in humans has yet to be determined, the suggested dose of air-dried FSC for the adult human is 0.5–1.5 g twice daily [93], which is equivalent to 20–60 mg of Sch B per day [94]. With this notion in mind, the recommended high dose of air-dried FSC (equivalent of 60 mg of Sch B per day in humans) is much lower than the low dose that induces hyperlipidemia in mice (which is equivalent to 973 mg of Sch B per day in the human). This suggests that the administration of Sch B-containing FSC at the recommended dose is likely to be safe. Until now, the safety and therapeutic action of Sch B in patients with NAFLD have not been investigated. *Schisandra Plus* (or Wei-Kang-Su (WKS) in Chinese) is a commercially available health product comprised of Ginseng Radix, Ophiopogonis Radix, and Schisandrae Fructus. A recent study has demonstrated that the recommended dose of WKS in humans can induce hepatoprotective effects in CCl_4- and ethanol-intoxicated rats [95, 96]. Taken together, the experimental results suggest that Sch B (given at low dose, long-term) has a great potential to be a therapeutic agent for use in patients with NAFLD. Since the estimation of the equivalent dose in the human is based on equations from an animal-human translation study [92], the optimal dose and duration of Sch B treatment need to be investigated in clinical studies in order to maximize efficacy and ensure safety.

3.2. Hepatoprotective Action of Sch B in Relation to the "Multiple Parallel Hits" Hypothesis of NAFLD. With regard to the attenuation of the "multiple parallel hits" hypothesis of NAFLD, Sch B possesses antioxidant, anti-inflammatory, anti-ER stress, and anticarcinogenic activity. Sch B induces a cellular/hepatic glutathione antioxidant response and protects against menadione-induced cytotoxicity in cultured AML12 hepatocytes [86], as well as hepatotoxicity in CCl_4-treated mice [97]. CCl_4 hepatotoxicity involves the release of TNF-α, nitric oxide, and TGF-α/β from Kupffer cells in the liver, resulting in the production of hepatic fibrosis [98]. The hepatoprotection against CCl_4 toxicity afforded by Sch B in rats is associated with enhancement of glutathione regeneration capacity [99]. The glutathione antioxidant system is regulated by nuclear factor erythroid 2-related factor-2 (Nrf2), the principal transcriptional regulator of cellular antioxidant genes, which in turn binds to its corresponding element, namely, the antioxidant response element (ARE), on DNA [100]. A recent study has elucidated the cytoprotective mechanism underlying the Sch-B-induced glutathione antioxidant response in cultured hepatocytes [101]. Sch B is metabolized by cytochrome P_{450} with concomitant production of ROS [101]. The ROS then activate the redox-sensitive ERK/Nrf2/ARE signaling cascade, with a resultant expression of an array of glutathione-related enzymes. Reduced glutathione (GSH) and glutathione-related antioxidant enzymes can operate in concert to ameliorate oxidative stress [101]. Given that oxidative stress is one of the risk factors for the development of NAFLD, the antioxidant properties of Sch B may be involved in its salutary effect in retarding the progression of NAFLD (particularly in the development of NASH and hepatic fibrosis). With the cross talk between the Nrf2-mediated antioxidant signaling pathway and the NF-κB-mediated proinflammatory signaling cascade [102], the induction of an antioxidant response may be protective by inhibiting proinflammatory factors. This postulate is supported by the observation that Sch B reduces the extent of inflammation in lipopolysaccharide-activated (LPS-activated) RAW264.7 macrophages [81], LPS/ATP-activated isolated peritoneal macrophages [87], and concanavalin A-activated isolated splenocytes [103], presumably *via* the induction of an Nrf2-mediated antioxidant response. With regard to the endotoxemia arising from the increased permeability of the intestine to gut microbiota in dysbiosis, the ability of Sch B to induce an anti-inflammatory response against LPS activation (LPS being a classical bacterial endotoxin) further strengthens its beneficial effect in NAFLD. In support of this, Song et al. have demonstrated that the daily intake of an aqueous extract of FSC (equivalent to 6.7 g dried FSC per day) for 12 weeks can modulate the composition of gut microbiota, which correlates well with some changes in various metabolic parameters (such as fat mass, ALT, AST, HDL, and fasting blood glucose) in obese women [104]. However, a tendency towards improvement in obesity-related parameters (such as waist circumference, body weight, body mass index, and fat mass) was observed in FSC-treated subjects, when compared with a placebo group [104]. A further clinical study is worthwhile to conduct in an effort to confirm the beneficial effect of aqueous extracts of FSC (as well as Sch B) in obese individuals. The ability of Sch B to ameliorate hepatic steatosis in cultured HepG2 hepatocytes and in C57BL/6 mouse liver in relation to the protection against ER stress has recently been reported [82]. Gomisin N (a stereoisomer of Sch B) was found to reduce the expression of ER stress markers (such as glucose-regulated protein-78, CCAAT/enhancer binding protein homolog protein, and X-box-binding protein-1), proinflammatory cytokines, and lipogenic enzymes and the level of TG in palmitate-challenged HepG2 cells [82]. Results from the cell-based study were further confirmed by an animal study, in which gomisin N was found to suppress the expression of ER stress markers and the levels of hepatic TG and TC in tunicamycin (an ER stress inducer)-injected mice [82]. In addition, Sch B was found to inhibit cell proliferation and induce apoptosis in cultured human hepatoma SMMC-7721 cells via a caspase-3-dependent pathway [88]. This finding

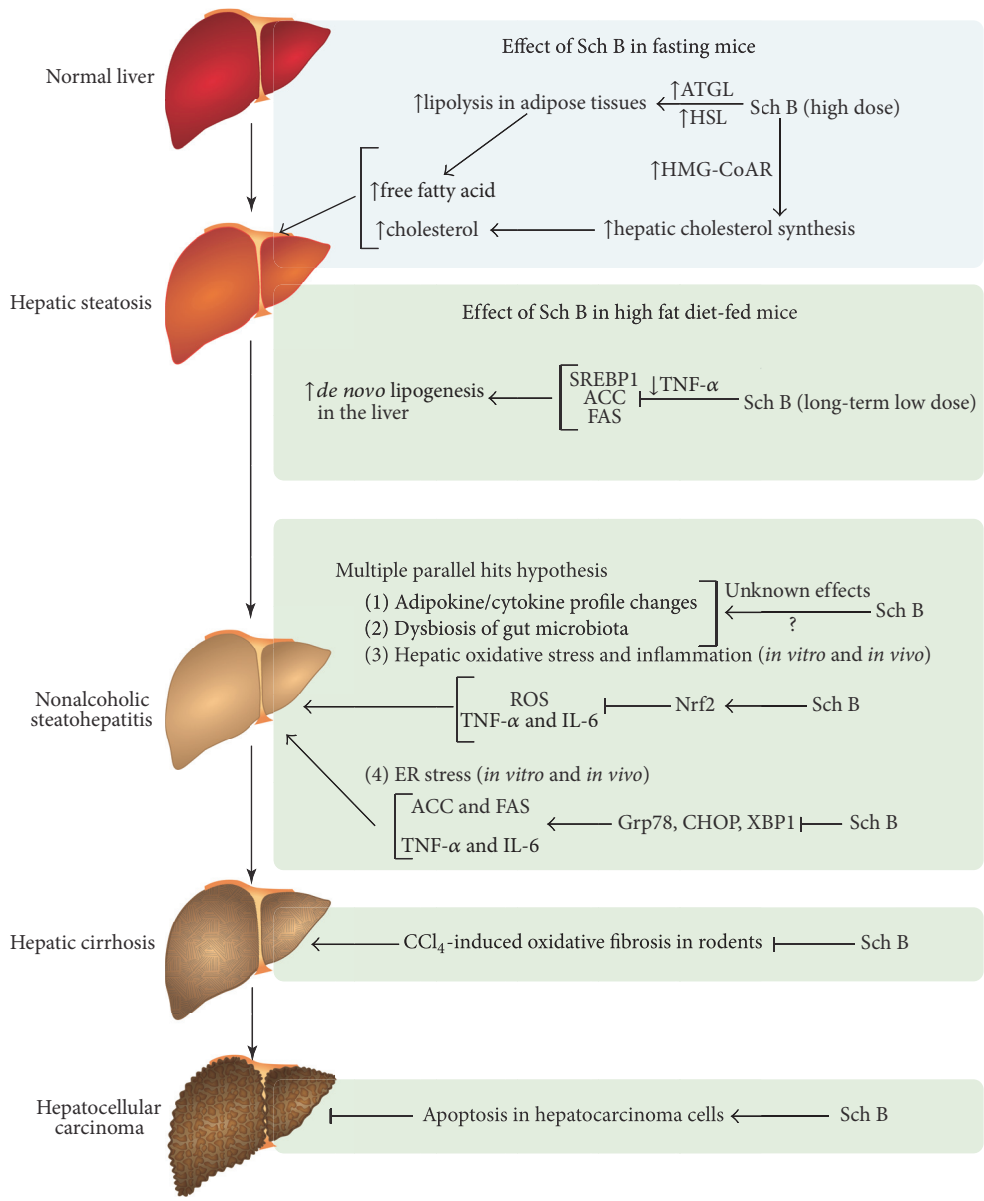

Figure 2: The hepatoprotection afforded by schisandrin B (Sch B) in relation to the pathogenesis of nonalcoholic fatty liver disease (NAFLD). The possible undesirable effect of Sch B is indicated in the blue box while the potential beneficial effects of Sch B are indicated in the green box. ATGL: adipose triglyceride lipase; HSL: hormone-sensitive lipase; HMG-CoAR: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; TNF-α: tumor necrosis factor-α; SREBP1: sterol regulatory element-binding protein-1; ACC: acetyl-CoA carboxylase; FAS: fatty acid synthase; Nrf2: nuclear factor (erythroid-derived 2)-like 2; ROS: reactive oxygen species; IL-6: interleukin-6; Grp78: glucose-regulated protein-78; CHOP: CCAAT/enhancer binding protein homologous protein; XBP1: X-box-binding protein-1; CCl_4: carbon tetrachloride.

suggests the possibility that Sch B may be effective in the treatment of hepatocarcinoma. However, the effect of Sch B (or a FSC extract) on the levels of adipokines in cultured adipocytes/obese animals has yet to be investigated.

4. Conclusion

The "multiple parallel hits" hypothesis for the pathogenesis of NAFLD suggests that obesity-associated hepatic steatosis is a "benign state," which is followed by "multiple parallel hits" including dysbiosis of gut microbiota, release of adipokines/cytokines from adipose tissue, and hepatic oxidative stress and/or inflammation. The differential action of Sch B on lipid metabolism in mice is likely related to a dose/time-dependent biphasic response, with a long-term low dose of Sch B being beneficial in HFD-fed obese mice (Table 2). The hepatoprotection afforded by Sch B against oxidative stress, inflammation, and ER stress has also been widely reported (Table 2). The possible effect of Sch B on the modulation of gut microbiota in relation to the pathological factors of NAFLD is deserving of further investigation. Taken together, currently available experimental evidence strongly suggests

Table 2: Pharmacological effects of schisandrin B on nonalcoholic fatty liver disease (NAFLD).

Effects on NAFLD	Experimental models	Concentration/dose	Pharmacological actions	References
Modulatory effects on lipid contents	Free fatty acid-induced steatotic L02 hepatocytes	1–100 μM	↓TG, ADRP, and SREBP-1	Chu et al. (2011) [75]
	High fat/cholesterol/bile salt-fed male ICR mice (for 7 days)	50–200 mg/kg/d × 6 doses p.o.	↓hepatic TC and TG	Pan et al. (2008) [76]
	Male ICR mice	0.2–1.6 g/kg × 1 dose p.o.	↑serum/hepatic TG, hepatic index; ↓hepatic TC and no changes in ALT & AST	Pan et al. (2011) [77]
	Male ICR mice (Sch B in combination with cholesterol/bile salt (2/0.5 g/kg))	1 g/kg × 1 dose	↑serum/hepatic TG, serum TC, and serum ALT and AST	Pan et al. (2013) [78]
	High fat diet-fed male C57BL/6 mice (for 20 days)	50 mg/kg/d × 14 doses	↓hepatic TG and palmitic acid; no changes in plasma TC	Kwan et al. (2015) [79]
	24 h fasting male C57BL/6 mice	0.8 g/kg × 1 dose	↑plasma TG and TC	Kwan et al. (2015) [79]
Antioxidant effects against fibrosis	CCl_4-induced hepatotoxicity in Balb/c mice	20 mg/kg × 15 doses p.o.	↓plasma SDH, hepatic mitochondrial MDA; ↑hepatic mitochondrial GSH	Leong et al. (2012) [80]
Anti-inflammatory activity	LPS-activated RAW264.7 macrophages	25–50 μM	↓TNF-α, IL-6, IL-1β, and NO	Leong et al. (2016) [81]
Anti-ER stress actions	Palmitic acid-induced steatotic HepG2 hepatocytes	10–100 μM	↓GRP78, CHOP, XBP1, and TG	Jang et al. (2016) [82]
	Tunicamycin-challenged C57BL/6 mice	1 and 30 mg/kg × 4 doses	↓GRP78, CHOP, XBP1, TC, and TG	Jang et al. (2016) [82]

TG: total triglyceride; ADRP: adipose differentiation-related protein; SREBP1: sterol regulatory element-binding protein-1; GSH: reduced glutathione; TC: total cholesterol; ALT: alanine transaminase; AST: aspartate aminotransferase; SDH: sorbitol dehydrogenase; MDA: malondialdehyde; Grp78: glucose-regulated protein-78; CHOP: CCAAT/enhancer binding protein homologous protein; XBP1: X-box-binding protein-1; CCl_4: carbon tetrachloride.

that Sch B may provide a potentially effective intervention for the prevention and/or treatment of NAFLD (Figure 2).

Competing Interests

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

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Research Article

Subnormothermic Perfusion in the Isolated Rat Liver Preserves the Antioxidant Glutathione and Enhances the Function of the Ubiquitin Proteasome System

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The reduction of oxidative stress is suggested to be one of the main mechanisms to explain the benefits of subnormothermic perfusion against ischemic liver damage. In this study we investigated the early cellular mechanisms induced in isolated rat livers after 15 min perfusion at temperatures ranging from normothermia (37°C) to subnormothermia (26°C and 22°C). Subnormothermic perfusion was found to maintain hepatic viability. Perfusion at 22°C raised reduced glutathione levels and the activity of glutathione reductase; however, lipid and protein oxidation still occurred as determined by malondialdehyde, 4-hydroxynonenal-protein adducts, and advanced oxidation protein products. In livers perfused at 22°C the lysosomal and ubiquitin proteasome system (UPS) were both activated. The 26S chymotrypsin-like (β5) proteasome activity was significantly increased in the 26°C (46%) and 22°C (42%) groups. The increased proteasome activity may be due to increased Rpt6 Ser120 phosphorylation, which is known to enhance 26S proteasome activity. Together, our results indicate that the early events produced by subnormothermic perfusion in the liver can induce oxidative stress concomitantly with antioxidant glutathione preservation and enhanced function of the lysosomal and UPS systems. Thus, a brief hypothermia could trigger antioxidant mechanisms and may be functioning as a preconditioning stimulus.

1. Introduction

A major clinical problem in liver surgery and transplantation is damage due to transient ischemia and reperfusion [1–3]. The pathological process involves a variety of cellular dysfunctions caused by increased production of reactive oxygen species and inflammatory responses [4]. Several studies suggest that the induction of mild (32–35°C) to moderate (28–32°C) hypothermia may attenuate the progression of liver damage against ischemia [1, 5, 6]. Moreover, perfusion in *ex vivo* machines resulted in improved viability at subnormothermic temperatures (20-21°C), in both livers from experimental animals [7–11] and from humans [12, 13]. These authors also found that many livers which would be discarded due to their low quality could be rescued for transplantation thanks to the restorative effects of subnormothermic perfusion.

There is growing interest in the use of hypothermia to prevent ischemic damage in clinical and experimental trials [14]. The protective effects of hypothermia are suggested to primarily be a consequence of decreased cellular metabolism, thus conserving ATP levels [15]. This proposal implies a passive method for hypothermia-induced protection and does not explain the wide role of therapeutic hypothermia against many injuries and in different tissues. Reduction of oxidative stress, as observed in ischemic cardiomyocytes

[16], and attenuation in the consumption of endogenous antioxidants, as seen in hypoxic brains [17, 18], could be two additional mechanisms.

In the liver, the observed hepatoprotective effects of hypothermia against ischemia include prolonging survival, attenuation of liver damage [1, 5], suppressed reactive oxygen species, and improved sinusoidal perfusion [19, 20]. Mild hypothermia also attenuated the progression of liver injury induced by other agents, such as acetaminophen in mice [21] and hepatocarcinogenesis in rats [22].

Cellular pathways affected by hypothermia have been reviewed by our group and others [23–25]. Most of the studies cited in these reviews focused on mechanisms related to inflammation, free radicals, or apoptosis, and consistently demonstrated that when hypothermia is induced before ischemic, hypoxic, or toxic episodes, it is able to reduce deleterious pathways while enhancing protective events.

These effects of hypothermia lead us to consider whether hypothermia could have a more active role in preventing damage through triggering antioxidative mechanisms of cell protection. Given the recent contributions that perfusion of isolated livers at subnormothermic temperature protects cellular integrity [9, 12], we investigated isolated perfused rat livers (IPRL) at a range of temperatures from normothermia (37°C) to subnormothermia (26°C and 22°C) for a brief period of time (15 minutes) and then investigated oxidative damage parameters and antioxidant defenses. Jung et al. [26] identified three lines of defense against oxidative stress: the first includes antioxidant molecules (such as glutathione), the second includes enzymatic antioxidants, and the third involves repair system proteins, including the proteolytic pathways. Of the two systems of intracellular proteolysis, the lysosomal system plays an important role in the degradation of membrane-bound proteins while the ubiquitin proteasome system (UPS) is widely recognized as the main system for degradation of cytosolic proteins [27, 28]. Our results suggest hypothermia limits ischemic damage by activating protective mechanisms in two of the oxidative stress defensive categories: antioxidant molecules and repair system proteins. We detected increased glutathione levels and proteolytic activity which may limit ischemia induced oxidative damage.

2. Materials and Methods

2.1. Animals and Liver Isolation and Perfusion. Adult male Sprague-Dawley rats (225–250 g body weight) were used in this study. Rats were fasted overnight and had free access to water. Rats were anesthetized with i.p. sodium pentobarbital (65 mg/Kg). Heart failure was induced by incision in the diaphragm and the liver was isolated and connected for perfusion in a nonrecirculating IPRL system at a flow rate of 3 mL/min/g liver with Krebs-Henseleit buffer (KHB) (mM): 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$, 20 Hepes (pH 7.4), aerated with 95% O_2, and 5% CO_2 [29]. The procedure was approved by the University of Barcelona Institutional Committee of Animal Care and Research and followed European Community guidelines.

Livers were perfused at 37°C for 15 min to stabilize. Then they were randomly distributed in the three experimental groups that were perfused at 37°C, 26°C, or 22°C for 15 min (total time of experiment lasting 30 min). As an index of cellular injury, plasma alanine aminotransferase (ALT) was measured in the effluent using a commercial kit (BioSystems, Barcelona, Spain). After perfusion, livers were frozen in liquid nitrogen and stored at −80°C until analysis.

Nitric oxide (NO) regulates the hepatic microvascular perfusion through its vasodilatory effect and through its anti-inflammatory actions [30]. NO levels were measured as nitrate plus nitrite in liver homogenates in 10% (w/v) PBS, centrifuged at 2000 ×g for 5 min, and ultrafiltered by means of a 30 kDa molecular weight cut-off filter. In the assay, nitrate was converted to nitrite using nitrate reductase and total nitrite was measured using the Griess reaction and a colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) and expressed as nmol/mg protein.

All other chemicals were purchased from Sigma-Aldrich Chemical.

2.2. Oxidant Assays. Lipid peroxidation in the liver was determined as the end product malondialdehyde (MDA) by thiobarbituric reactive substances (TBARS) assay [31]. Liver was homogenized with a teflon bar in 10% (w/v) RIPA solution, (Tris 50 mM pH 7.4, 1% Triton X-100, NaCl 150 mM, NaF 5 mM, 0.1% sodium dodecyl sulphate, and 1% sodium deoxycholate) with antiprotease solution (aprotinin at 1.7 mg/mL, 2 μg/mL pepstatin, 2 μg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride, and sodium orthovanadate at 1 mM). The suspension was centrifuged at 2000 ×g for 5 min and the pellet discarded. The formation of MDA-TBA adduct was fluorometrically measured at an excitation wavelength of 515 nm and an emission wavelength of 550 nm. The calibration curve was determined using tetraethoxypropane. Values are expressed as TBARS in nmol/mg protein.

Advanced oxidation protein products (AOPP) have been identified as a biomarker of oxidative damage to proteins, detecting dityrosine-containing and cross-linking protein products, but also a novel mediator of inflammation [32]. AOPP in liver homogenates were assayed by a modification of Witko–Sarsat's method [32, 33]. The formation of AOPP was spectrophotometrically measured at 340 nm and results were obtained through a standard calibration curve using 100 μL of chloramine-T solution (0–100 μmol/L). AOPP concentration was expressed as μmol/L of chloramine-T equivalents.

2.3. Total Protein Determination. The total liver proteins were determined using the Bradford protein assay [34].

2.4. Antioxidant Assays. Reduced (GSH) and oxidized glutathione (GSSG) were measured in the liver extracts using the procedure described by Hissin and Hilf [35] and modified by Alva et al. [36]. Tissue was homogenized in cold buffer containing 5 mm phosphate-EDTA buffer (pH 8.0) and 25% HPO_3. The homogenates were ultracentrifuged at 100,000 ×g and 4°C for 30 min, and the resulting supernatant was used to determine GSH and GSSG concentrations, using the fluorescent probe *o*-phthalaldehyde. Fluorescence was determined at a wavelength emission of 420 nm and excitation at 350 nm.

Glutathione reductase (GSH-R, EC 1.6.4.2) is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). For GSH-R determination, tissue was homogenized in cold buffer (50 mM potassium phosphate, pH 7.5, 1 mM EDTA) and centrifuged at 10,000 ×g for 15 min (4°C). The resulting supernatant was used to measure GSH-R activity with Cayman Chemical Glutathione Reductase Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA) by measuring the rate of NADPH oxidation. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. Results of GSH-R activity are expressed as mU/mg protein.

2.5. Proteolytic Activity Measurement of Cathepsin B and Cathepsin L and 26S Proteasome. Proteolytic activities were measured as previously described by our laboratory [37–39]. Cathepsin B and Cathepsin L assays were carried out in a total volume of 100 μL per well in black 96-well plates. For the cathepsin B activity, protein samples (25 μg) were incubated with 100 μM substrate Z-Arg-Arg-AMC (Biomol) in 44 mM potassium phosphate buffer, 6 mM sodium phosphate, 0.67 mM EDTA, and 1.35 mM cysteine (pH 6.0). For cathepsin L activity, protein samples (25 μg) were incubated with 100 μM substrate Z-Phe-Arg-AMC (Peptides International, Louisville, KY, USA) in 100 mM sodium acetate buffer, with 1 mM EDTA and 1 mM DTT (pH 5.5). The assay was conducted in the absence and presence of a specific inhibitor to determine specific activity: for cathepsin B, 10 μM CA-074 (Enzo, Life Sciences, Farmingdale, NY, USA) was used, and to inhibit cathepsin L, 10 μM Cathepsin L inhibitor I (Calbiochem, La Jolla, CA, USA) was used. Released AMC was measured using a Fluoroskan Ascent fluorometer (Thermo Electron) at 390 nm (excitation wavelength) and 460 nm (emission wavelength) for up to 120 min.

The ATP-dependent 26S proteasome activities were measured in the presence of 0.1 mM ATP [40]. Liver cell lysates were prepared by homogenization in 50 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, and 1 mM DTT, pH 7.5. The samples were then centrifuged at 12,000 ×g for 15 min (4°C) and the supernatants were collected. To analyze the 26S proteasome activity of liver homogenates (24 μg/sample), the fluorescent substrates Z-LLE-AMC 100 μM for β1, Boc-LSTR-AMC for β2, and Suc-LLVY-AMC 100 μM for β5 were used. Each assay was conducted in the absence and presence of a specific proteasome inhibitor Z-Pro-Nle-Asp-H (Enzo) 40 μM for β1, epoxomicin (Peptides International) 40 μM for β2, and epoxomicin 10 μM for β5 to determine proteasome-specific activity. All the assays were carried out in a total volume of 100 μL. Released AMC was measured using a Fluoroskan Ascent fluorometer (Thermo Electron) at an excitation wavelength of 390 nm and an emission wavelength of 460 nm.

2.6. Western Blot Analysis and Quantification. Whole liver cell lysates were prepared by homogenizing the livers in: 50 mM Tris (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, supplemented with DTT, and protease inhibitors (final concentrations were 10 μg/mL aprotinin, 2 μg/mL pepstatin, 2 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The samples were then centrifuged at 12,000 ×g for 10 min and the protein concentration measured in the supernatant. Supernatants were treated with Laemmli loading buffer and 50 μg of proteins resolved on SDS-polyacrylamide (10%) gels and transferred to nitrocellulose. Membranes were then blocked for 1 h with 3% nonfat dry milk (NFM) in Tris-buffered saline (TBS) (pH 7.4) containing 0.05% (w/v) Tween 20 (TTBS). The membranes were washed three times in TTBS and probed overnight with the following primary antibodies: anti-4-HNE conjugates (Novus Biologicals, Littleton, CO, USA), anti-ubiquitin (Sigma-Aldrich), anti-Rpt6, phospho S120 Rpt6 (affinity purified rabbit antibody commercially made by 21st Century Biochemicals), anti-Rpt1, and 20S core subunits (α5/α7, β1, β5, β5i, and β7) (Enzo life sciences). Detection was performed with anti-IgG-HRP (Santa Cruz Biotechnology, Inc., Heidelberg, Germany). The blots were visualized with enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, Rockford, IL, USA) and detected and scanned on Fujifilm LAS-3000 Imager (Fujifilm Corporation, Tokyo, Japan). Digital images were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA) and normalized to β-actin (Sigma-Aldrich, MO, USA). Ponceau S staining as a loading control was used for anti-Rpt6 and phospho S120 Rpt6 western blots due to high protein load (80 μg) [41].

2.7. Statistical Analysis. Results are expressed as means ± SEM of six animals. Data were processed using the statistical software GraphPad InStat (GraphPad Software, Inc., San Diego, CA, USA). The means of the experimental groups were analyzed by two-way ANOVA using the Student–Newman–Keuls test to identify significant differences (when $P < 0.05$) between the groups.

3. Results

3.1. Subnormothermic Perfusion Preserves Liver Function but Increases Oxidative Stress and Protein Oxidation. Our results showed that subnormothermic perfusion preserves liver integrity and function, as reflected by decreased ALT levels in the perfusate (Figure 1(a)), and increased NO in liver (Figure 1(b)). Because NO has an extremely short half-life, we measured it indirectly by quantifying the final products of its reaction, nitrates, and nitrites. These levels increased from 1.48 ± 0.14 at 37°C to 2.27 ± 0.17 at 26°C ($P < 0.01$) and 1.945 ± 0.14 at 22°C ($P < 0.05$). However subnormothermia resulted in higher levels of oxidative stress markers and protein oxidation. One of the effects of oxidative stress is lipid peroxidation which involves the interaction of free radicals with polyunsaturated fatty acids. The end products of lipid peroxidation are reactive aldehydes, such as malondialdehyde (MDA) measured as thiobarbituric reactive substances (TBARS) and 4-hydroxynonenal (HNE). Proteins are particularly susceptible to changes caused by HNE, and HNE-protein adducts formation plays a significant role in many cellular processes [42]. We have found increased levels of TBARS after subnormothermic perfusion at 26°C ($P < 0.05$) (Figure 2(a)) and increased HNE-protein adducts at both subnormothermic perfusion temperatures ($P < 0.05$)

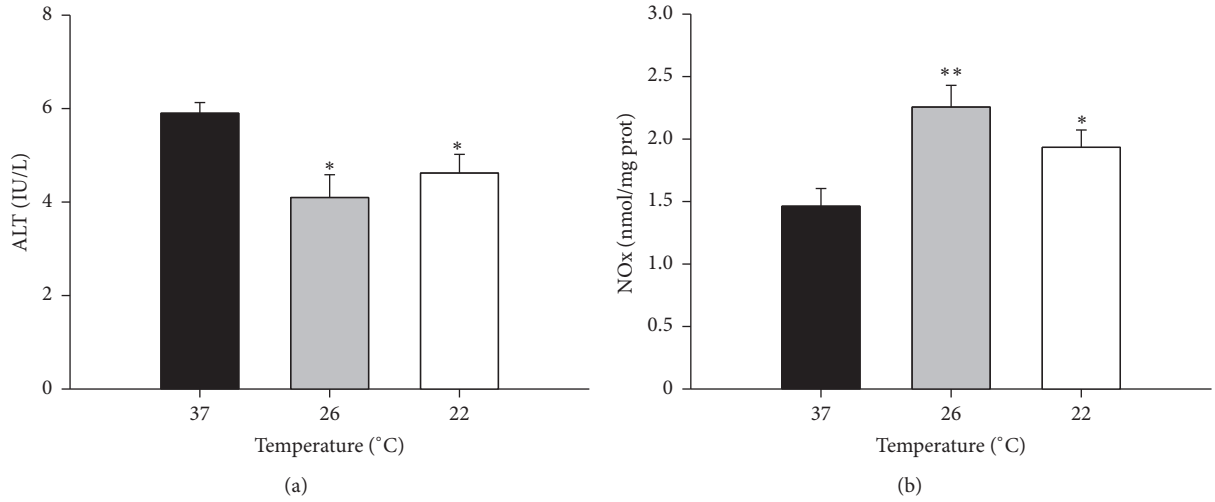

Figure 1: Temperature effects on the hepatic function of isolated perfused rat (IPR) livers. IPR livers were perfused in a nonrecirculating system with Krebs-Henseleit buffer at 37°C for 15 min, for stabilization, and then at the indicated temperatures for 15 min more. Results show cell viability by ALT assay in the perfusate (a) and NO production in liver homogenates (b). Values expressed as mean ± SEM of $n = 6$. Significant differences from livers perfused at 37°C: $^{*}P < 0.05$ and $^{**}P < 0.01$.

(Figure 2(b)). Oxidation induces several modifications in proteins that can lead to new compounds and modified structures. AOPP, which measures dityrosine-containing and cross-linking protein products, increased by 27% in livers from both subnormothermic perfusion ranges ($P < 0.05$) (Figure 2(c)).

3.2. Subnormothermic Perfusion and Antioxidant Status. Previous results show that the hepatic GSH [36] and the hepatic reduced/oxidized glutathione ratio (GSH/GSSG) increased in hypothermic rats [43] and in *ex vivo* subnormothermic rat liver [9]. Therefore, we measured the levels of oxidized and reduced glutathione and the activities of the enzyme responsible for the formation of reduced glutathione, glutathione reductase (GSH-R). No differences were observed in GSH levels, GSH/GSSG ratio, and GSH-R activity at 26°C (Figures 3(a), 3(b), and 3(c)). However, at 22°C GSH and the GSH/GSSG ratio rised, and the activity of GSH-R increased by 18%.

3.3. Subnormothermic Perfusion Increased the Activity of Cathepsin B and Cathepsin L. The largest group of hydrolases in lysosomal compartments are the cathepsin proteases. Investigation of two cathepsins, B and L, showed that the enzymatic activity of these proteases increased by 26-27% in subnormothermic livers perfused at 22°C ($P < 0.05$) (Figures 4(a) and 4(b)) compared to the normothermic group (37°C) suggesting that the lysosomal activity is increased.

3.4. Subnormothermic Perfusion Increased Ubiquitinated Proteins and the Activity of 26S Proteasomes. Investigation of the three proteolytic activities of the proteasome, the caspase-like β1, trypsin-like β2, and chymotrypsin-like β5 activities, indicated that proteasome activity was increased. While the 26S (ATP-dependent) caspase-like and trypsin-like activities were not significantly affected by the subnormothermic perfusion (Figures 5(a) and 5(b)), the chymotrypsin-like proteasome activity was significantly increased at 26°C (46%) and at 22°C (42%) ($P < 0.01$) (Figure 5(c)). This is especially interesting since the expression of several 20S core subunits (Figure 6(b)) and the expression of Rpt1 ATPase of the 19S proteasome subunit (Figure 6(a)) were not affected by subnormothermic perfusion. These results suggest that the increased proteasome activity may be due to posttranslational modifications or changes in the levels of associating partners on the proteasomes rather than its expression. We have previously shown that proteasomes from different tissues have different levels of associating partners which can affect proteasome activity [39].

To determine if the levels of polyubiquitinated proteins are affected by subnormothermic perfusion we utilized western blotting and observed that subnormothermic perfusion resulted in increased levels of polyubiquitinated proteins (Figure 6(c), $P < 0.001$). Since ubiquitinated proteins are selectively degraded by the 26S proteasome and Rpt6 S120 phosphorylation has been shown to increase 26S proteasome activity [44], the levels of Rpt6 S120 phosphorylation were investigated. A significant increase in the ratio of phospho-Rpt6/Rpt6-ATPase of the 19S proteasome subunit, from 0.59± 0.06 at normothermia to 1.06 ± 0.09 ($P < 0.05$) at 26°C and 1.24±0.1 ($P < 0.01$) at 22°C (Figure 6(d)), was detected. These results suggest that the increased activity of the proteasome that occurs with subnormothermic perfusion may be due to increased phosphorylation of the serine 120 subunit of Rpt6.

4. Discussion

There is widespread consensus that treatment with hypothermia protects against various types of damage in many organs

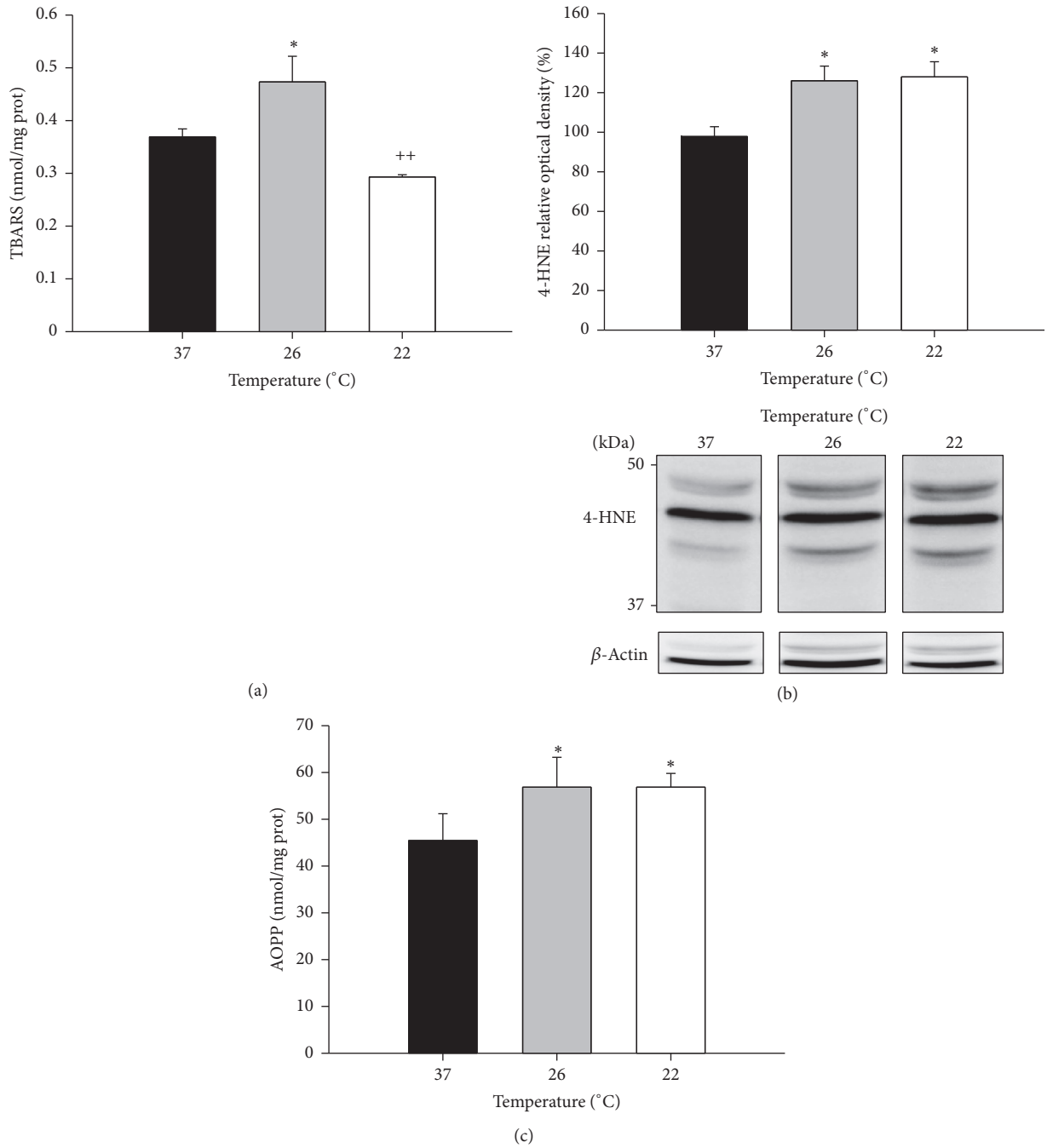

Figure 2: Lipid and protein oxidation in isolated perfused rat livers. IPR livers were perfused in a nonrecirculating system with Krebs-Henseleit buffer at 37°C for 15 min, for stabilisation, and then at the indicated temperatures for 15 min more. Lipid peroxidation was measured as TBARS (a) and by western blotting of HNE-protein adducts (b), and advanced oxidation protein products (dityrosine-containing and cross-linking protein products) measured spectrophotometrically at 340 nm (c). Values expressed as mean ± SEM of $n = 6$. Significant differences from livers perfused at 37°C: $^{*}P < 0.05$. Significant differences from livers perfused at 26°C: $^{++}P < 0.01$.

[25, 45–47]. Even so, the early events triggered by hypothermic treatment are not yet well defined. Potential mechanisms suggested for such protection include reduction of oxidative stress [16], increased endogenous antioxidants [17, 18], reduction of inflammatory mediators [48], and decreased apoptosis [49]. These effects of hypothermia, together with the improved viability seen in subnormothermic perfused livers [7, 9, 12], lead us to consider whether perfusion at subnormothermic temperatures for a limited period of time could trigger antioxidant mechanisms that confer cellular protection.

The present study demonstrated that liver integrity was retained at subnormothermic temperatures despite increased lipid and protein oxidation levels. Preserved cellular integrity

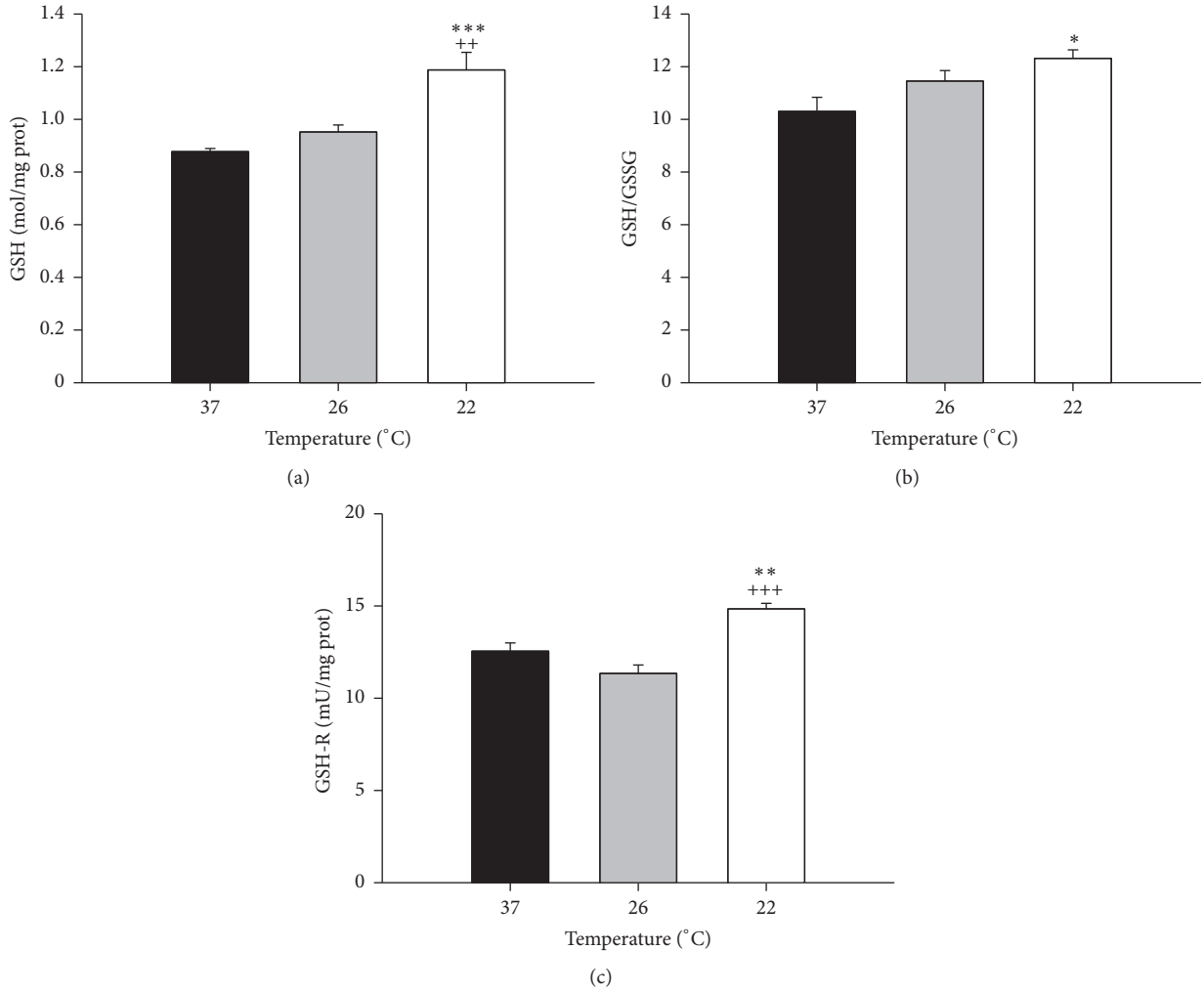

Figure 3: Temperature effects on antioxidant status in isolated perfused rat livers. IPR livers were perfused at 37°C for 15 min and then at the indicated temperatures for 15 min more. Bar graphs show hepatic GSH (a), GSH/GSSG ratio (b), and GSH-reductase activity (c). Values expressed as mean ± SEM of $n = 6$. Significant differences from livers perfused at 37°C: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Significant differences from livers perfused at 26°C: $^{++}P < 0.01$ and $^{+++}P < 0.001$.

was reflected by low ALT levels in the perfusate and increased NO in the liver. Increased synthesis of NO has been shown to be protective, as it reduces neutrophil adhesion and platelet aggregation while preventing microcirculatory disturbances in liver blood flow [50, 51]. NO has also been proposed to play a key role in both initiating and mediating ischemic preconditioning [30] and is involved in the preconditioning response for ischemia-reperfusion injury in fatty livers [50, 52]. Despite these protective effects, we also measured increased oxidation levels: lipid peroxidation, such as malondialdehyde MDA (measured as TBARS) and protein oxidation (measured by AOPP and HNE-protein adducts) were increased in subnormothermic perfused livers.

Although some results showed that in liver the protein expression of antioxidant enzymes and their activity were relatively unaffected by one hour of cold exposure in rats [53] and in hibernating animals [54], the GSH reductase activity was previously shown to significantly increase after cold exposure in rat livers [55]. Furthermore, the hepatic GSH [36] and reduced/oxidized glutathione ratio (GSH/GSSG) [9, 43] were shown to increase after one to six hours at subnormothermic temperature in rats. However, the early effects of brief exposure to subnormothermic temperature on antioxidant enzymes have not been previously reported. In the present work we found increased levels of GSH, the GSH/GSSG ratio, and the activity of GSH-R in livers perfused at 22°C during 15 min. Although increased levels of GSH and GSH-R activity suggest increased antioxidant capability of the liver, the increased levels of lipid and protein oxidation suggest that the higher antioxidant capacity is at least initially unable to completely prevent lipid peroxidation and the resulting protein damage.

The lipid peroxidation process is a chain reaction that produces multiple breakdown molecules, such as the aldehydes MDA and 4-HNE [56]. 4-HNE appears to be the most toxic product of lipid peroxidation [57]. 4-HNE can

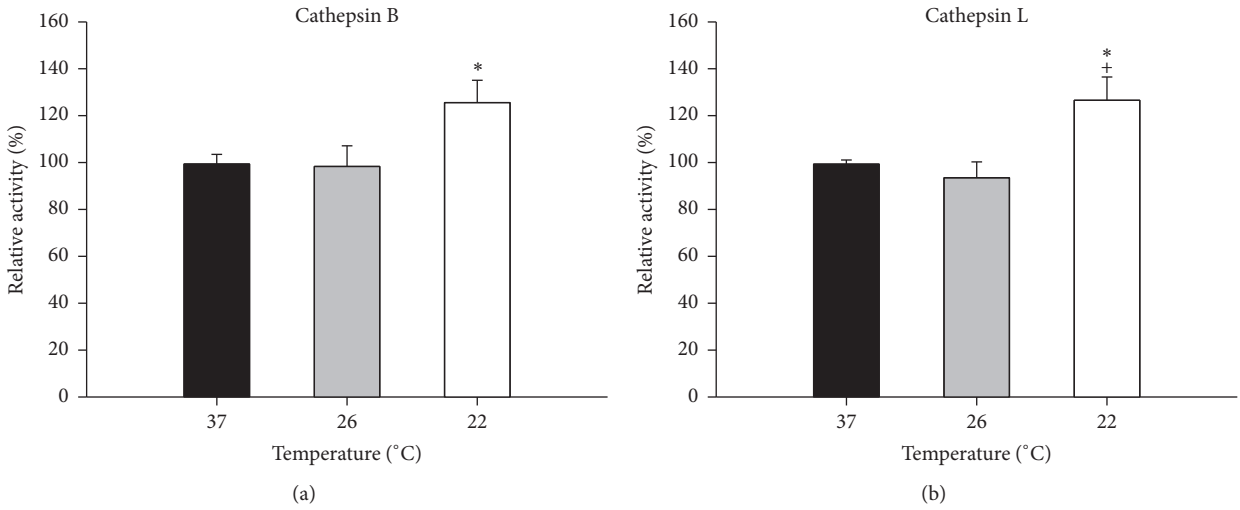

Figure 4: Lysosomal activity in isolated perfused rat livers. IPR livers were perfused at 37°C for 15 min and then at the indicated temperatures for 15 min more. To independent lysosomal enzymes, cathepsin B (a) and cathepsin L (b) were measured to determine if lysosomal activity was affected. Data is expressed as mean ± SEM of $n = 6$. Significant differences from livers perfused at 37°C: $^{*}P < 0.05$. Significant differences from livers perfused at 26°C: $^{+}P < 0.05$.

modify proteins by forming covalent adducts, accelerating protein aggregation [42]. Oxidative modifications of proteins can affect their physiological activity and typically increase their degradation rate. Several reports have highlighted that mild oxidation of proteins increases their susceptibility to proteasomal degradation [58, 59]. The excessive accumulation of such abnormal proteins can exacerbate the apoptotic or necrotic pathways in the cell [60]. As such, removing abnormal proteins may prevent oxidative stress induced cell death. This function, largely due to the proteasome, has been suggested to be part of the antioxidant defense [26].

The mechanisms by which lipid peroxidation modified proteins are removed from the cell have been studied in different models. 4-HNE exposure increases ubiquitination of adiponectin [61] and the UPS was shown to be the predominant proteolytic enzyme involved in the removal of 4-HNE adducts of alcohol dehydrogenase [62]. In kidney homogenates, HNE-modified proteins were shown to be specifically degraded by the UPS [63]. However, in lens epithelial cells, HNE-modified proteins are ubiquitinated but degraded by the ubiquitin-dependent lysosomal pathway rather than by the proteasome [64]. Moreover, electron micrographs of 4-HNE-treated cells showed extensive vacuolization and treatment with lysosomal inhibitors induced cell death. These findings suggest that lipid peroxidation-derived aldehydes also stimulate autophagy [65]. The present study implicates elevated activity in both the UPS and lysosomal pathways.

The lysosome system in the liver is involved in the degradation of membrane-bound or organelle-associated proteins and aggregates. The largest group of hydrolases in lysosomal compartments are the cathepsin proteases, essential for the proteolysis of protein substrates [66]. Cathepsins are responsible for intracellular degradation of Advanced Glycation End Products- (AGEs-) modified proteins [67] and following oral administration of AGEs, upregulation of cathepsins B and L activities was reported in *Drosophila melanogaster* [68]. In our model, isolated liver perfusion at 22°C (but not at 26°C) resulted in increased activity of the two lysosomal proteases investigated (cathepsins B and L) when compared to control temperatures (37°C). This increased lysosomal activity may be important in limiting lipid peroxidation damage and removing some of the oxidized proteins (HNE-protein adducts and AOPP). Increased lysosomal activity (lysosomal degradation of autophagosomes) has been shown to protect cardiac myocytes against ischemia/reperfusion injury [69]. Hence it is possible that the increased lysosomal activity at 22°C may be protective against ischemia/reperfusion injury.

Oxidized proteins can also be removed by the proteasome [37, 59] via ubiquitin independent degradation as well as ubiquitin tagging and subsequent targeting by the 26S proteasome [28]. Conversely, it has been suggested that an excess of HNE may directly form adducts on the three proteolytic subunits of the proteasome, thereby reducing its enzymatic activity and contributing to the accumulation of modified proteins [70]. Oxidative damage to various subunits of the 26S proteasome during ischemia and reperfusion has also been reported [60]. Seemingly in support of this data, we observed higher levels of polyubiquitinated proteins at 26°C and 22°C when compared to 37°C. Similarly, ubiquitin conjugated proteins increased 2-3-fold during torpor in the liver of hibernating squirrels [71].

The intact 26S proteasome is composed of one or two 19S regulatory particles at each end of a cylindrical 20S core particle. Each protein particle has a complex quaternary structure consisting of many (19–28) subunits. Degradation of protein substrates requires the 19S particle to recognize the ubiquitin-conjugated protein and regulate the entry of

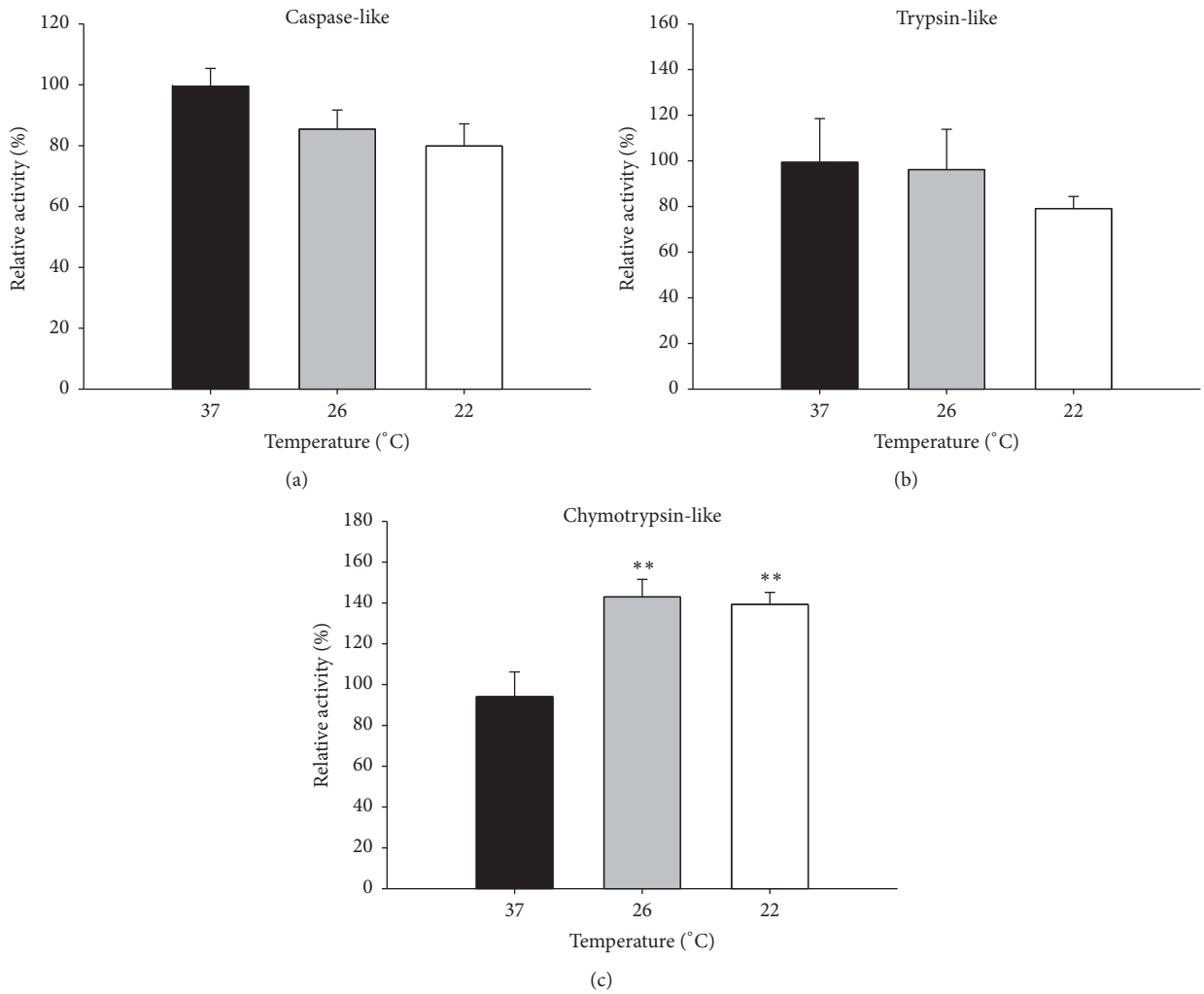

Figure 5: Proteasomal activity of 26S proteasome in isolated perfused rat livers. IPR livers were perfused at 37°C for 15 min and then at the indicated temperatures for 15 min more. The caspase-like β1 (a), trypsin-like β2 (b), and chymotrypsin-like β5 (c) activities of the proteasome were determined. Data is expressed as mean ± SEM of $n = 6$. Significant differences from livers perfused at 37°C: $^{**}P < 0.01$.

the substrate in the proteolytic cavity of the 20S core particle. The expression of several 20S and 19S subunits of the proteasome was not affected by subnormothermic temperature in our study, indicating no effect on the number of proteasomes assembled. However, we found a significant increase in the phosphorylation of the 19S subunit Rpt6 at serine 120. Previous studies have revealed that phosphorylation of proteasome subunit Rpt6 increases 26S proteasome activity and that Rpt6 phosphorylation may be an important regulatory mechanism for proteasome-dependent control [44, 72, 73]. Covalent regulation via phosphorylation allows for a quick increase in the activity of the UPS independent of increasing the overall number of proteasomes. Consistent with an increase in the levels of phosphorylated Rpt6, the 26S chymotrypsin-like activity of the proteasome was significantly increased in livers perfused at both 26°C and 22°C subnormothermic temperatures. Considering these results and the findings that ischemic preconditioning also enhances proteasome function [74], it seems probable that the increased proteasome activity is protective. Additionally, elevated proteasome activity has been shown to be associated with long-lived humans (centenarians) [75] and very long-lived animals such as the naked mole rat [76].

5. Conclusions

Overall, our results suggest that the early events initiated by hypothermia include the induction of oxidative stress and concurrent stimulation of several potential mechanisms of cell protection, including increased NO and GSH levels and the activation of lysosomal and proteasomal systems to repair oxidative damage. The increased proteasomal activity is likely due, in part, to phosphorylation of serine 120 on the proteasome Rpt6 subunit. Hypothermia may be acting as a preconditioning stimulus which could explain its protective role against ischemic, hypoxic, and toxic damage.

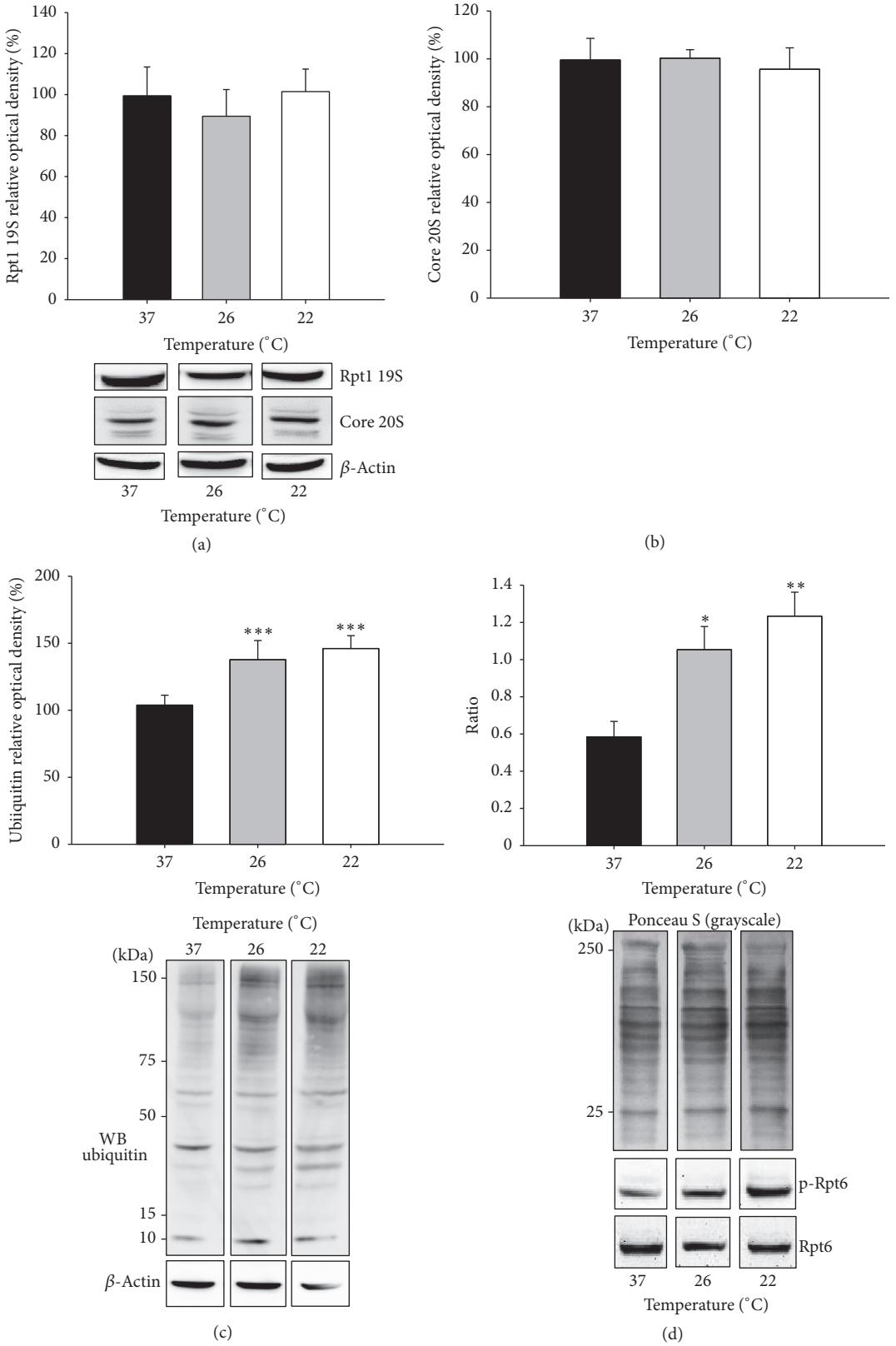

Figure 6: Expression of ubiquitin proteasome system in isolated perfused rat livers. IPR livers were perfused at 37°C for 15 min and then at the indicated temperatures for 15 min more. Results show western blots and densitometric analysis for Rpt1 19S (a) and 20S core subunit (b). (c) Levels of polyubiquitinated proteins; densitometry includes all polyubiquitinated bands detected. β-Actin was used as a normalization control for the western blots. (d) Ponceau S (total protein) staining was used as loading control for phospho S120 Rpt6 and Rpt6 densitometric analyses of western blots; values were then used to calculate p-Rpt6/Rpt6 ratios. Results are expressed as mean ± SEM of $n = 3$ to 6 independent samples per group. Significant differences from livers perfused at 37°C: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Abbreviations

ALT: Alanine aminotransferase
AOPP: Advanced oxidation protein products
GSH: Reduced glutathione
GSH-R: Glutathione reductase
GSSG: Oxidized glutathione
HNE: 4-Hydroxynonenal
IPRL: Isolated perfused rat livers
MDA: Malondialdehyde
NO: Nitric oxide
TBARS: Thiobarbituric reactive substances
UPS: Ubiquitin proteasome system.

Competing Interests

The authors declare no competing or financial interests.

Acknowledgments

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Research Article

Hyperglycemia Aggravates Hepatic Ischemia Reperfusion Injury by Inducing Chronic Oxidative Stress and Inflammation

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Aim. To investigate whether hyperglycemia will aggravate hepatic ischemia reperfusion injury (HIRI) and the underlying mechanisms. *Methods*. Control and streptozotocin-induced diabetic Sprague-Dawley rats were subjected to partial hepatic ischemia reperfusion. Liver histology, transferase, inflammatory cytokines, and oxidative stress were assessed accordingly. Similarly, BRL-3A hepatocytes were subjected to hypoxia/reoxygenation (H/R) after high (25 mM) or low (5.5 mM) glucose culture. Cell viability, reactive oxygen species (ROS), and activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) and nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) were determined. *Results*. Compared with control, diabetic rats presented more severe hepatic injury and increased hepatic inflammatory cytokines and oxidative stress. HIRI in diabetic rats could be ameliorated by pretreatment of N-acetyl-L-cysteine (NAC) or apocynin. Excessive ROS generation and consequent Nrf2 and NF-κB translocation were determined after high glucose exposure. NF-κB translocation and its downstream cytokines were further increased in high glucose cultured group after H/R. While proper regulation of Nrf2 to its downstream antioxidases was observed in low glucose cultured group, no further induction of Nrf2 pathway by H/R after high glucose culture was identified. *Conclusion*. Hyperglycemia aggravates HIRI, which might be attributed to chronic oxidative stress and inflammation and potential malfunction of antioxidative system.

1. Introduction

Hepatic ischemia reperfusion injury (HIRI) is a major cause for acute postoperative liver dysfunction and liver failure. It is common in major hepatic surgery including liver transplantation and partial hepatectomy. The mechanisms of HIRI consist of multiple complicated pathophysiological processes including mitochondrial energy exhaustion, excessive reactive oxygen species (ROS) production, calcium overload, leukocyte aggregation, cytokine release, and microcirculation dysfunction [1–5]. Of note, the outburst of oxidative and proinflammatory factors is crucial to initiating HIRI [6]. During reperfusion, enormous ROS are generated due to sudden restoration of oxygen and exert direct deleterious effects on cells through lipid peroxidation, protein degradation, and DNA damage [1].

Diabetes is a major risk factor for many surgical complications [7–9]. It is particularly associated with poor prognosis of ischemia reperfusion (IR) injury. Many have reported that IR injury of heart, kidney, and neuron tissues tends to be more severe in diabetic patients [10–12]. Hyperglycemia is a major manifestation of diabetes and it contributes to the progression of many diabetic complications [13].

Hyperglycemia could increase basal ROS levels in cardiomyocytes, renal mesenchymal cells, and endothelial cells, resulting in a state of chronic oxidative stress [14–16]. The increase in baseline ROS generation has been reported to be an important factor to aggravate cardiac IR injury [17]. Furthermore, chronic oxidative stress would diminish the protection of anesthetic preconditioning and ischemic preconditioning upon acute oxidative stress in IR [18, 19].

Chronic oxidative stress may promote the onset or progression of chronic liver diseases including nonalcoholic fatty liver disease (NAFLD), cirrhosis, hepatitis, and hepatic carcinoma, which if not treated properly are likely to advance to end-stage liver diseases requiring surgical intervention [20, 21]. However, little is known about the impact of chronic oxidative stress in diabetes on HIRI. The evidence of correlation between diabetes and negative outcome of HIRI is accumulating [22, 23]. For instance, the risk of liver graft failure is higher in diabetic patients compared with nondiabetic patients after liver transplant [24]. Consistently, diabetes is a risk factor for unexpected readmission in patients receiving hepatic surgery [25]. Since liver plays a central role in glucose metabolism, deterioration of liver function would in turn worsen diabetes [26]. The underlying mechanisms of diabetes and poor outcome of hepatic surgery remain unknown.

As the incidence of diabetes rises rapidly [27], there is a growing need to focus on HIRI in diabetic patients. By establishing in vivo HIRI model on streptozotocin- (STZ-) induced type 1 diabetic rats and in vitro hypoxia/reoxygenation (H/R) model on hepatocytes, we intend to explore the impact of hyperglycemia or high glucose condition upon HIRI or H/R injury and the possible mechanisms.

2. Methods

2.1. Animal Models of Diabetes. This study was approved by the Animal Ethical and Welfare Committee of Sun Yat-sen University (approval number IACUC-DB-16-0202, Guangzhou, China). Diabetes was induced by intraperitoneal administration of 50 mg/kg STZ (Sigma-Aldrich, St. Louis, MO) dissolved in citrate buffer solution (0.1 mM, pH 4.5) to male Sprague-Dawley (SD) rats weighing 220 g~280 g [28], which were fasted overnight. A week after STZ administration, rats were fasted for 6 hours and blood was obtained by cutting tail tips to measure the glycemic level with a blood glucose meter (Abott). Rats were considered diabetic when fasting blood glucose levels were >14 mmol/L. Diabetic rats were fed with sufficient normal diet and water ad libitum and raised for another 8 weeks before being subjected to surgery.

2.2. In Vivo Experiment Protocol. To illustrate HIRI under hyperglycemic settings, normal and diabetic rats of the same weight range (140–150 g) were randomly distributed into 4 groups ($n = 9$ per group): (1) normal sham group; (2) diabetic sham group; (3) normal HIRI group; (4) diabetic HIRI group.

To determine the role of ROS during HIRI in diabetic rats, two classic antioxidants, NAC (300 mg/kg) and NAPDH oxidase inhibitor apocynin (2.5 mg/kg), were dissolved in normal saline and were administered intraperitoneally 1 h before the surgery separately. Confirmed diabetic rats were randomly distributed into 4 groups ($n = 5$ per group): (1) diabetic sham group; (2) diabetic HIRI group; (3) diabetic NAC group; (4) diabetic apocynin group.

2.3. Partial Hepatic Ischemia Reperfusion. General anesthesia was induced in all the rats by continuous spontaneous inhalation of 2%~5% volatile anesthetic isoflurane via a mask. After laparotomy, all vessels (hepatic artery, portal vein, and bile duct) to the left and median liver lobes were clamped, according to a previously described method [29]. After 60 min of liver partial ischemia, these vessels were unclamped, and then the abdomen was closed and rats were revived to allow hepatic circulation to be restored for a reperfusion period of 6 hours. Rats were then anesthetized again before being sacrificed by withdrawal of blood from abdominal aorta. The blood collected was centrifuged at 3000 rpm for 10 minutes for serum. The left and median liver lobes were collected for histology and protein extraction described in the following paragraphs.

2.4. Histology. Liver paraffin-embedded sections stained were prepared as described previously [30]. Histological severity of HIRI was graded using Suzuki's criteria, in which sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration are grated from 0 to 4. The absence of necrosis, congestion, or centrilobular ballooning is given a score of 0, whereas severe congestion, ballooning degeneration, and >60% lobular necrosis are given a value of 4 [31].

2.5. Serum Transferase Activities. The serum alanine transferase (AST) and aspartate transferase (ALT) activities were measured by using the Infinity™ AST (GOT) Liquid Stable Reagent and Infinity ALT (GPT) Liquid Stable Reagent (Thermo Fisher Scientific Inc.), respectively, according to the manufacturer's instructions. Briefly, 30 μL of serum was mixed with 300 μL of reagent in a 96-well plate and the absorbance at 340 nm was measured with a microplate reader from 1 to 3 minutes. The AST/ALT activity was calculated with the following formula: the activity in U/L = (Δ Absorbance/min) $\times$ 1746.

2.6. Serum H_2O_2 Activities. Serum H_2O_2 activities were assessed using KeyGEN H_2O_2 Assay Reagent Kit (KGT018, KeyGEN, Nanjing, China) according to manufacturer's instruction. Briefly, 100 μL of sample, distilled water, or standard H_2O_2 solution was, respectively, mixed with the reagent provided in the kit in a required order and reacted for 1 minute. Absorbance at 405 nm was measured with a microplate reader and the H_2O_2 activity was calculated with the following formula: H_2O_2 (mmol/L) = [Abs (sample) − Abs (water)]/[Abs (standard) − Abs (water)] $\times$ 163.

2.7. Whole Cell Lysate (WCL). WCL of liver and hepatocytes were extracted using KeyGEN WCL Assay Kit according to the manufacturer's instruction (KGP2100, KeyGEN, Nanjing, China). Lysis was performed on ice for 15 min, and cell debris was removed by centrifugation at 13,000 $\times$g at 4°C for 10 min (5810R, Eppendorf AG). The supernatant was recovered as WCL and stored at −80°C.

2.8. Hepatic Malondialdehyde (MDA). Hepatic MDA was assessed with WCL of liver tissues following the instruction of the manufacturer of KeyGEN MDA Assay Reagent Kit (KGT004, KeyGEN, Nanjing, China). Briefly, 200 μL of homogenized liver samples was mixed with the reagent provided in the kit and was processed in boiled water and

then ice as stated in the instruction. The sample was then centrifuged and the supernatant was collected for measurement. Absorbance at 532 nm was obtained by a microplate reader.

2.9. Immunohistochemistry Staining. Liver paraffin-embedded sections were stained for 8-hydroxydeoxyguanosine (8-OHdG) to evaluate oxidative stress within hepatocytes. The samples were deparaffinized and incubated with monoclonal mouse 8-OHdG Ab (sc-66036, Santa Cruz Biotechnology, Inc.). The integrated optical intensity (IOD) of 8-OHdG (+) within the nuclei of hepatocytes was evaluated by Image-Pro Plus 6.0 (Media Cybernetics, Inc.).

2.10. Cell Culture. Normal rat hepatocytes BRL-3A were obtained from the Cell Bank of Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences (category number GNR10) and cultured in low glucose DMEM (Hyclone) containing 10% fetal bovine serum and supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere in 5% CO_2 at 37°C. Cells were plated and incubated overnight, before being used in experiment.

2.11. Hypoxia/Reoxygenation (H/R). Cells were seeded and incubated overnight. The medium was replaced by serum-free nonglucose DMEM (Gibco, Thermo Fisher Scientific Inc.). Cellular hypoxic conditions were created and maintained in an airtight incubator (Galaxy 48R, Eppendorf Company) by continuous gas flow with a 95% N_2 and 5% CO_2 gas mixture, enabling the percentage of O_2 to fall to 1% in 15 minutes. After 4 hours of hypoxia, 4 hours of reoxygenation was achieved by continuously flushing with a 95% air and 5% CO_2 gas mixture and the glucose in the medium was restored.

2.12. High Glucose Culture. 24 hours after cell plating, the culture media were replaced with a serum-free medium containing 5.5 mM or 25 mM D-glucose. The low glucose culture medium containing 5.5 mM D-glucose was supplemented with 19.5 mM mannitol (Amresco) to adjust the total osmotic pressure to 25 mM. For various purposes, the incubation continued for 6, 24, 48, 72, 96, 120, or 144 hours. As for H/R experiment, cells were incubated in serum-free mediums containing either 5.5 mM or 25 mM D-glucose (the osmotic pressure of which had been balanced as previously stated) for 6 hours before being subjected to hypoxia. At the end of treatment the cells or medium were harvested.

2.13. Nuclear Extract Preparation. Cells were washed twice with ice-cold PBS and scraped off from dishes with a rubber cell lifter. Nuclear extracts were prepared according to the instruction of NE-PER Nuclear & Cytoplasmic Extraction Reagent Kit (78833, Thermo Fisher Scientific Inc.). After being extracted, the nuclear and cytoplasmic protein was stored at −80°C.

2.14. Cell Viability. Cell viability was assessed by the CCK-8 test (KGA317, KeyGEN, Nanjing, China). Cells were seeded at 1×10^5 into 96-well-plates. After the indicated treatments, 10 μL CCK-8 solution at a 1/10 dilution was added to each well and then the plate was incubated for 2 hours in the incubator. Absorbance at 450 nm was measured with a microplate reader: cell viability (%) = (OD treatment group/OD control group) × 100%.

2.15. Cell Cytotoxicity. Cell cytotoxicity was assessed by determining released lactate dehydrogenase (LDH) into the medium by necrotic cells, using Pierce LDH Cytotoxicity Assay Kit (88953, Thermo Fisher Scientific Inc.) according to manufacturer's instruction. Briefly, cultured cells underwent previously stated glucose culture and H/R procedure and subsequently released LDH into the medium after H/R. The medium was transferred to a new plate and mixed with reaction mixture. After 30-minute room temperature incubation, reactions were stopped by adding Stop Solution. Absorbance at 490 nm and 680 nm was measured using a plate-reading spectrophotometer to determine LDH activity. To determine LDH activity, the 680 nm absorbance value (background signal from instrument) was subtracted from the 490 nm absorbance.

2.16. ROS. ROS levels were determined using 6-carboxy-2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (D6883, Sigma-Aldrich, St. Louis, MO). Cells were washed with ice-cold PBS and incubated with 10 μM DCFH-DA for 30 min. Then, the medium was discarded and cells were washed with ice-cold PBS in the dark, and ROS generation was evaluated by the fluorescence intensity measured by fluorescence spectrometry (SpectraMax M5, Molecular Devices, USA). The excitation wavelength was 504 nm and emission wavelength was 529 nm.

2.17. Enzyme-Linked Immunosorbent Assay (ELISA). Rat-specific ELISA kits were applied to determine hepatic and medium tumor necrosis factor α (TNF-α, SEA133Ra, USCN, Cloud-Clone Corp., Wuhan, China), monocyte chemokine protein-1 (MCP-1, SEA087Mi, USCN, Cloud-Clone Corp., Wuhan, China), and hepatic interleukin-6 (IL-6, SEA079Ra, USCN, Cloud-Clone Corp., Wuhan, China) and levels according to the manufacturer's instructions. Absorbance all measured at 450 nm. Hepatic 8-isoprostane level was determined using Cayman: 8-Isoprostane EIA Kit (number 516351, Cayman Chemical, USA) according to the manufacturer's instruction. The absorbance was measured at a wave length of 420 nm.

2.18. Western Blotting Assays. Western blotting assays were performed as described before [32]. Briefly, liver or hepatocyte WCL or nucleus extract were collected and the protein concentration was measured by Bradford assay. The samples were subjected to electrophoresis and transformation to polyvinylidene fluoride membranes. These membranes were blocked in 5% milk for 1 h, incubated with different primary antibodies overnight at 4°C, and then rinsed and incubated with secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. Antigen antibody complexes were then visualized using ECL kit (35050, Thermo Scientific Inc.).

The primary antibodies used here include those against nuclear factor-erythroid 2-related factor 2 (Nrf2, 1 : 1000,

Table 1: Fasting, random, and preoperative glycemic level of normal and diabetic rats. $^{\triangle\triangle\triangle}P < 0.001$ versus normal sham group; $^{\#\#\#}P < 0.001$ versus normal HIRI group. All glycemic measurement was obtained from blood dripped from cut tail tip and determined by a blood glucose meter (Abott). Fasting glucose was measured when rats were fasted for 6 hours. Random glucose was measured without deliberate interruption of food and water supply. Preoperative glucose was measured after the rats were anesthetized and before laparotomy.

Glycemia (mmol/L)	Normal		Diabetic	
	Sham ($n = 9$)	HIRI ($n = 9$)	Sham ($n = 9$)	HIRI ($n = 9$)
Fasting	4.53 ± 0.35	4.52 ± 0.34	$14.48 \pm 0.61^{\triangle\triangle\triangle}$	$14.55 \pm 0.56^{\#\#\#}$
Random	7.20 ± 0.57	8.62 ± 0.68	$23.60 \pm 2.29^{\triangle\triangle\triangle}$	$22.55 \pm 1.89^{\#\#\#}$
Preoperative	4.40 ± 0.38	4.43 ± 0.36	$14.43 \pm 0.62^{\triangle\triangle\triangle}$	$14.40 \pm 0.48^{\#\#\#}$

Abcam, Cambridge, MA), nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB, 1 : 1000, Cell Signaling Technology (CST), Danvers, MA), total NF-κB inhibitor, alpha (IκBα, 1 : 2000, CST), phosphor-IκBα (1 : 500, CST), heme oxygenase-1 (HO-1, 1 : 250, Santa Cruz), and NAD(P)H:quinone oxidoreductase 1 (NQO1, 1 : 250, Santa Cruz).

2.19. Statistical Analysis. Results were processed by SPSS 13.0 (SPSS Inc.). Measurable data are expressed as means ± standard error of the mean (SEM). Pathological scores are expressed as median with interquartile range. Statistical analyses of measurable data were performed with the independent t-test, and we performed Mann-Whitney U test to analyze pathological scores. A P value less than 0.05 was considered significant.

3. Results

3.1. Hyperglycemia Aggravates HIRI in Diabetic Rats. Behrends et al. have proved that acute hyperglycemia induced by single intraperitoneal injection of glucose could worsen HIRI [33]. To establish a more stable hyperglycemia model, we adopted STZ-induced type 1 diabetes model to intensively study the effect of glucose overload. We measured the glycemic level of normal and diabetic rats subjected to sham or partial hepatic I/R, respectively, and confirmed consistent significant hyperglycemia in STZ-induced diabetic rats compared to normal rats (Table 1). There were no significant differences with regard to hepatic histological findings between diabetic sham group and normal sham group (Figures 1(a) and 1(b)). However, when subjected to IR, more severe hepatocyte necrosis, sinus congestion, and hepatocyte ballooning were observed in diabetic group (Figures 1(a) and 1(b), $P < 0.05$). The trend of serum ALT and AST was consistent with histological findings (Figures 1(c) and 1(d), $P < 0.05$), which also indicated that hyperglycemia resulted in more serious HIRI.

3.2. Hyperglycemia Increases Baseline Lipid Peroxidation and Inflammatory Cytokines in Diabetic Rats and Further Aggravated Them after HIRI. The unifying hypothesis of diabetic complication places ROS at the center of the deleterious effect of hyperglycemia [13]. Liver is particularly prone to oxidative/nitrosative stress due to its high metabolic rate and because hepatocytes are rich in ROS/RNS-producing mitochondria, cytochrome P450 (CYP) enzymes, and inducible nitric oxide synthase (iNOS). Increased oxidative/nitrosative stress has been a key contributor of hepatopathology relevant to diabetes such as NAFLD, viral hepatitis, and alcoholic fatty liver disease [34]. So we found it necessary to determine the extent of oxidative stress between diabetic and normal rats.

As shown in Figure 2, serum H_2O_2 and nuclear 8-OHdG were higher in diabetic HIRI group compared to normal HIRI group (Figures 2(a) and 2(b), $P < 0.05$) which implicated excessive ROS generation and consequent aggravated DNA injury in diabetic rats. The baseline level of lipid peroxidation product, hepatic 8-isoprostane and MDA, increased significantly in diabetic rats compared to normal rats and soared after IR (Figures 2(c) and 2(d), $P < 0.05$).

Subclinical inflammation has also been reported among the basic changes within diabetic patients [35]. We determined several common inflammatory cytokines including MCP-1, IL-6, and TNF-α in hepatic WCL, and it turned out they were also significantly higher in diabetic rats and further increased after IR (Figure 2(e), $P < 0.05$).

3.3. High Glucose Culture before H/R Increased the Vulnerability of BRL-3A Hepatocytes to H/R Injury. We subjected BRL-3A hepatocytes to 5.5 mM and 25 mM of D-glucose culture for 6–96 hours, and it turned out that the cell viability of BRL-3A was similar between 2 groups in normoxic environment (Figure 3(a)). However, after H/R, the viability of BRL-3A hepatocytes was significantly hampered after 6, 24, 48, 72, and 96 hours of 25 mM glucose pretreatment (Figure 3(b), $P < 0.05$). H/R could comparably increase the LDH leakage in both groups (Figure 3(c)).

3.4. High Glucose Culture Increased ROS and Cytokines under Normoxic Settings and Further Elevated Them after H/R. It has been proposed that excessive glucose induces ROS in endothelial cells mainly by activation of protein kinase C isoforms, increased formation of glucose-derived advanced glycation end-products (AGEs), and increased glucose flux through the aldose reductase pathway or polyol pathways [36, 37]. Here we demonstrated in hepatocytes that ROS generation was also increased after 25 mM glucose culture. H/R stress resulted in excessive production of ROS in both 5.5 mM and 25 mM glucose pretreated group. However, the fluorescent intensity of ROS was significantly higher and further enhanced after H/R in 25 mM group compared to

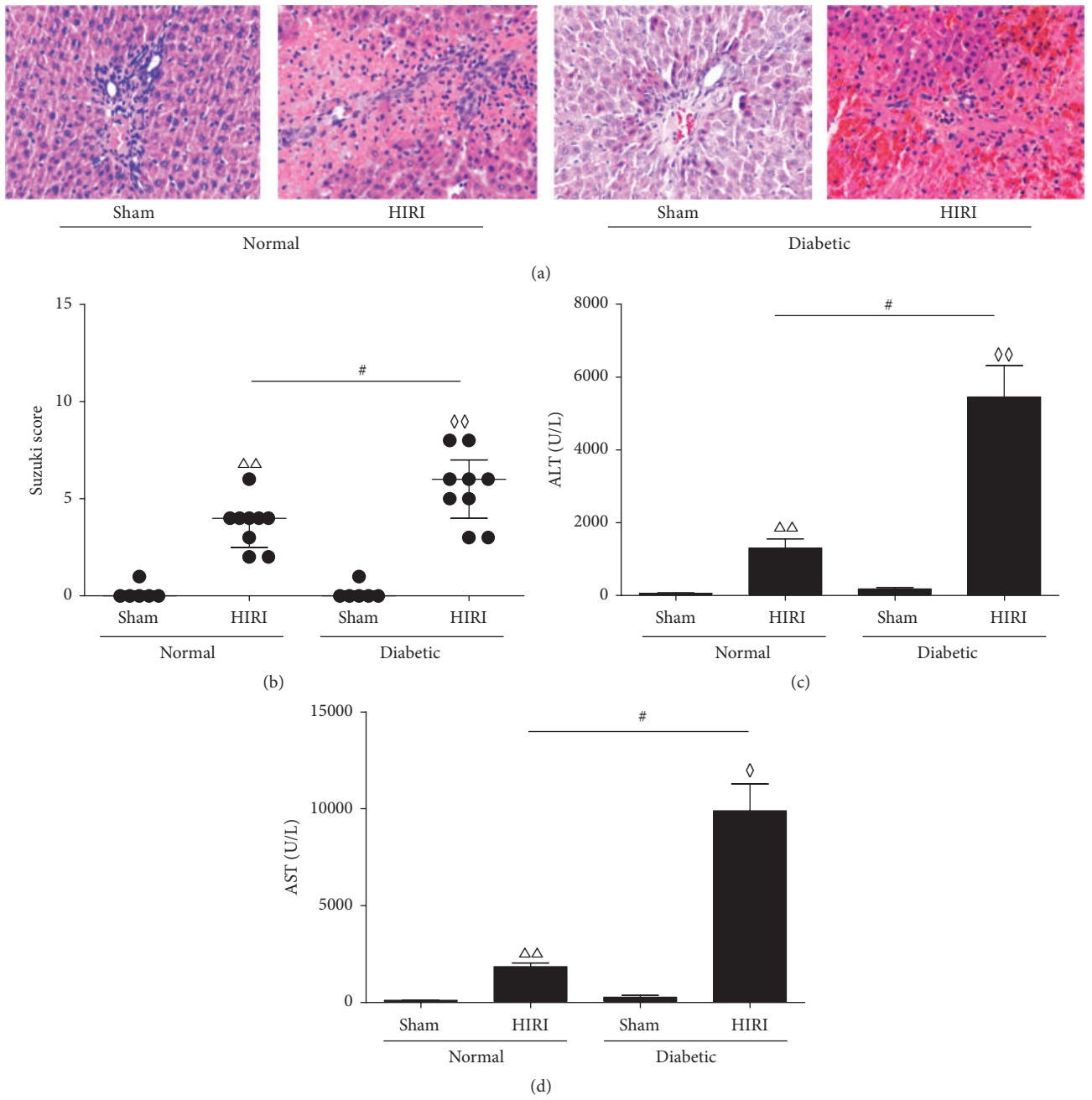
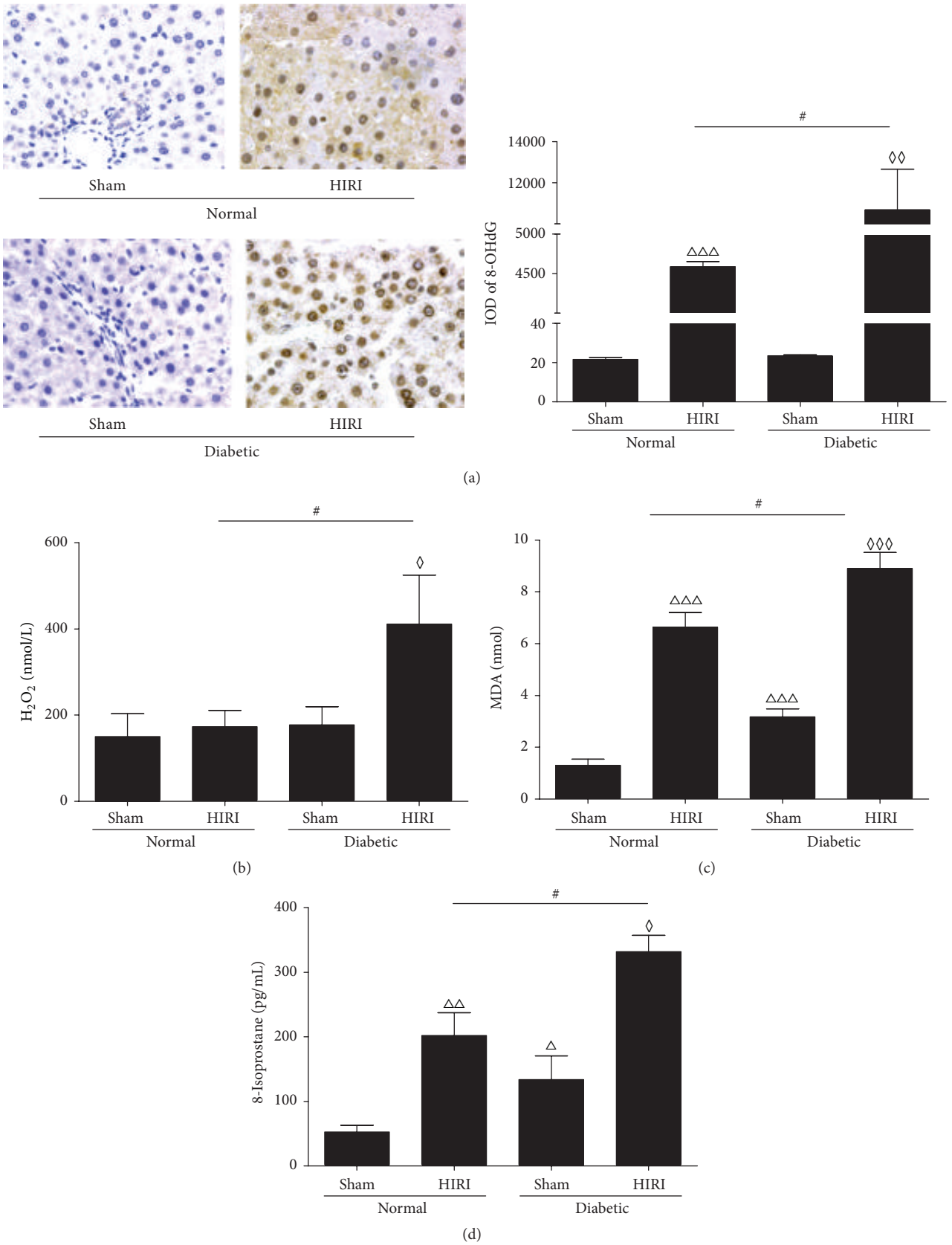


Figure 1: HIRI after 60 minutes of ischemia followed by 6 hours of reperfusion in control or diabetic rats. ((a), (b)) Hepatic pathological sections (200x) and pathology score by Suzuki's criteria. ((c), (d)) Serum ALT and AST levels. Measurable data are expressed as mean ± SEM ($n = 9$ per group). Pathology scores are expressed as medium with interquartile range. $^{\lozenge}P < 0.05$ versus diabetic sham group; $^{\lozenge\lozenge}P < 0.01$ versus diabetic sham group; $^{\#}P < 0.05$ versus normal HIRI group. HIRI: hepatic ischemia reperfusion injury; ALT: alanine aminotransferase; AST: aspartate aminotransferase; sham: sham operating group.

5.5 mM group (Figures 4(a) and 4(b), $P < 0.05$). This implicates that the stress of acute H/R can amplify existing oxidative stress induced by excessive glucose, therefore causing more severe HIRI compared to control.

As has been observed in in vivo HIRI models, inflammatory cytokines, including MCP-1 and TNF-α in culture medium, were both significantly increased after exposure to 25 mM D-glucose under normoxic settings as well (Figures 4(c) and 4(d), $P < 0.05$). After H/R, in 25 mM pretreated cells, TNF-α was significantly higher compared to 5.5 mM group (Figure 4(c), $P < 0.05$).

3.5. High Glucose Culture Triggered NF-κB Translocation within Normoxic and Hypoxic-Redox BRL-3A Cells. Besides direct damage to cell components, ROS are also pivotal signals in various cellular pathways including NF-κB



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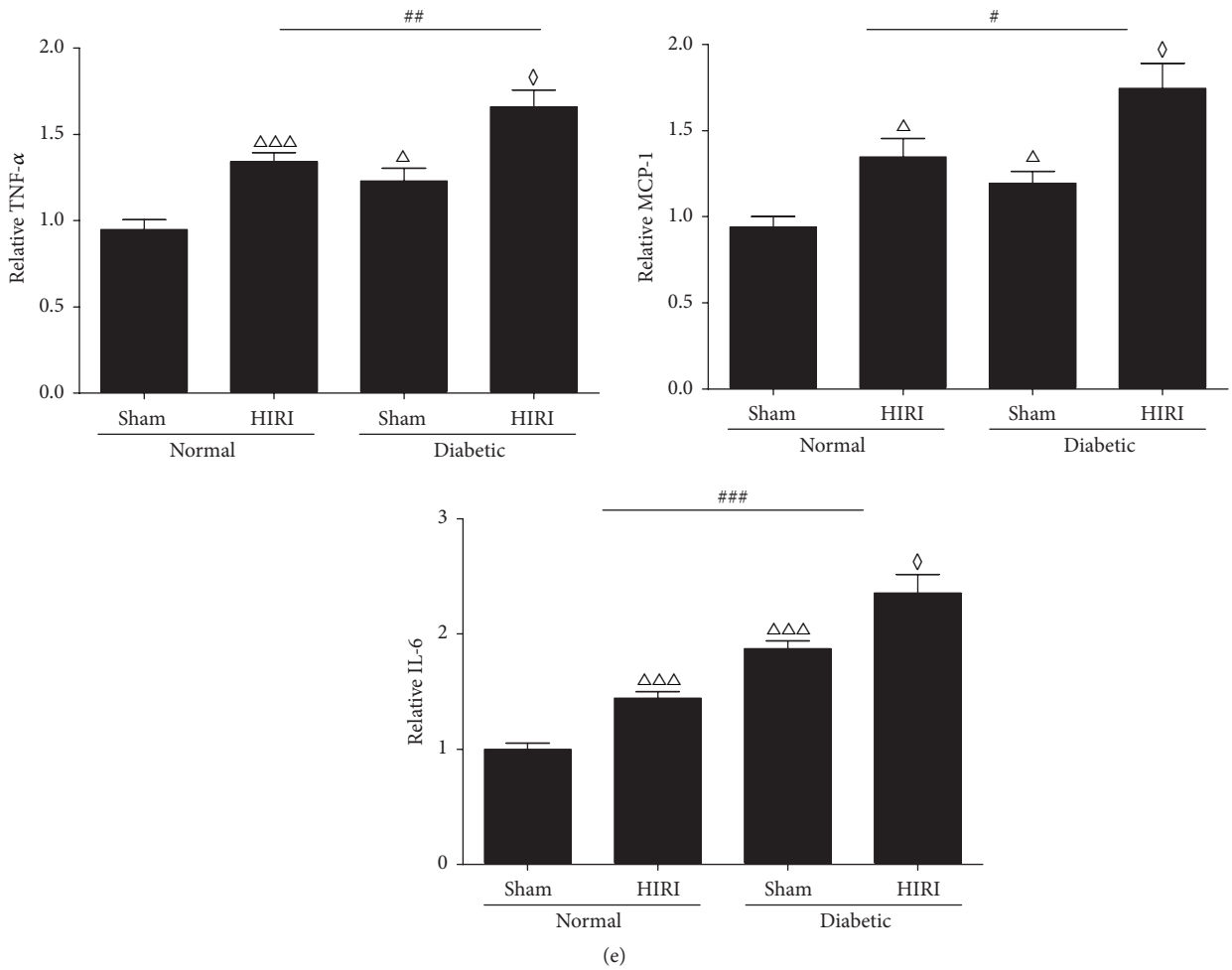

Figure 2: Hepatic oxidative stress and inflammation in normal or diabetic rats. (a) Nuclear 8-OHdG (400x) detected by IHC staining and IOD assessed by Image-J. (b) Serum H_2O_2 levels. (c) Hepatic MDA levels. (d) Hepatic 8-isoprostane determined by ELISA. (e) Hepatic inflammatory cytokines determined by ELISA. Data are expressed as mean ± SEM ($n = 9$ per group). $^{\triangle}P < 0.05$ versus normal sham group; $^{\triangle\triangle}P < 0.01$ versus normal sham group; $^{\triangle\triangle\triangle}P < 0.001$ versus normal sham group; $^{\Diamond}P < 0.05$ versus diabetic sham group; $^{\Diamond\Diamond}P < 0.01$ versus diabetic sham group; $^{\Diamond\Diamond\Diamond}P < 0.001$ versus diabetic sham group; $^{\#}P < 0.05$ versus normal HIRI group; $^{\#\#}P < 0.01$ versus normal HIRI group; $^{\#\#\#}P < 0.001$ versus normal HIRI group. HIRI: hepatic ischemia reperfusion injury; 8-OHdG: 8-hydroxydeoxyguanosine; IOD: integrated optical intensity; IHC: immunohistochemistry; H_2O_2: hydrogen peroxide; MDA: malondialdehyde; TNF-α: tumor necrosis factor α; MCP-1: monocyte chemokine protein-1; IL-6: interleukin-6; ELISA: enzyme-linked immunosorbent assay; sham: sham operating group.

pathway [38]. NF-κB is an important transcription factor that reacts to redox signals and regulates the expression of many inflammatory cytokines including MCP-1, IL-6, and TNF-α [39, 40]. Compared with 5.5 mM group, IκBα phosphorylation and nuclear NF-κB were higher before H/R after 6 hours of 25 mM D-glucose culture (Figure 5(a), $P < 0.05$), which explained the consistently increased cytokines in medium. After H/R, the nuclear NF-κB in 25 mM H/R group was significantly increased compared to 5.5 mM H/R group (Figure 5(b), $P < 0.05$). Thus, high glucose culture before H/R not only could accelerate NF-κB activation but also increased the translocation of NF-κB after H/R. The subsequently upregulated proinflammatory cytokines could activate neutrophils and Kupffer cells to trigger profound cytotoxic immune response [41].

3.6. High Glucose Culture Activated Nrf2 Translocation within Normoxic and Hypoxic-Redox BRL-3A Cells without Affecting the Expression of NQO1 and HO-1. Nrf2 is another redox-sensitive transcription factor that mainly regulates the expression of antioxidases including HO-1 and NQO1 to counter harmful stimuli including ROS [42]. Here we demonstrated in BRL-3A hepatocytes that nuclear Nrf2 was significantly higher before H/R after 6 hours of 25 mM D-glucose culture compared with 5.5 mM group (Figure 6(a), $P < 0.05$). Nevertheless, as downstream oxidase of Nrf2, the expression of NQO1 and HO-1 was comparable between low and high glucose pretreated groups. Consistently, while NQO1 and HO-1 were upregulated after H/R in 5.5 mM H/R group compared to normoxic control, they remained unaffected in 25 mM H/R group (Figure 6(b)).

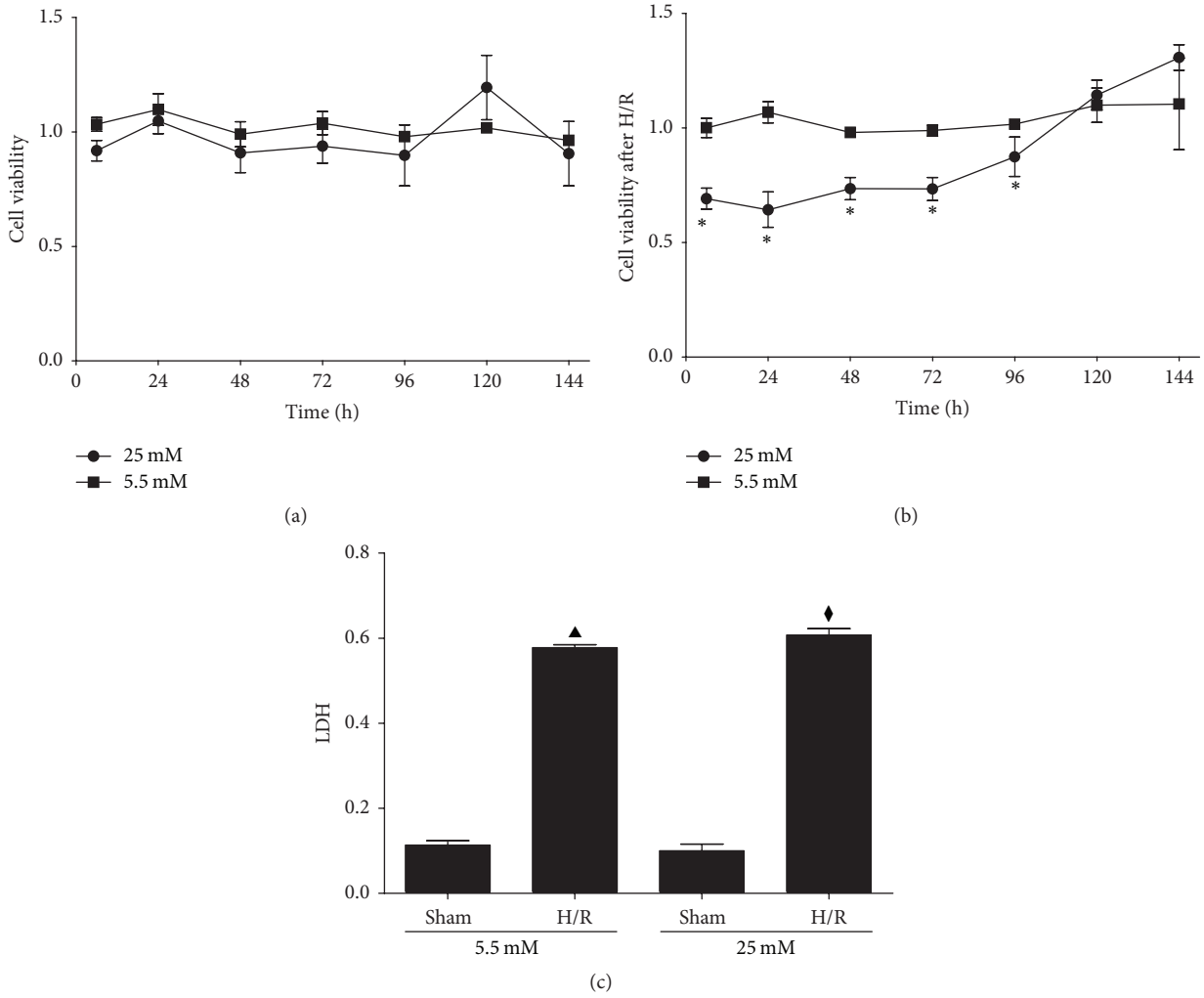

Figure 3: High glucose culture exerted deleterious effect upon BRL-3A hepatocytes after hypoxia/reoxygenation. (a) Cell viability under normoxic circumstances after being treated with serum-free medium containing 5.5 Mm and 25 mM D-glucose, respectively. (b) Cell viability under redox circumstances after being treated with serum-free medium containing 5.5 mM and 25 mM, respectively. (c) LDH levels in the medium after hypoxia/reoxygenation. $^{*}P < 0.05$ versus 5.5 mM D-glucose group; $^{\blacktriangle}P < 0.05$ versus 5.5 mM control group; $^{\blacklozenge}P < 0.05$ versus 25 mM control group. H/R: hypoxia/reoxygenation; LDH: lactate dehydrogenase.

3.7. NAC and Apocynin Pretreatment Ameliorated HIRI and Oxidative Stress and Inflammation. With these findings, we postulate that ROS lies in the center of the amplified inflammation cascades and aggravated HIRI. ROS scavengers including NAC and apocynin have been suggested to be a solution to ameliorate or reverse the deleterious effect of overly triggered oxidative stress [43, 44]. N-Acetyl-cysteine serves as a prodrug to L-cysteine, which is a precursor to the biologic antioxidant glutathione. Hence, administration of NAC replenishes reduced glutathione (GSH) [45]. The GSH synthesis is controlled by the activity of γ-glutamyl cysteine ligase (γ-GCL) and by the availability of cysteine. γ-GCL is composed of two subunits: the glutamate-cysteine ligase complex modifier subunit (GCLM) and the GCL catalytic subunit (GCLC). Nrf2 regulates the expression of these subunits [42]. Apocynin is an inhibitor of NADPH oxidase activity and thus is effective in preventing reduction of O_2 to superoxide ($O_2^{-\bullet}$), in human neutrophilic granulocytes. We intended to examine whether HIRI in diabetic rats could be ameliorated by either partial restoration of hampered Nrf2 pathway or suppression of ROS generation.

In the present study, we found out that NAC and apocynin administered intraperitoneally before IR could both ameliorate HIRI in diabetic rats, indicated by lower pathological scores and lower serum ALT and AST (Figures 7(a) and 7(b), $P < 0.05$). NAC and apocynin pretreatment could also lower the expressions of hepatic IL-6, MCP-1, and TNF-α (Figure 7(c), $P < 0.05$) compared with HIRI group. The nuclear 8-OHdG significantly decreased in NAC and apocynin pretreated group compared with HIRI group (Figures 7(d) and 7(e), $P < 0.05$). The same went for hepatic 8-isoprostane and MDA (Figures 7(f) and 7(g), $P < 0.05$), which indicated a decrease in lipid peroxidation.

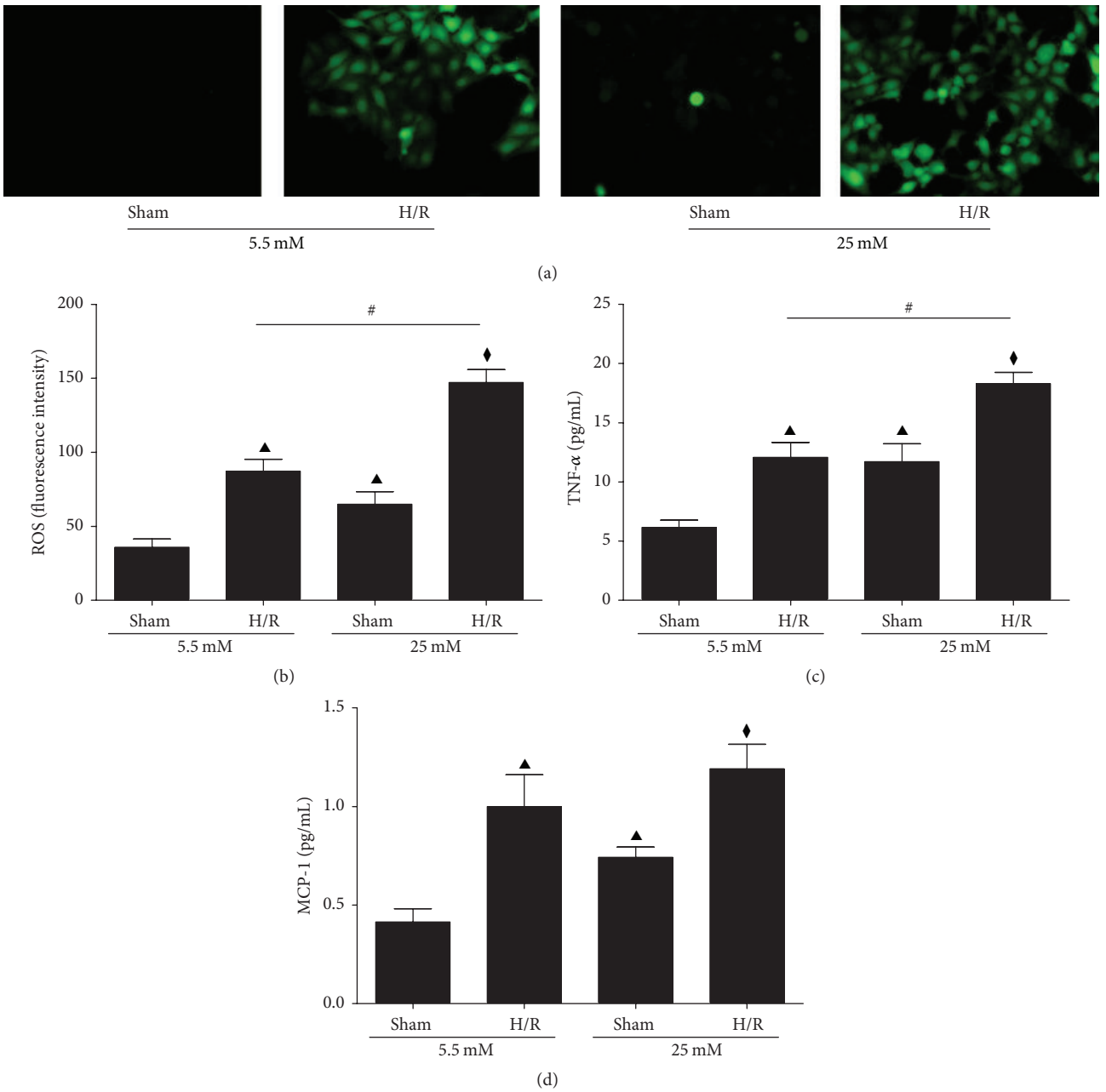

Figure 4: High glucose culture increases ROS generation and cytokines release within BRL-3A hepatocytes. ((a), (b)) Intracellular ROS stained by 6-carboxy-2′7′-DCFH-DA (200x) and the fluorescence intensity was detected by fluorescence spectrophotometer. ((c), (d)) TNF-α and MCP-1 in the culture medium. $^{\blacktriangle}P < 0.05$ versus 5.5 mM control group; $^{\blacklozenge}P < 0.05$ versus 25 mM control group; $^{\#}P < 0.05$ versus 5.5 mM H/R group. H/R: hypoxia/reoxygenation; ROS: reactive oxygen species; TNF-α: tumor necrosis factor α; MCP-1: monocyte chemokine protein-1.

4. Discussion

Hyperglycemia alone under normoxic condition does not significantly impact the viability of hepatocytes. Chen et al. demonstrated that Chang liver cells in mannitol-balanced 5.5 mM, 25 mM, and 100 mM glucose media after at least 3 weeks showed no significant variation in viability and apoptosis among the three culture conditions [21]. However, hyperglycemia is associated with chronic oxidative stress [13]. In the cardiac tissues of both type 1 and type 2 diabetes, an elevated baseline of ROS induced by hyperglycemia was identified [46]. Glucose induce ROS mainly by activation of protein kinase C isoforms, increased formation of glucose-derived advanced glycation end-products (AGEs), and increased glucose flux through the aldose reductase pathway or polyol pathways [36, 37]. Consistently, the present

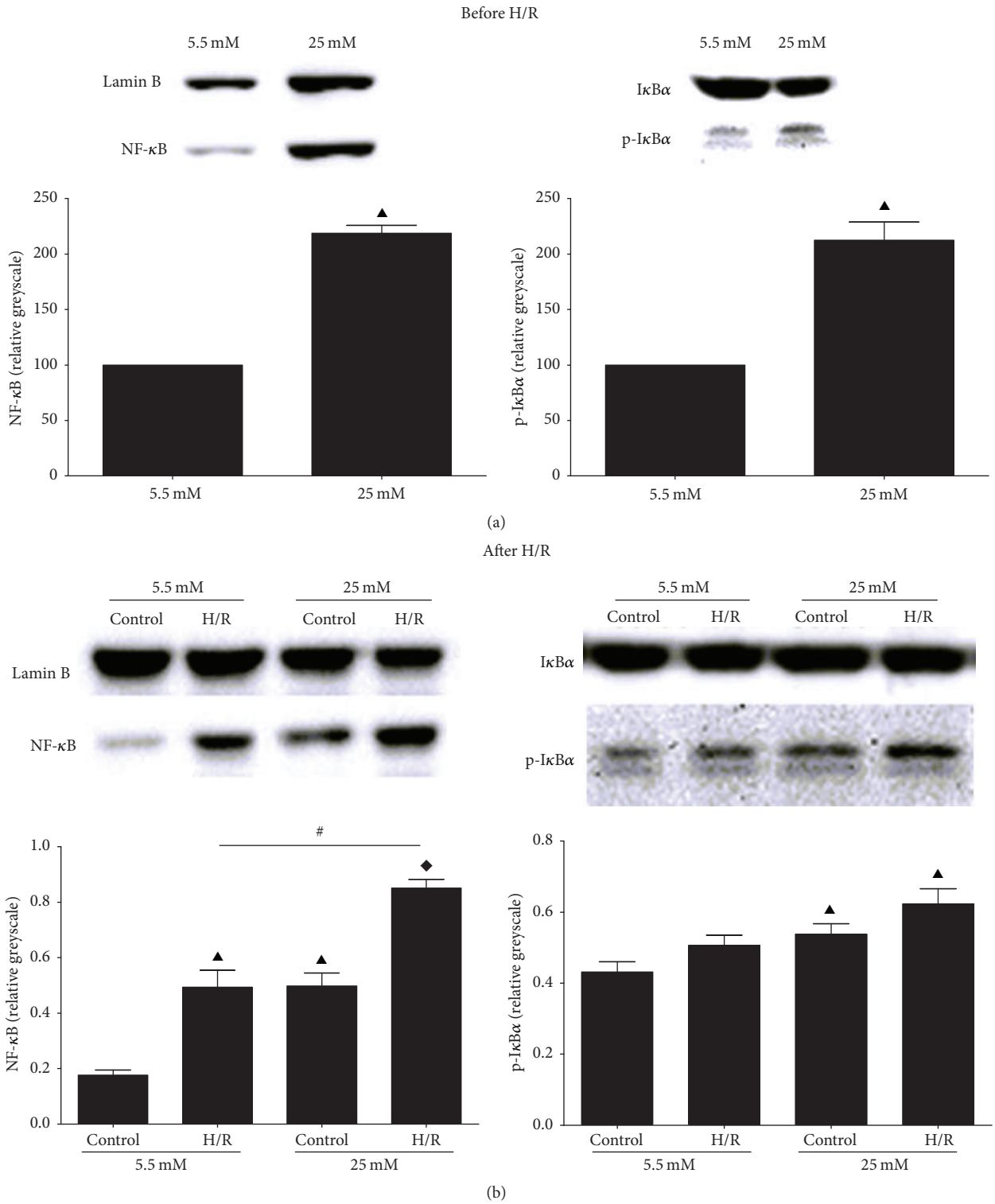

FIGURE 5: NF-κB signaling related protein alterations determined by western blotting before and after H/R. (a) NF-κB and its cytoplasm inhibitor IκBα after 6 hours of 5.5 mM or 25 mM D-glucose culture before H/R. (b) NF-κB and IκBα after H/R. $^{\blacktriangle}P < 0.05$ versus 5.5 mM control group; $^{\blacklozenge}P < 0.05$ versus 25 mM control group; $^{\#}P < 0.05$ versus 5.5 mM H/R group. H/R: hypoxia/reoxygenation; NF-κB: nuclear factor of kappa light polypeptide gene enhancer in B-cells; IκBα: total inhibitor alpha; p-IκBα: phosphor-IκBα.

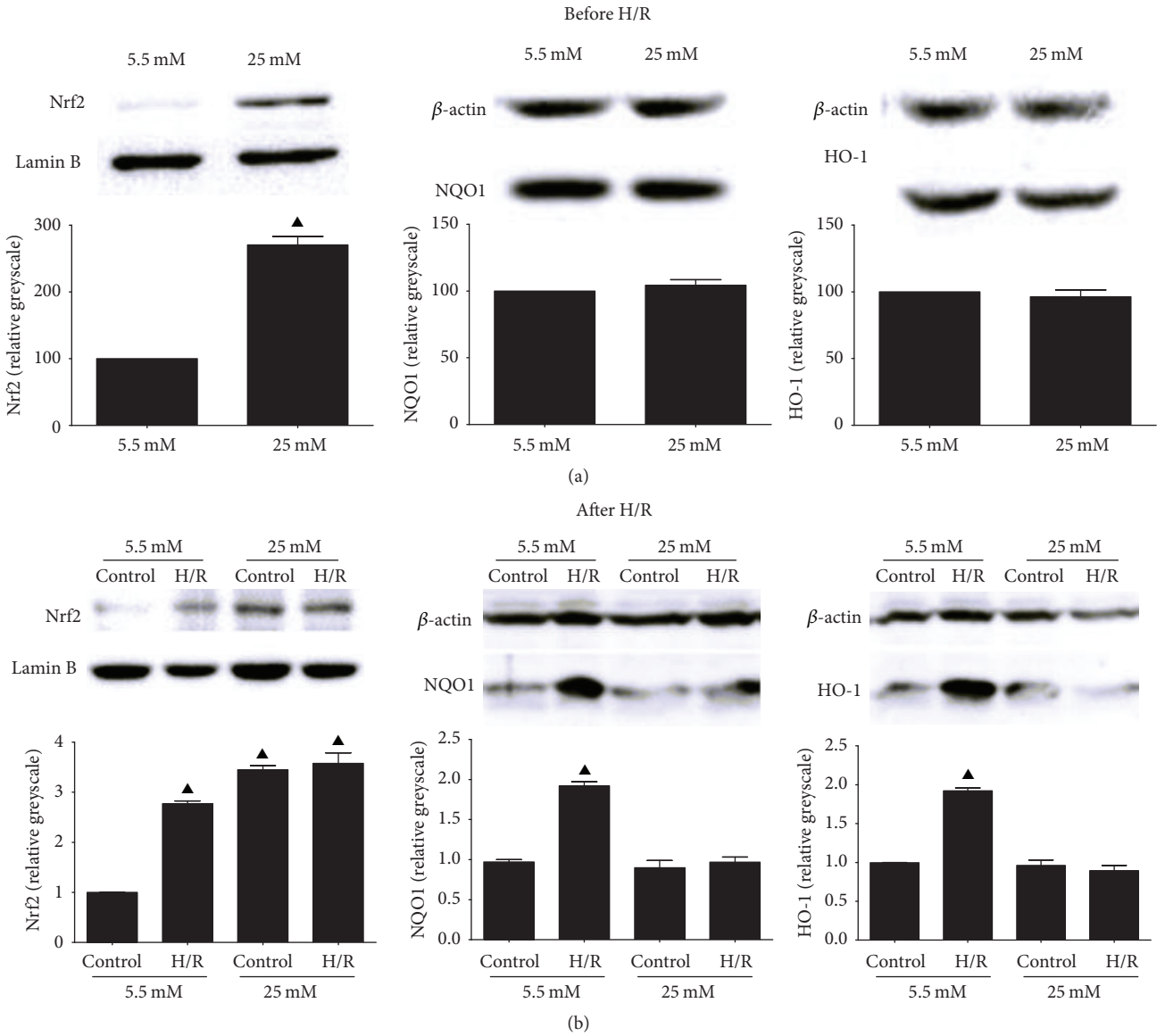

Figure 6: Nrf2 signaling related protein alterations determined by western blotting before and after HR. (a) Nrf2 and its downstream antioxidases NQO1 and HO-1 after 6 hours of 5.5 mM or 25 mM D-glucose culture before H/R. (b) Nrf2, NQO1, and HO-1 after H/R. ▲$P < 0.05$ versus 5.5 mM control group. H/R: hypoxia/reoxygenation; Nrf2: nuclear factor-erythroid 2-related factor 2; HO-1: heme oxygenase-1; NQO1: NAD(P)H:quinone oxidoreductase 1.

study demonstrated that both in vivo and in vitro high glucose exposure could increase baseline hepatic oxidative stress.

Chronic oxidative stress may promote the onset or progression of chronic liver diseases which if not treated properly are likely to advance to end-stage liver diseases requiring surgical intervention [20, 21]. However, little is known about the impact of chronic oxidative stress caused by hyperglycemia on HIRI. During reperfusion, enormous ROS are generated due to sudden restoration of oxygen and exert direct deleterious effects on cells through lipid peroxidation, protein degradation, and DNA damage [1]. The present study demonstrated that hepatic oxidative stress was further exacerbated after HIRI or H/R in both in vivo and in vitro high glucose settings. In line with soaring ROS generation, hepatocytes in high glucose environment predisposed to HIRI or H/R injury demonstrated by higher pathological score and serum transferase or lower cell viability. This implicates that the stress of acute IR can amplify existing oxidative stress induced by glucose overloads, therefore causing more severe HIRI compared to control.

In addition to direct damage to cell components, ROS can also activate various cellular pathways including NF-κB pathway [38]. The inactive NF-κB is sequestered in the cytoplasm with an inhibitor protein, IκB. In a conventional activation pathway, IκB is phosphorylated by IκB kinases (IKKs) in response to different activators, including ROS, and subsequently degraded thus liberating the active NF-κB

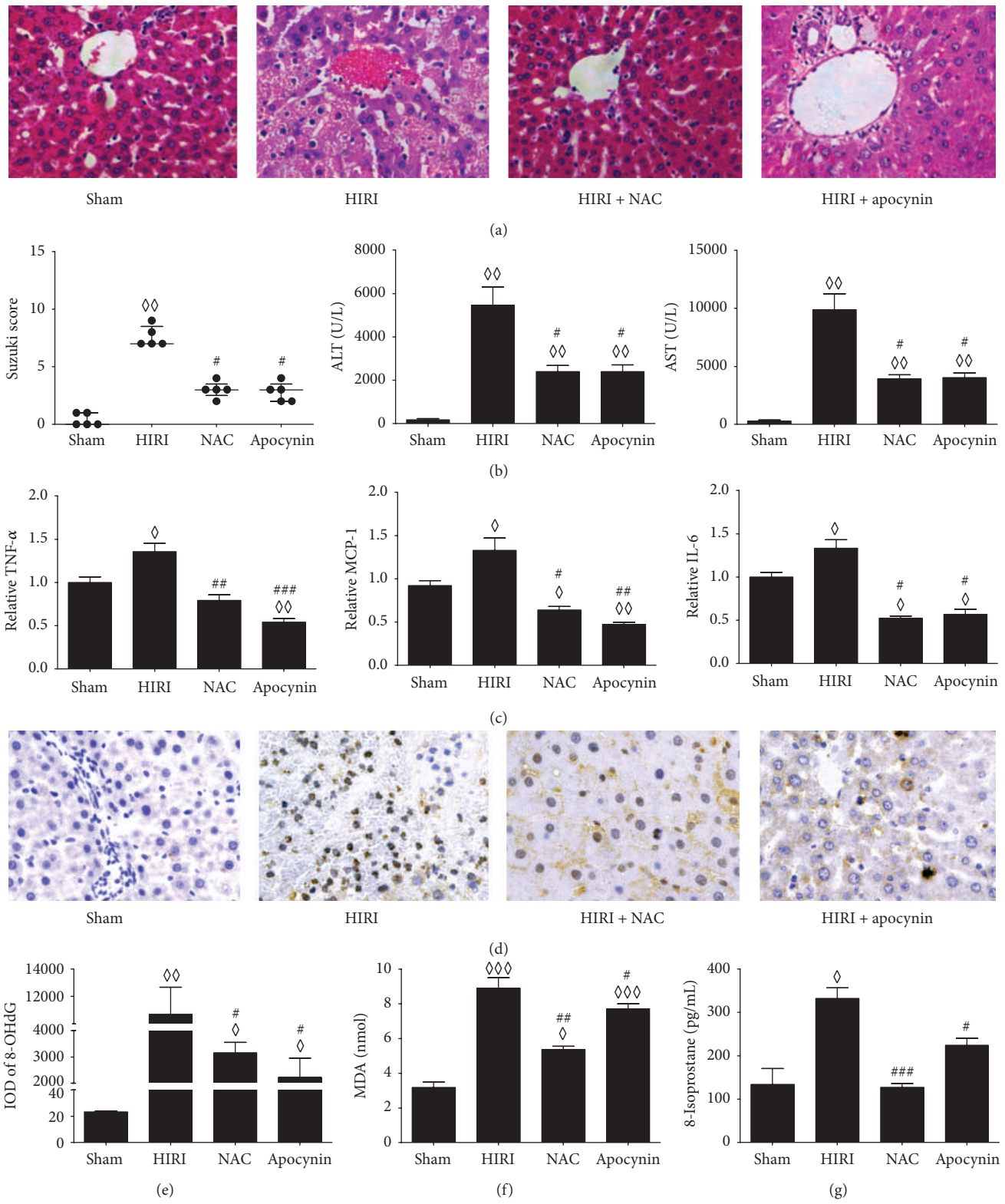

Figure 7: Hepatic ischemia reperfusion injury, oxidative stress, and inflammation in NAC or apocynin pretreated diabetic rats. ((a), (b)) Hepatic pathology sections (200x), pathological scores, and serum transferase. (c) Hepatic inflammatory cytokines levels. ((d), (e)) Nuclear 8-OHdG (400x) detected by IHC staining and IOD assessed by Image-J; (f) MDA levels; (g) 8-isoprostane levels by ELISA. Measurable data are expressed as mean ± SEM ($n = 5$ per group). Pathology scores are expressed as medium with interquartile range. $^{\lozenge}P < 0.05$ versus sham group; $^{\lozenge\lozenge}P < 0.01$ versus sham group; $^{\lozenge\lozenge\lozenge}P < 0.001$ versus sham group; $^{\#}P < 0.05$ versus HIRI group; $^{\#\#}P < 0.01$ versus HIRI group; $^{\#\#\#}P < 0.001$ versus HIRI group. HIRI: hepatic ischemia reperfusion injury; NAC: N-acetyl-L-cysteine; IHC: immunohistochemistry; 8-OHdG: 8-hydroxydeoxyguanosine; IOD: integrated optical intensity; MDA: malondialdehyde; TNF-α: tumor necrosis factor α; MCP-1: monocyte chemokine protein-1; IL-6: interleukin-6; ELISA: enzyme-linked immunosorbent assay; sham: sham operating group.

complex to nucleus [39]. We observed phosphorylated IκBα, increased NF-κB translocation into nucleus after high glucose culture before H/R, which explained the consistently increased cytokines in medium. The result of in vitro study was similar to that of in vivo study where hepatic cytokines increased at baseline and strikingly rose after IR in diabetic rats, suggesting high glucose induced activation of NF-κB might also be involved in HIRI in diabetic rats. Clinically NF-κB related cytokines, TNF-α, IL-6, and MCP-1, were found increased in patients with type 2 diabetes [35]. These proinflammatory cytokines could activate neutrophils and Kupffer cells to trigger profound cytotoxic immune response [41].

Nrf2 is another redox-sensitive transcription factor that mainly regulates the expression of antioxidases including HO-1 and NQO1 to counter harmful stimuli including ROS [42]. It has been reported that Nrf2 pathway activates in high glucose stimulated cardiomyocytes endothelial cells and renal mesenchymal cells as intrinsic defense against oxidative stress [42, 47, 48]. However, in our present study, high glucose could increase the Nrf2 translocation in hepatocytes but did not alter the expression of HO-1 and NQO1. Moreover, in contrast to its further increase and consequent upregulation of HO-1 and NQO1 after H/R in low glucose pretreated hepatocytes, nuclear Nrf2 in high glucose pretreated hepatocytes was not significantly increased following H/R and neither were its downstream antioxidases. The results implicate that the antioxidative ability of Nrf2 pathway might be hampered after high glucose exposure.

Recent evidence indicates that NF-κB may directly repress Nrf2 signaling at the transcription level [49, 50]. DNase I footprint assays using purified transcription factors revealed the presence of NF-κB and AP-2 binding sites in the proximal part of the promoter region of the human HO-1 gene [51]. Therefore, it is reasonable to postulate that ROS overproduction by high glucose could initiate both NF-κB and Nrf2 activation, while the former prevailed and frustrated the function of Nrf2. However, the exact mechanisms of NF-κB and Nrf2 interaction under hyperglycemic settings remain to be further elucidated.

With these findings, we postulate that ROS lies in the center of the amplified inflammation cascades and aggravated HIRI. ROS scavengers including NAC and apocynin have been suggested to be a solution to ameliorate or reverse the deleterious effect of overly triggered oxidative stress [43, 44]. NAC and apocynin administered intraperitoneally before IR could ameliorate hepatic injury in diabetic rats along with decreased inflammatory cytokines, attenuated lipid peroxidation, and ROS-inflicted DNA injury compared with the HIRI controls. The present results are consistent with those of other researchers which reported the long-term oral administration of NAC and Allopurinol showed synergistic protective effect on diabetic ischemic cardiac tissues [52]. It is therefore a promising strategy to alleviate HIRI by countering preoperative chronic oxidative stress in diabetes before IR.

One of the limitations of this study may lie in the choice of animal models used to represent chronic hyperglycemia. STZ-induced diabetes is a model of type 1 diabetes mellitus, within which the cause of hyperglycemia is attributed to insulin insufficiency due to STZ toxicity upon islet β-cells [53]. The dosage, approach, and frequency of STZ administration vary in a broad range [54]. In the current study, we chose to give a single dose of 50 mg/kg of STZ administered intraperitoneally, which is a considerably mild dose compared to those used in other studies. We provided sufficient normal diet and water for the confirmed diabetic rats and measured the fasting, random, and preoperative glycemic levels to prevent hypoglycemia. Since persistent hyperglycemia is a major manifestation of all kinds of diabetes, the primary aim of our study was to examine the impact of glucose excess on HIRI.

Another issue that might be of concern is the potential protection of insulin in hyperglycemia induced aggravation of HIRI. Insulin is undoubtedly the first-line treatment for preoperative and intraoperative hyperglycemia. Preoperative insulin treatment is definitely effective in compensating β-cell function and lowering blood glucose in STZ-induced type 1 diabetic models. Rocha et al. demonstrated that insulin led to the significant reduction of the serum concentration of AST, ALT, gamma-glutamyl transferase (GGT), and LDH in rat HIRI model. Nevertheless, these beneficial effects were found to be independent from blood glucose levels and may have been attributed to inhibition of GSK-3β [55]. Our focus of the current study, however, was mainly on the role of hyperglycemia, as an initial trigger of chronic oxidative stress, which will lead to further exacerbation of I/R injury. However, persistent hyperglycemia is very common when surgical stress is imposed upon diabetic patients, sometimes even in normal patients, since the secretion of catecholamine would aggravate insulin resistance, thus reducing the efficacy of exogenous insulin infusion. Moreover, the attempt to lower blood glucose might not be enough or in time to reverse the deleterious effects, for instance, the formation of excessive ROS and amplified inflammation, which are already imposed on the rendered liver. Therefore, we intended to find a solution to ameliorate the consequence caused by high glucose as it may be more practical in the context of surgery. Nevertheless, we cannot deny the potential protection offered by insulin during the preoperative preparation, which has proven to be very meaningful for the timing of elective surgery on diabetic patients. We will conduct more researches to study the effect of insulin-related pathways on diabetic HIRI in the future.

5. Conclusion

In summary, we demonstrated that hyperglycemia could aggravate HIRI, and the exacerbation was related to chronic oxidative stress and inflammation induced by excessive glucose (Figure 8). The amplified inflammation may be attributed to NF-κB translocation most probably induced by chronic oxidative stress. Hampered antioxidative ability of Nrf2 pathway may also be involved in aggravated HIRI after high glucose exposure. Inhibitory interaction between NF-κB and Nrf2 at transcription level may be a possible explanation, though the exact mechanisms of the interaction of these two transcription factors under hyperglycemic settings remain

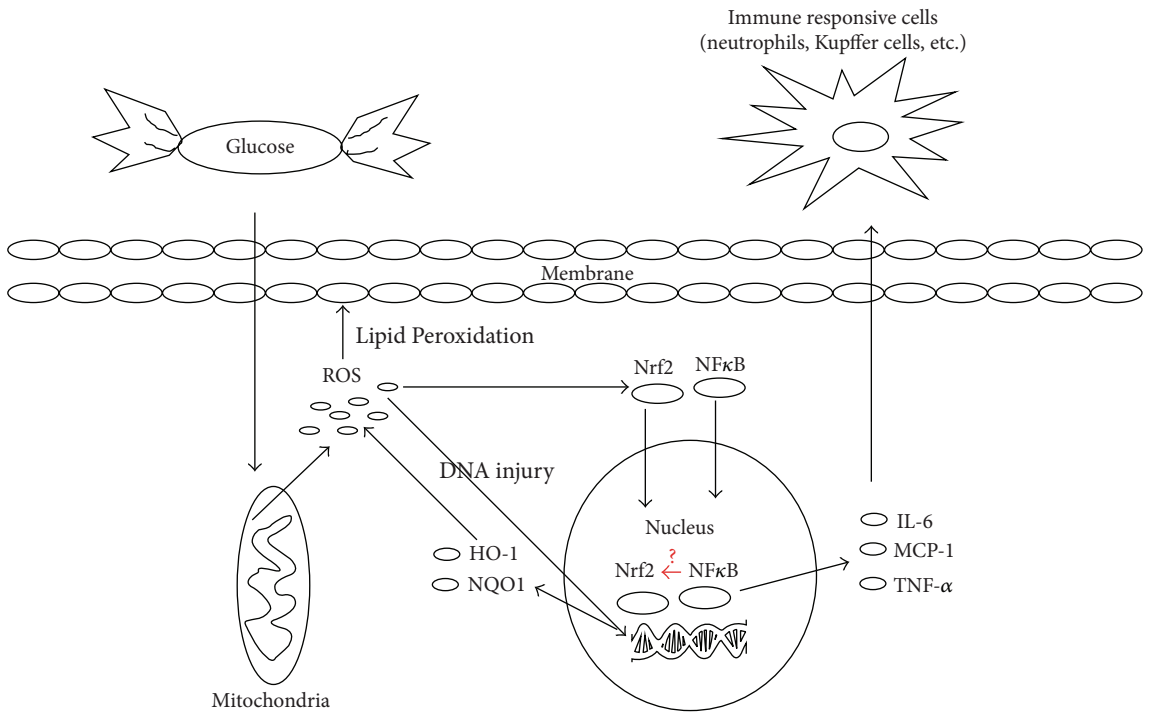

Figure 8: The proposed mechanisms of hyperglycemia induced aggravation of HIRI. Chronic oxidative stress represented by generation of ROS was induced by excessive glucose. The amplified inflammation may be attributed to NF-κB translocation most probably induced by chronic oxidative stress. Hampered antioxidative ability of Nrf2 pathway may also be involved in aggravated HIRI after high glucose exposure. The potential inhibitory interaction between NF-κB and Nrf2 at transcription level may be a possible explanation.

to be studied. Antioxidant precondition may be a potential therapy to alleviate diabetic HIRI.

Competing Interests

There are no conflicts of interest for any of the authors.

Authors' Contributions

Ziqing Hei and Xinjin Chi contributed equally as corresponding authors. Ziqing Hei, Xinjin Chi, and Yong Huang worked jointly in designing this experiment. Yihan Zhang and Dongdong Yuan contributed equally in major experiment procedure and thesis writing. Yihan Zhang, Weifeng Yao, and Xi Chen cooperated in the establishment of animal models and consequent assessment. Dongdong Yuan, Yue Liu, and Fei Huang were major participants of in vitro researches. Yihan Zhang and Qianqian Zhu were mainly responsible for data analysis. All authors have read and approved the final manuscript.

Acknowledgments

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