Oxidative Medicine and Cellular Longevity

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Lead Guest Editor: Ayman M. Mahmoud
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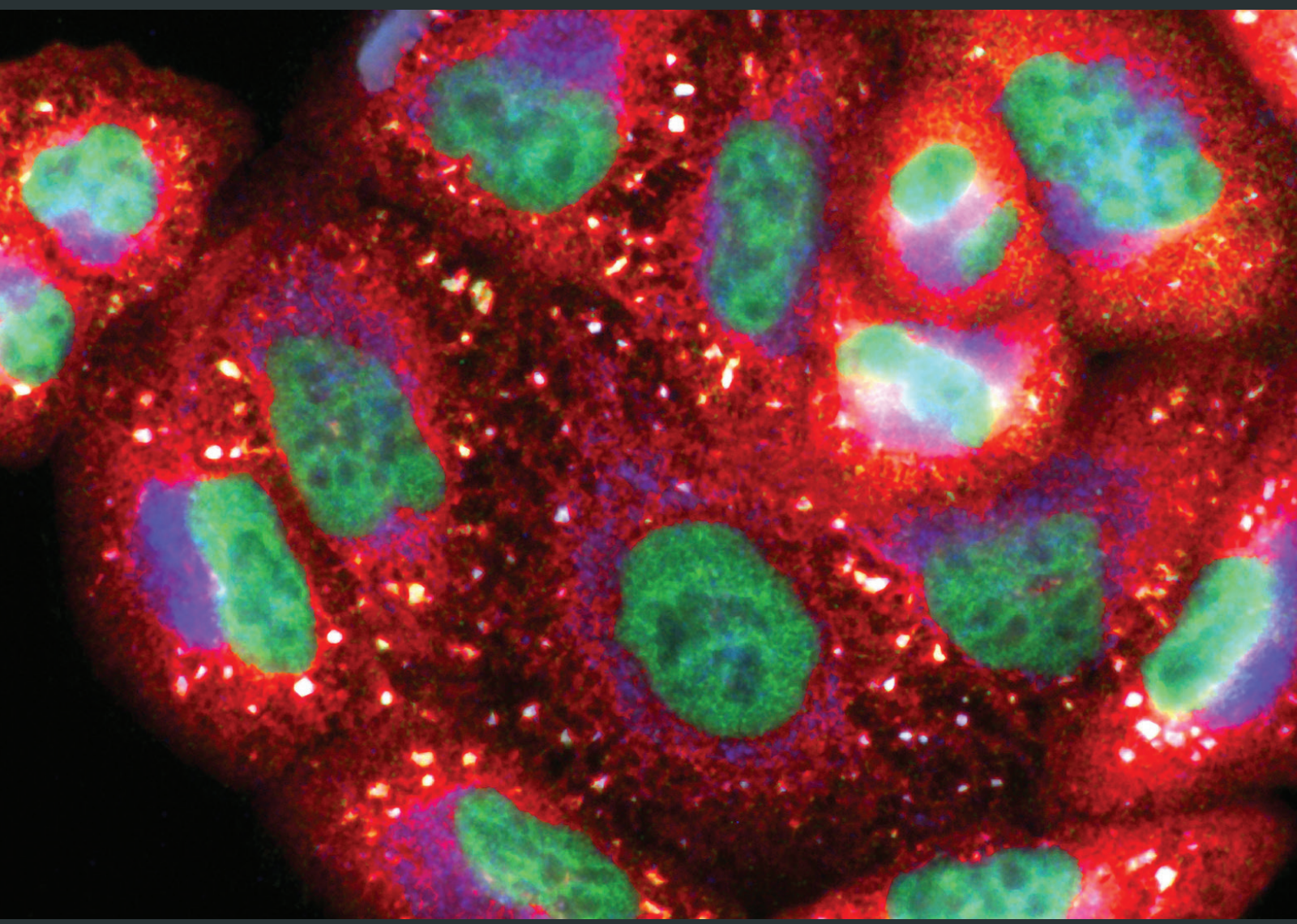

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Editorial Board

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Editorial

Oxidative Stress in Metabolic Disorders and Drug-Induced Injury: The Potential Role of Nrf2 and PPARs Activators

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Oxidative stress plays a major role in metabolic disorders and a wide range of chronic diseases such as diabetes mellitus, obesity, metabolic syndrome, aging, cancer, osteoporosis, rheumatoid arthritis, cardiovascular diseases, and neurodegenerative disorders. In addition, drug-induced organ injury is well known to be associated with oxidative stress and inflammation. Considerable evidence indicates that oxidative stress and inflammation are the key pathophysiological processes underpinning these disorders. Therefore, modulation of oxidative stress represents an important strategy for the treatment of multiple human diseases.

The transcription factor nuclear factor erythroid 2 related factor 2 (Nrf2) is the master regulator of the basal and inducible expression of a large network of cytoprotective and antioxidant genes [1]. Under basal conditions, Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1) which functions as a sensor protein against electrophiles and reactive oxygen species (ROS). Upon cell stimulation, Nrf2 dissociates from Keap1 and activated Nrf2 is translocated into the nucleus where it binds to the antioxidant response element (ARE) and leads to expression of target genes including heme oxygenase-1, NAD(P)H:quinone oxidoreductase 1, superoxide dismutase, catalase, glutathione peroxidase, and glutathione-S-transferase [2]. Thus, Nrf2 plays a role as a multiorgan protector against oxidative stress via inducing target genes. In recent years, Nrf2 has shown promise as a novel therapeutic target in diseases with underlying oxidative and inflammatory stress components [3–6].

Peroxisome proliferator-activated receptors (PPARs) are proteins that belong to the nuclear receptor family of ligand-activated transcription factors. The three main forms of peroxisome proliferator-activated receptors (PPARα, PPARβ/δ, and PPARγ) belong to a superfamily of nuclear receptors that function as transcription factors regulating the expression of multiple genes. Upon ligand binding, they form heterodimers with retinoid X receptor (RXR) and result in modulation of gene transcription [7]. PPARs regulate a variety of biological processes in various tissues. Among their effects, PPARα controls lipid metabolism and inflammatory processes [8], PPARβ/δ regulates glucose utilization, cell differentiation, and inflammation [9], and PPARγ is involved in adipocyte differentiation, glucose metabolism, and inflammatory pathways [10]. Upon activation, PPARs are known to exert anti-inflammatory and antioxidant properties via suppressing nuclear factor-κB, decreasing ROS production, and upregulating the expression of antioxidant enzymes [11].

Recent reports point to coactivation and possible interaction between PPARs and Nrf2 through multiple mechanisms. Coactivation of PPARγ and Nrf2 has been shown to protect against oxidative stress, inflammation, and carcinogenesis [4, 5, 12–14]. Ongoing and future research will probably provide efficient PPARs and Nrf2 modulating agents for preventing and treating metabolic and other common disorders.

This special issue encompasses cutting edge research and review articles focusing on the role of Nrf2 and PPARs in modulating oxidative stress and inflammation. It includes 8 novel research articles and 3 reviews describing the role of Nrf2 and PPARs in various pathological conditions, summarized as follows:

(1) Drug-induced oxidative stress and hepatotoxicity

Research article: "Gamma-Glutamylcysteine Ethyl Ester Protects against Cyclophosphamide-Induced Liver Injury and Hematologic Alterations via Upregulation of PPARγ and Attenuation of Oxidative Stress, Inflammation, and Apoptosis." In this article, S. Alqahtani and A. M. Mahmoud introduced evidence demonstrating the involvement of PPARγ in mediating the hepatoprotective effect of the synthetic glutathione precursor gamma-glutamylcysteine ethyl ester. Activation of PPARγ resulted in enhancement of antioxidant defenses and attenuation of cyclophosphamide-induced oxidative stress, inflammation, and apoptosis.

Review article: "Collaborative Power of Nrf2 and PPARγ Activators against Metabolic and Drug-Induced Oxidative Injury." C. Lee reviewed the general features of PPARγ and Nrf2 signaling pathways in the context of oxidative stress conditions. One of the main sections of this review was the role of natural and synthetic Nrf2 and PPARγ activators and the crosstalk between Nrf2 and PPARγ in alleviating drug-related oxidative stress and damage.

(2) Endocrine system and diabetes

Research article: "NRF2 Plays a Critical Role in Both Self and EGCG Protection against Diabetic Testicular Damage." This study by C. Pan et al. aimed to evaluate the protective role of epigallocatechin gallate (EGCG) against diabetic testicular damage and addressed the requirement of Nrf2. Eight-week-old normal and diabetic male C57BL/6 wild-type and Nrf2 knockout mice were treated with EGCG for 24 weeks. Nrf2 knockout abrogated both self and EGCG protection against diabetes-induced testicular weight loss, reduction in spermatozoa count, apoptotic cell death, endoplasmic reticulum (ER) stress, inflammation, and oxidative damage. Therefore, this study provides evidence that Nrf2 plays a central role in mediating the protective effect of EGCG against diabetic-induced testicular damage.

Research article: "Activation of the Nrf2-Keap 1 Pathway in Short-Term Iodide Excess in Thyroid in Rats." The effect of normal and high iodide intake on the antioxidative action of sulfredoxin (Srx) and peroxiredoxin 3 (Prx 3) via Nrf2-Keap 1 pathway has been investigated in the thyroid of rats. The expression of Srx and Prx 3 are known to be regulated via Nrf2. Srx is a member of the oxidoreductase family that contributes to cellular redox balance, and Prx 3 is a critical scavenger for mitochondrial ROS. The results showed that the activation of Nrf2 signaling, Srx, and Prx 3 may play a key role in protecting the thyroid gland from excess iodide-induced oxidative stress.

Research article: "Antioxidant Treatment Induces Hyperactivation of the HPA Axis by Upregulating ACTH Receptor in the Adrenal and Downregulating Glucocorticoid Receptors in the Pituitary." J. P. Prevatto et al. tested the hypothesis that an imbalance in the redox system not only increases ROS production but also alters the homeostasis of the hypothalamus-pituitary-adrenal (HPA) axis culminating in its hyperactivation. The results showed activated HPA axis, increased levels of systemic glucocorticoids, decreased expression of Nrf2 and HO-1 in the pituitary, upregulated adrenocorticotropic hormone (ACTH) receptors in the adrenal gland, and downregulated glucocorticoid receptors in the pituitary. Therefore, the indiscriminate use of antioxidants may represent a risk to develop several morbidities related to persistent hypercorticoidism.

(3) Nonalcoholic steatohepatitis

Research article: "Genetic Nrf2 Overactivation Inhibits the Deleterious Effects Induced by Hepatocyte-Specific c-met Deletion during the Progression of NASH." Based on the previous findings that overexpression of Nrf2 was able to reduce triglyceride accumulation and ROS production and suppress the levels of liver steatosis and fibrosis in c-met-deficient hepatocytes, P. Ramadori et al. provided *in vivo* evidence for the role of Nrf2 in preventing the deleterious effects induced by hepatocyte-specific c-met deletion during the progression of nonalcoholic steatohepatitis (NASH). In c-met/Keap1 knockout mice fed a methionine-choline-deficient (MCD) diet, Nrf2 overexpression reduced triglycerides accumulation, dampened the exacerbation of oxidative stress, drastically reduced the number of apoptotic cells, decreased the influx of infiltrating inflammatory cells, and attenuated the enhanced development of fibrosis.

(4) Hepatic encephalopathy

Research article: "*Commiphora molmol* Modulates Glutamate-Nitric Oxide-cGMP and Nrf2/ARE/HO-1 Pathways and Attenuates Oxidative Stress and Hematological Alterations in Hyperammonemic Rats." In a rat model of hyperammonemia, a serious complication of liver disease which may lead to encephalopathy and death, A. M. Mahmoud et al. investigated the effect of *Commiphora molmol* resin extract on the glutamate-NO-cGMP and Nrf2/ARE/HO-1 signaling pathways. Activation of Nrf2 by *C. molmol* resin extract protected against excess ammonia via attenuation of oxidative stress and inflammation and modulation of the glutamate-NO-cGMP signaling pathway. In addition,

C. molmol prevented hematological alterations and ameliorated both the activity and the expression of cerebral Na^+/K^+-ATPase and therefore might be a promising protective agent against hyperammonemia.

(5) Chronic kidney disease

Research article: "Expression of the *NRF2* Target Gene *NQO1* Is Enhanced in Mononuclear Cells in Human Chronic Kidney Disease." Reduced Nrf2 activity has been reported in models of chronic kidney disease (CKD). In this study, J. Shen et al. quantified the NQO1 gene expression as a readout parameter for Nrf2 signaling in monocytes of patients with CKD with and without dialysis therapy. When compared to healthy control subjects, CKD patients showed an upregulated gene expression of Nrf2 and NQO1 and a slight increase in the NQO1 protein content in monocytes from these patients. The study concluded that Nrf2 activation in monocytes of CKD patients is modulated through an influence on both gene expression and protein content of Nrf2 targets in a complex way.

(6) Cardiovascular function and disease

Review article: "The Role of Nrf2 in Cardiovascular Function and Disease." In this review article, S. Satta et al. summarized the mechanisms regulating the activity of Nrf2 and the role of Nrf2 in preventing mitochondrial dysfunction in cardiovascular disease. The authors highlight the central role of Nrf2 signaling in endothelial dysfunction, atherosclerosis, vascular calcification, hypertension, diabetic cardiomyopathy, and in the aging heart. In the last section of this review, the authors provide a summary of the role of Nrf2 activators in the treatment of cardiovascular disease.

Research article: "Probucol Protects Rats from Cardiac Dysfunction Induced by Oxidative Stress Following Cardiopulmonary Resuscitation." The objective of this study was to investigate the protective effect of the lipid-lowering agent probucol on cardiac injury induced by cardiac arrest (CA) in rats. CA is one of the most critical cardiovascular phenomena. Probucol protected against CA in rats as evidenced by the improved restoration of spontaneous circulation (ROSC) rate, alleviated oxidative stress, prolonged survival time, and improved hemodynamic parameters, and cardiac function. These protective effects of probucol are mediated through activating Nrf2 signaling.

(7) Pregnancy disorders

Review article: "Modulatory Mechanism of Polyphenols and Nrf2 Signaling Pathway in LPS Challenged Pregnancy Disorders." In this review article, T. Hussain et al. focused on the modulatory activity of flavonoids on oxidative stress-mediated pregnancy insults. They describe the role of Nrf2 activation in cases of pregnancy disorders.

The editors anticipate this special issue to be of interest to the readers and expect researchers to benefit in making further progress in the understanding of Nrf2 and PPARs activators.

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M. Yvonne Alexander
Yusuf Tutar
Fiona L. Wilkinson
Alessandro Venditti

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Research Article

Probucol Protects Rats from Cardiac Dysfunction Induced by Oxidative Stress following Cardiopulmonary Resuscitation

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Objective. To investigate the protective effect of probucol on induced cardiac arrest (CA) rats and possible mechanisms. *Methods*. Sprague Dawley rats were orally administrated with probucol at different dosage or vehicle for 5 days and subjected to a CA model by electrical stimulation, followed by cardiopulmonary resuscitation (CPR). The return of spontaneous circulation (ROSC) rate, antioxidant enzyme activities, and lipid oxidation markers were measured in serum and myocardium. Hemodynamic parameters and myocardial functions of animals were analyzed. Expression of erythroid-derived 2-like 2 (NFE2L2) and Kelch-like ECH-associated protein 1 (KEAP1) in the myocardium were examined with immunohistochemistry. *Results*. Probucol treatment significantly increased the ROSC rate and survival time of CA-induced rats. After ROSC, levels of oxidation-specific markers were decreased, while activities of antioxidant enzymes were increased significantly in probucol treatment groups. The probucol treatment improves hemodynamic parameters and myocardial functions. These parameter changes were in a dose-dependent manner. In the probucol treatment groups, the expression of KEAP1 was downregulated, while that of NFE2L2 was upregulated significantly. *Conclusion*. In the CA-induced rat model, probucol dose dependently improved the ROSC rate, prolonged survival time, alleviated oxidative stress, and improved cardiac function. Such protective effects are possibly through regulations of the KEAP1-NFE2L2 system.

1. Introduction

Cardiac arrest (CA) is one of the most critical cardiovascular phenomena. It is also the major cause of sudden cardiac death [1, 2]. During CA, the heart fails to pump blood around the body, and this results in ischemic damage. If the blood supply is not restored quickly, irreversible alterations to the endocrine system and the neural and/or humoral mechanisms will take place [3]; this leads to organ dysfunction and even failure of vital organs such as the heart, kidney, and brain. The severity of organ damage is related to the duration of ischemia and hypoxia and the time to restoration of spontaneous circulation (ROSC) [4]. Cardiopulmonary resuscitation (CPR) is the most effective first-aid measure for CA. However, significant improvements in resuscitation success rates, as well as in the long-term survival and quality of life of patients, have not been observed. In addition, following successful CPR, ROSC occurs, and large amounts of oxygen reactive species (ROS) are generated by mitochondria in ischemic neuronal tissues resulting in oxidative stress and extensive reperfusion injury [5]. Therefore, adequate post-ROSC reperfusion of vital organs is crucial to improving resuscitation success rates and injury reduction [6]. To date, treatments that can positively improve organ function and patient prognosis remain unavailable [7] Over recent years, many studies have focused on using drugs to alleviate oxidative stress and ischemic reperfusion injury. In particular, drugs used against oxidative stress play an important role in improving the success rate of CPR. Probucol is a lipid-lowering agent usually used to treat hypercholesterolemia.

It also exhibits a potent action against oxidative stress [8, 9]. It is still unclear whether probucol affects post-CA ROSC and CA-related injury. In the present study, we observed how probucol treatment changed oxidative stress, hemodynamics, myocardial function, and short-term survival rate in rats following ROSC and analyzed the protective role of probucol in oxidative stress-induced cardiac dysfunction and the mechanisms involved.

2. Materials and Methods

2.1. Ethics Statement. This project was approved by the Ethics Committee of the Hospital of the University of Electronic Science and Technology of China and Sichuan Provincial People's Hospital, Chengdu, Sichuan, China. The animal study was approved by Animal Experimentation Ethics Committee. All rats were first acclimatized and housed at the research animal laboratory during the experimental period. All experiments were performed in accordance with relevant guidelines and regulations, including any relevant details.

2.2. Animals. Fifty Sprague Dawley, specific-pathogen-free rats weighing 300 ± 20 g (25 males and 25 females) were provided by the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, People's Republic of China). All diet used met the National Research Council (NRC) nutrient specifications. The rats were fed a standard granulated feed and allowed to drink purified water ad libitum. Before the experiments, all the rats were fed with different dosage of probucol or vehicle through gastric gavage for 5 days. The night before an experiment, the rats were fasted but allowed to drink water ad libitum.

For each set of experiments, rats were divided into five groups: sham-operated ($n = 10$); animal model ($n = 10$); low-dose probucol (4 mg/kg) ($n = 10$); medium-dose probucol (8 mg/kg) ($n = 10$); and high-dose probucol (16 mg/kg) ($n = 10$). In the sham-operated group, the procedure used was the same as with the other groups, but CA was not triggered. Unlike the three treatment groups (low-, medium-, and high-dose probucol), the rats in the CA model group were not treated with probucol.

2.3. Materials and Equipment. Probucol (0.125 g tablets) was obtained from Qilu Pharmaceutical Co. Ltd. (Shandong Sheng, People's Republic of China). Before feeding, probucol was suspended in 2 ml of 0.5% carboxymethyl cellulose sodium salt (CMC-Na) solution. The assay kits for MDA, catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH), and superoxide dismutase (SOD) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, People's Republic of China). The rabbit anti-murine KEAP1 (primary antibody), the rabbit anti-murine NFE2L2 (primary antibody) antibodies, and the rabbit two-step kit (secondary antibody) were purchased from Bioss (Beijing, People's Republic of China). Other reagents used had a known high standard of purity (analytical grade). The TKR-200C small animal ventilator was provided by Beijing Yatai Kelong Instrument Technology Co. Ltd. (Beijing, People's Republic of China). An automatic biochemistry analyzer was obtained from Shenzhen iCubio Biomedical Technology Co. Ltd. (Shenzhen, People's Republic of China), and the BL-420F Data Acquisition & Analysis System was obtained from Chengdu Techman Software Co. Ltd. (Chengdu, People's Republic of China).

2.4. Methods

2.4.1. Animal Model of Cardiac Arrest (CA). The animal model of CA was established with electrical stimulation of the esophagus. In brief, each rat was anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg). Then, the rat was placed on a small animal ventilator (tidal volume: 7.5 ml/kg; breathing rate: 80 breaths/min) and underwent a tracheotomy. A catheter was inserted into the right femoral artery to monitor blood pressure, and a probe was inserted into each of the upper limbs to record the standard lead II electrocardiogram. Once the typical left ventricular pressure waveform appeared, a bipolar pacing electrode lead was inserted into the rat's mouth and then the esophagus. The rat rested for 10 min; this rest period was followed by electrical stimulation of the esophagus (frequency: 50 Hz; pulse width: 15 ms; amplitude: 6 mA; duration: 120 s). CA lasted for 5 min, and CPR was initiated when the systolic arterial pressure dropped to 20 mmHg. During CPR, cardiac compression was performed, and pure oxygen was given. If a spontaneous cardiac rhythm was not restored within 1 min, epinephrine was injected. ROSC was defined as the restoration of a spontaneous cardiac rhythm, the presence of a supraventricular rhythm, and a mean arterial pressure (MAP) ≥ 60 mmHg persisting for 10 min. If ROSC was not achieved after 10 min of CPR, the rat was considered dead and was excluded from the study. The rate of ROSC was calculated as follows:

$$\text{ROSC rate } (\%) = \frac{n}{N} \times 100\%, \quad (1)$$

where n = the number of rats with ROSC and N = the total number of rats with CA.

2.4.2. Determination of Biochemistry Parameters. All rats were sacrificed 12 hours after ROSC. The serum samples were collected from right ventricle blood immediately after animal death. Then, the rat hearts were harvested, and the left ventricular myocardial tissue was collected. For each sample, half of the myocardial tissue was homogenized and filtered to collect fluid for biochemistry assay following manufacturer's protocols. The remainder of the tissue was fixed in 4% paraformaldehyde and used for immunohistochemistry analysis.

Ten [10] of random serum and myocardial tissue extract samples from each experimental group were used to measure a panel of major antioxidant enzymes including catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) for the antioxidant actions of probucol. We also measured the malondialdehyde (MDA) levels. All the biochemistry assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, People's Republic of China).

2.4.3. Hemodynamic Monitoring. Twelve hours after ROSC, hemodynamic monitoring for heart rate (HR), MAP, maximal rate of rising of left ventricular pressure ($+dP/dt_{max}$), and maximal rate of fall of left ventricle pressure ($-dP/dt_{max}$) was conducted.

2.4.4. Myocardial Function Monitoring. Twelve hours after ROSC, the following parameters were measured with ultrasonography: left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular ejection fraction (LVEF), and short-axis shortening.

2.4.5. Immunohistochemistry Assay of KEAP1 and NFE2L2 Expression. Streptavidin-biotin-peroxidase immunohistochemistry was used to assay KEAP1 and NFE2L2; immunoreactive cells were defined as those with light brown or brown granules present in the cytoplasm. Immunoreactive cells were counted in five visual fields (×200 magnification) randomly selected in each slide. The staining intensity of slides was determined as follows: 0—immunoreactive cell count ≤ 10%; 1 point—immunoreactive cell count = 11–30%; 2 points—immunoreactive cell count = 31–50%; 3 points—immunoreactive cell count = 51–70%; and 4 points—immunoreactive cell count > 71%. The staining intensity of immunoreactive cells was determined as follows: 0—same color as the background or no stain; 1 point—light brown; 2 points—pale brown; and 3 points—brown.

2.4.6. Western Blot. Experimental rats were sacrificed 12 hours after ROSC. Then, the rat hearts were harvested and the left ventricular myocardial tissue was collected, homogenized, and lysed with lysis buffer (Cell Signaling Technology, Boston, MA, USA) containing 0.5 mM of phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA). Protein concentration was determined by BCA protein assay (Thermo Fisher Scientific, Grand Island, NY, USA). Samples of 25 μg protein were fractionated by SDS-PAGE in 4–20% gradient Tris-glycine precast gels (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was incubated for 1 hour in blocking solution containing 5% nonfat milk powder and 0.1% Tween-20, pH 7.6. This was followed by an overnight incubation at 4°C in blocking solution containing rabbit primary antibodies against KEAP1 (D6B12, Cell Signaling Technology, Boston, MA, USA). Subsequently, the labeled proteins were visualized by incubation with a horseradish peroxidase- (HRP-) conjugated anti-goat or rabbit IgG (1 : 2000; Santa Cruz Biotechnology) followed by development with a chemiluminescence substrate for HRP (Thermo Fisher Scientific). The images of Western blots were captured by GE imageQuant.

2.5. Data Analysis. Measurement data are expressed as mean ± standard deviation ($\overline{x} \pm s$); enumeration data are expressed as percentages. All data were analyzed using SPSS 20.0 software (IBM Corporation, Armonk, New York, USA). Differences among groups were analyzed with a one-way ANOVA; differences between groups were analyzed with a t-test. Enumeration data were analyzed with a Chi-square test. $P < 0.05$ was deemed statistically significant.

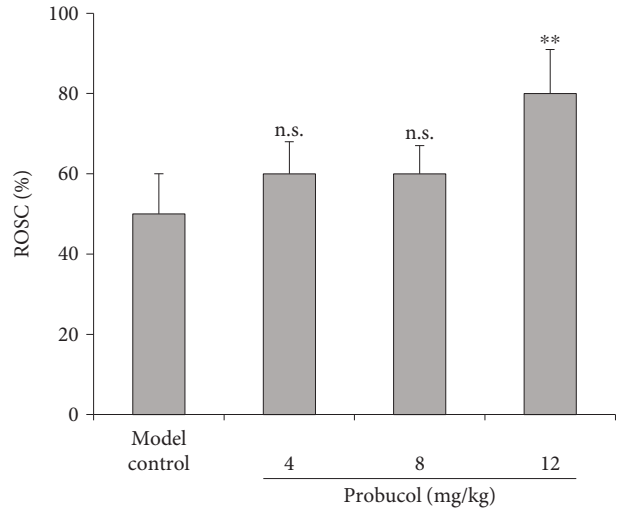

Figure 1: Effect of different dosage of probucol on the return of spontaneous circulation (ROSC) rate in rats after induced cardiac arrest (CA) and cardiopulmonary resuscitation (CPR). Data are from three sets of experiments and expressed in relative percentage ROSC rate and as mean ± SD. $^{**}P < 0.001$. n.s.: not significant.

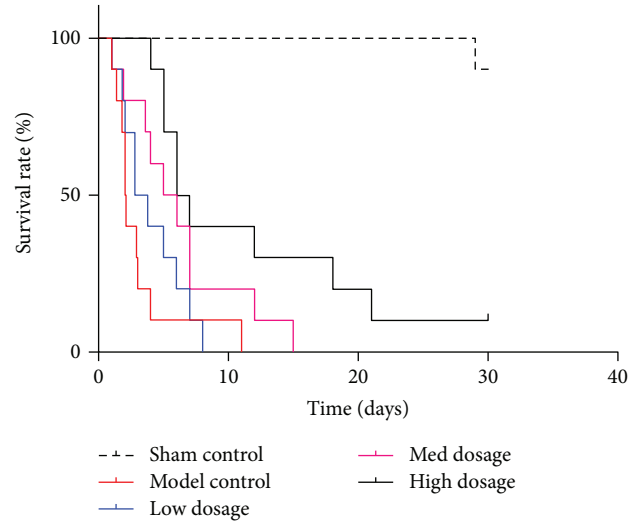

Figure 2: Effect of different dosage of probucol on survival time following ROSC of CA model-induced rats. Data use death as an end point and are expressed as Kaplan–Meier plot with the time (days) against relative percentage survival rate.

3. Results

3.1. Effect of Probucol on ROSC Rate. The average of success rate of CA was 80% (40/50). As shown in Figure 1, ROSC rates after CPR in CA-induced rats were 60%, 60%, and 80% in the three probucol treatment groups. In the CA model group without probucol treatment, the ROSC rate was 50%.

Table 1: Effect of probucol on serum MDA and antioxidant enzyme activities 12 hours after ROSC ($\overline{x} \pm s$; $n = 10$).

Group	MDA (mmol/l)	CAT (U/l)	GPx (U/l)	GSH (U/l)	SOD (U/l)
Sham operated	1.16 ± 0.12	2.32 ± 0.76	1.62 ± 0.47	1.47 ± 0.45	1.80 ± 0.43
CA model	$3.07 \pm 0.67^{**}$	$0.97 \pm 0.32^{**}$	$0.75 \pm 0.23^{**}$	$0.65 \pm 0.10^{**}$	$0.75 \pm 0.21^{**}$
CA + low dose	2.98 ± 0.93	1.04 ± 0.43	$1.11 \pm 0.61^{\dagger}$	0.83 ± 0.25	$1.03 \pm 0.31^{\dagger}$
CA + medium dose	$1.94 \pm 0.45^{\ddagger\S}$	$1.77 \pm 0.55^{\ddagger\S}$	$1.42 \pm 0.40^{\ddagger}$	$1.04 \pm 0.52^{\dagger\S}$	$1.33 \pm 0.53^{\dagger}$
CA + high dose	$1.45 \pm 0.46^{\ddagger\P\dagger\dagger}$	$2.02 \pm 0.74^{\ddagger\P\dagger\dagger}$	$1.50 \pm 0.35^{\ddagger\S}$	$1.28 \pm 0.40^{\ddagger\S}$	$1.49 \pm 0.35^{\ddagger\S}$

$^{**}P < 0.01$ versus the sham-operated group; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.01$ versus the animal CA model group; $^{\S}P < 0.05$ and $^{\P}P < 0.01$ versus the low-dose group; $^{\dagger\dagger}P < 0.05$ versus the medium-dose group. CAT: catalase; GPx: glutathione peroxidase; GSH: glutathione; MAD: malondialdehyde; ROSC: return of spontaneous circulation; SOD: superoxide dismutase.

Table 2: Effect of probucol on myocardial MDA and antioxidant enzyme activities 12 hour after ROSC ($\overline{x} \pm s$; $n = 10$).

Group	MDA (mmol/l)	CAT (U/l)	GPx (U/l)	GSH (U/l)	SOD (U/l)
Sham operated	3.18 ± 0.33	3.75 ± 0.82	26.06 ± 3.47	23.53 ± 2.54	1.94 ± 0.61
CA model	$5.55 \pm 1.07^{**}$	$1.82 \pm 0.40^{**}$	$15.76 \pm 3.02^{**}$	$13.65 \pm 2.11^{**}$	$0.84 \pm 0.34^{**}$
CA + low dose	4.80 ± 0.76	1.99 ± 0.34	$19.56 \pm 2.64^{\dagger}$	$17.43 \pm 2.91^{\dagger}$	$1.21 \pm 0.42^{\dagger}$
CA + medium dose	$3.43 \pm 0.83^{\dagger\S}$	$2.54 \pm 0.82^{\ddagger\S}$	$19.34 \pm 2.79^{\ddagger}$	$19.98 \pm 3.02^{\dagger\S}$	$1.47 \pm 0.60^{\ddagger}$
CA + high dose	$3.29 \pm 0.46^{\ddagger\P}$	$3.21 \pm 0.75^{\ddagger\P\dagger\dagger}$	$22.00 \pm 2.35^{\ddagger\S\dagger\dagger}$	$19.28 \pm 1.45^{\ddagger\S}$	$1.71 \pm 0.63^{\ddagger\S}$

$^{**}P < 0.01$ versus the sham-operated group; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.01$ versus the animal model group; $^{\S}P < 0.05$ and $^{\P}P < 0.01$ versus the low-dose group; $^{\dagger\dagger}P < 0.05$ versus the medium-dose group. CAT: catalase; GPx: glutathione peroxidase; GSH: glutathione; MAD: malondialdehyde; ROSC: return of spontaneous circulation; SOD: superoxide dismutase.

Table 3: Effect of probucol on hemodynamic parameters 12 hours after ROSC ($\overline{x} \pm s$; $n = 10$).

Group	HR/min	MAP (mmHg)	$+dP/dt_{max}$	$-dP/dt_{max}$
Sham operated	420.31 ± 17.41	134.44 ± 4.91	6145.63 ± 348.09	4903.41 ± 278.45
CA model	$336.63 \pm 20.31^{**}$	$94.62 \pm 7.64^{**}$	$3533.01 \pm 135.81^{**}$	$2698.25 \pm 199.32^{**}$
CA + low dose	$391.34 \pm 18.76^{\ddagger}$	103.71 ± 4.92	$3865.41 \pm 219.76^{\dagger}$	$2937.49 \pm 301.76^{\dagger}$
CA + medium dose	$394.20 \pm 17.76^{\ddagger}$	105.29 ± 6.29	$3904.03 \pm 178.62^{\ddagger\S}$	$3231.01 \pm 210.93^{\ddagger\S}$
CA + high dose	$408.54 \pm 16.29^{\ddagger}$	$114.94 \pm 7.09^{\ddagger\S\dagger\dagger}$	$4376.29 \pm 185.73^{\ddagger\P\ddagger\ddagger}$	$3813.13 \pm 108.82^{\ddagger\P\ddagger\ddagger}$

$^{**}P < 0.01$ versus the sham-operated group; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.01$ versus the animal model group; $^{\S}P < 0.05$ and $^{\P}P < 0.01$ versus the low-dose group; $^{\dagger\dagger}P < 0.05$ and $^{\ddagger\ddagger}P < 0.01$ versus the medium-dose group. $+dP/dt_{max}$: maximal rate of rise of left ventricular pressure; $-dP/dt_{max}$: maximal rate of fall of left ventricular pressure HR: heart rate; MAP: mean arterial pressure; ROSC: return of spontaneous circulation.

The ROSC rate differed significantly between the high-dose group and the animal model group ($P = 0.001$).

3.2. Effect of Probucol on Short-Term Survival Rate. As shown in Figure 2, the median survival time differed significantly between the probucol treatment groups (3.3, 5.5, and 6.5 d, resp.) and the animal model group (2.1 d) ($P = 0.01$ with one-way ANOVA). Survival was significantly more prolonged in the high-dose group than in the other two treatment groups.

3.3. Effect of Probucol on Plasma MDA Level and Antioxidant Enzymes Post-CA ROSC

3.3.1. Effect of Probucol on Systemic Oxidative Parameters. Twelve hours after ROSC, compared with the sham-operated group, the animal model groups displayed significant increase in serum MDA levels ($P < 0.01$) but significant decreases in the activity of antioxidant enzymes: CAT, GPx, GSH, and SOD ($P < 0.01$) (Table 1). Compared to the untreated animal CA model group, the probucol treatment group, especially the medium- and high-dose groups, displayed significant decrease in serum MDA levels ($P < 0.01$). In contrast, compared to the untreated CA model group, the probucol treatment groups displayed significant increases in the activity of CAT, GPx, GSH, and SOD ($P < 0.05$ or $P < 0.01$). These changes followed a dose-dependent pattern across the probucol treatment groups, that is, some parameters differed significantly between the medium- and high-dose and low-dose groups ($P < 0.05$ or $P < 0.01$) and between the high-dose and medium-dose groups ($P < 0.05$).

3.3.2. Effect of Probucol on Myocardial Oxidative Stress Parameters. Twelve hours after ROSC, compared with the sham-operated group, the animal model groups displayed significant increases in myocardial MDA levels

Table 4: Effect of probucol on myocardial function 12 hours after ROSC ($\overline{x} \pm s$; $n = 10$).

Group	LVESD/mm	LVEDD/mm	LVEF (%)	Short-axis shortening (%)
Sham operated	3.31 ± 0.31	4.57 ± 0.54	83.23 ± 3.80	50.54 ± 3.14
CA model	$5.27 \pm 1.01^{**}$	$7.98 \pm 0.64^{**}$	$62.78 \pm 4.30^{**}$	$36.25 \pm 2.42^{**}$
CA + low dose	5.02 ± 0.78	$6.76 \pm 0.13^{\dagger}$	65.74 ± 4.21	37.59 ± 2.51
CA + medium dose	$4.50 \pm 0.21^{\dagger}$	$5.89 \pm 0.59^{\ddagger}$	$68.43 \pm 2.96^{\dagger}$	$41.45 \pm 3.49^{\dagger\S}$
CA + high dose	$3.27 \pm 0.56^{\ddagger\P\dagger\dagger}$	$4.94 \pm 0.70^{\ddagger\S\dagger\dagger}$	$73.29 \pm 2.08^{\ddagger\P\dagger\dagger}$	$48.81 \pm 3.45^{\ddagger\P\dagger\dagger}$

$^{**}P < 0.01$ versus the sham-operated group; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.01$ versus the animal model group; $^{\S}P < 0.05$ and $^{\P}P < 0.01$ versus the low-dose group; $^{\dagger\dagger}P < 0.05$ versus the medium-dose group. LVEDD: left ventricular end-diastolic diameter; LVEF: left ventricular ejection fraction; LVESD: left ventricular end-systolic diameter; ROSC: return of spontaneous circulation.

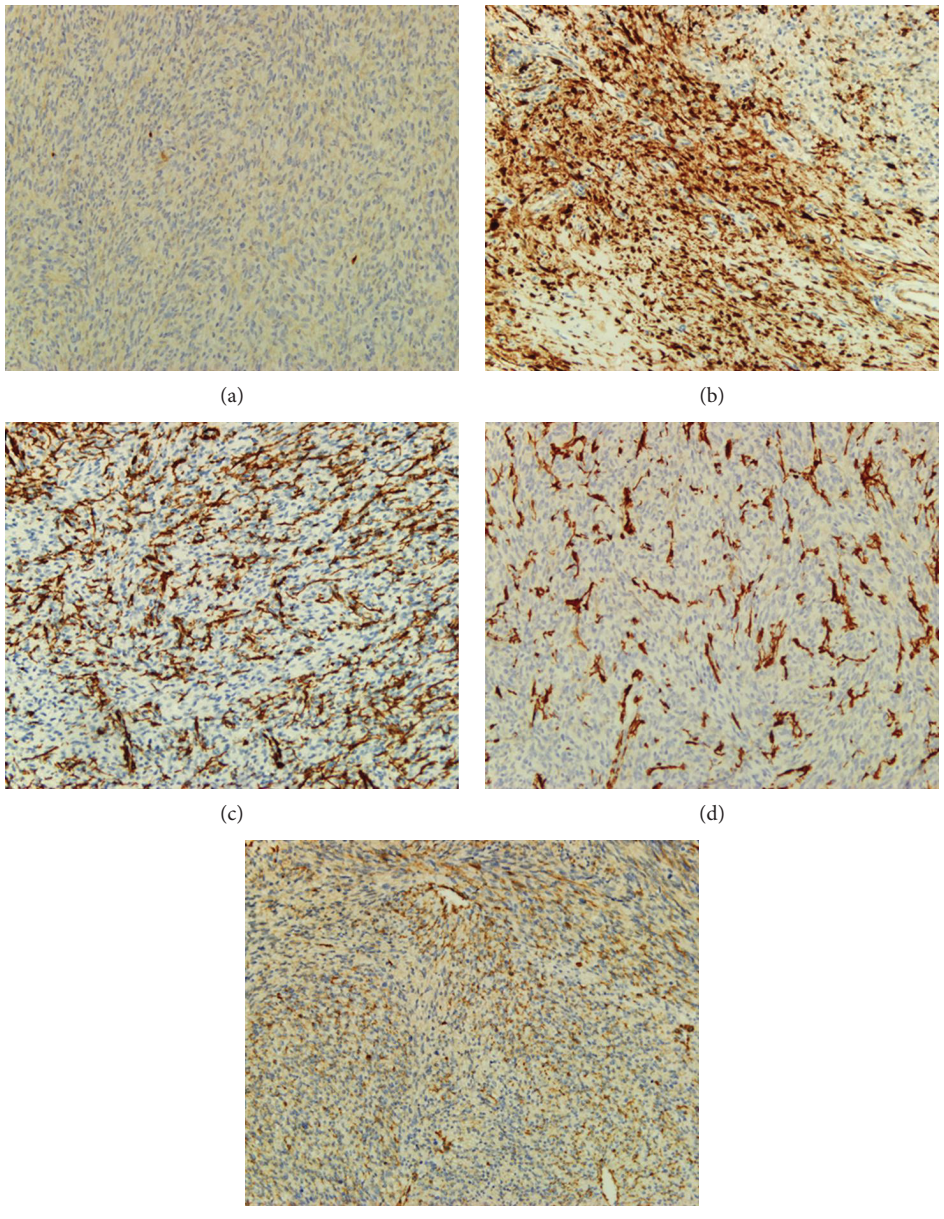

Figure 3: Effect of different dosage of probucol on myocardial KEAP1 expression in CPR model rats. Representative pictures of immunohistochemistry with anti-KEAP1 as shown as: (a) sham-operated group, (b) animal model group, (c, d, e) low-, medium-, and high-dose probucol groups, respectively. CPR = cardiopulmonary resuscitation; KEAP1 = Kelch-like ECH-associated protein 1.

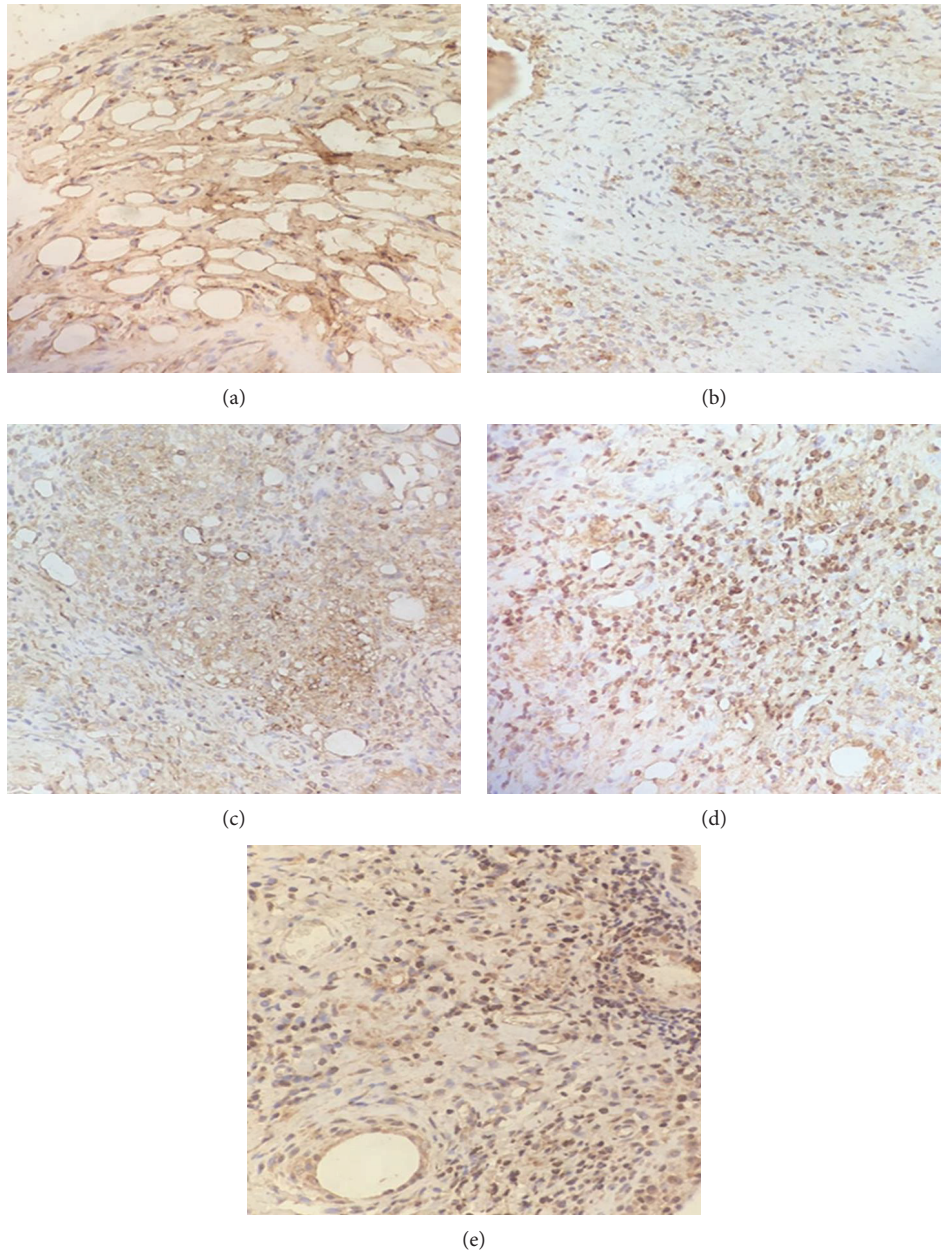

Figure 4: Effect of different dosage of probucol on myocardial KEAP1 and NFE2L2 expression in CPR model rats. Representative pictures of immunohistochemistry with anti-KEAP1 as shown as: (a) sham-operated group, (b) animal model group, (c, d, e) low-, medium-, and high-dose probucol groups, respectively. CPR = cardiopulmonary resuscitation; NFE2L2 = nuclear factor, erythroid-derived 2-like 2.

and significant decreases in the myocardial activity of CAT, GPx, GSH, and SOD ($P < 0.01$) (Table 2). Among the animal model groups, myocardial MDA levels decreased significantly, but the myocardial activity of CAT, GPx, GSH, and SOD increased significantly ($P < 0.05$ or $P < 0.01$) in the probucol treatment groups. These changes followed a dose-dependent pattern across the probucol treatment groups.

3.4. Effect of Probucol on Hemodynamics. The hemodynamic parameters at baseline did not differ significantly between the study groups before the CA model was established. Twelve hours after ROSC, compared with the sham-operated group, the animal model groups displayed significant decreases in HR, MAP, $+dP/dt_{max}$, and $-dP/dt_{max}$ ($P < 0.01$) (Table 3). Compared to the untreated CA model group, the probucol treatment groups displayed significant improvements in HR, MAP, $+dP/dt_{max}$, and $-dP/dt_{max}$. These indicators showed an upward trend, and the difference was statistically significant ($P < 0.05$ or $P < 0.01$). In particular, $+dP/dt_{max}$ and $-dP/dt_{max}$ changed more significantly and in a dose-dependent manner across the treatment groups ($P < 0.05$ or $P < 0.01$).

Table 5: Effect of probucol on myocardial KEAP1 and NRF2 expression 12 h after ROSC ($\bar{x} \pm s$; $n = 10$).

Group	Protein expression level	
	KEAP1	NFE2L2
Sham operated	2.18 ± 0.67	5.67 ± 0.09
CA model	$11.53 \pm 2.48^{**}$	$4.28 \pm 0.11^{**}$
CA + low dose	$7.36 \pm 1.83^{\ddagger}$	$4.46 \pm 0.10^{\dagger}$
CA + medium dose	$7.03 \pm 1.38^{\ddagger}$	$5.13 \pm 0.09^{\ddagger}$
CA + high dose	$5.43 \pm 1.44^{\ddagger\P\dagger\dagger}$	$5.35 \pm 0.16^{\ddagger\$}$

$^{**}P < 0.01$ versus the sham-operated group; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.01$ versus the CA model group; $^{\$}P < 0.05$ and $^{\P}P < 0.01$ versus the low-dose group; $^{\dagger\dagger}P < 0.01$ versus the medium-dose group. KEAP1: Kelch-like ECH-associated protein 1; NFE2L2: nuclear factor, erythroid-derived 2-like 2; ROSC: return of spontaneous circulation.

3.5. Effect of Probucol on Myocardial Function. Twelve hours after ROSC, compared with the sham-operated group, the animal model groups displayed significant increases in LVESD and LVEDD ($P < 0.01$) and significant decreases in LVEF and short-axis shortening ($P < 0.01$) in the animal model group (Table 4). Compared to the untreated CA model group, the high-dose group displayed significant decreases in LVESD and LVEDD ($P < 0.05$ or $P < 0.01$) and significant increases in LVEF and short-axis shortening ($P < 0.05$ or $P < 0.01$). In addition, these parameters changed in a dose-dependent manner across the probucol treatment groups.

3.6. Effect of Probucol on the Myocardial Expression of KEAP1 and NFE2L2. As shown in Figures 3 and 4, KEAP1 and NFE2L2 protein expression tended to show an opposite trend in each group. Compared to the sham-operated group, the KEAP1 expression was upregulated significantly; but NFE2L2 expression was downregulated significantly in the animal model groups ($P < 0.01$) (Table 5). Compared to untreated CA model group, KEAP1 expression was downregulated significantly, but NFE2L2 expression was upregulated significantly in the probucol treatment groups ($P < 0.05$ or $P < 0.01$) and more significantly so in the high-dose group ($P < 0.01$). Such an effect followed a dose-dependent pattern.

To confirm on Table 5, we performed Western blotting analysis using myocardial tissue lysates from the experimental rats. Figure 5 showed that comparing to the sham control (lane b), the CA rat model myocardial tissue contains relatively much more KEAP1 protein level (lane c). The treatment with probucol has dose-dependent reduction of the KEAP1 level 12 hours after ROSC (lanes d–f). Furthermore, the KEAP1 activity is primarily in the cytosolic fraction of the myocardial tissue (lanes g and h). These data are in agreement with the immunohistochemistry results.

4. Discussion

Cardia arrest (CA) is a serious threat to health and a major social and economic burdens. With continuous improvement in CPR techniques, ROSC rates continue to increase; nevertheless, the overall post-ROSC mortality rate remains at around 70% [10]. In the clinical setting, it is difficult to increase the postresuscitation survival rate and improve the long-term prognosis of patients affected by CA. CA usually results from malignant arrhythmias secondary to heart disease [11]. Therefore, early control of arrhythmias, especially ventricular fibrillation, is critical to the management of CA. Postcardiac arrest syndrome (PCAS) describes the spectrum of organ dysfunction following ROSC in patients suffering CA; it is the most dangerous consequence of CA. PCAS involves important pathological processes, such as oxidative stress, cardiac dysfunction, hemodynamic changes, and reperfusion injury; these processes are closely associated with the low survival rate after ROSC [12–14]. In clinical settings, epinephrine is the most potent vasoconstrictor agent, and it can increase mean arterial pressure (MAP), promote blood redistribution, and maintain blood supply to vital organs during CPR [15]. Epinephrine has been shown to increase the success rate of CPR significantly, although it has an adverse effect on microcirculation perfusion and the long-term survival rate of patients [16]. Hence, finding drugs that alleviate disturbances in microcirculation after ROSC and improve patient prognosis is key to the prevention and management of PCAS. Here, we tested probucol, an effective lipid-lowering agent that possesses potential antioxidative action and may help the body fight against high levels of reactive oxygen species [17].

In the present study, we established a rat model of CA by electrically stimulating the esophagus. This model was able to achieve more than 80% of success rate in rats. Among those CA-induced rats, the ROSC rate increased in all the probucol treatment groups, and it differed significantly between the high-dose group and the untreated CA model group. Previously, some antioxidative agents have been shown to alleviate post-ROSC injury significantly, although they failed to affect the ROSC rate significantly [18, 19]. Treatment with probucol was also thought to bring bigger benefits to CA patients [20]. The present study found that the survival time of rats after CPR was prolonged significantly in all the probucol treatment groups, compared to the untreated CA model group. This demonstrated that probucol provides a potential benefit in patient care especially following CPR treatment.

We also tested the systemic oxidative stress level by examination of serum malondialdehyde (MDA). When systemic oxidative stress level is high, it will generate more products of lipid peroxidation, including MDA. The MDA epitopes on LDL particles are now widely accepted as the measurement of systemic oxidative stress markers [21–24]. To test the antioxidant actions of probucol, we also measured a panel of major antioxidant enzymes including catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) [25–28] Due to their antioxidation functions, levels of these enzymes are usually inversely correlated with oxidative stress status of the host. In our results, compared to the sham-operated group, the levels of MDA increased significantly in both serum and myocardium of CA-induced rats, while the activity of CAT, GPx, GSH, and SOD decreased significantly in these CA-induced rats, suggesting oxidative stress following CA induction and ROSC, as

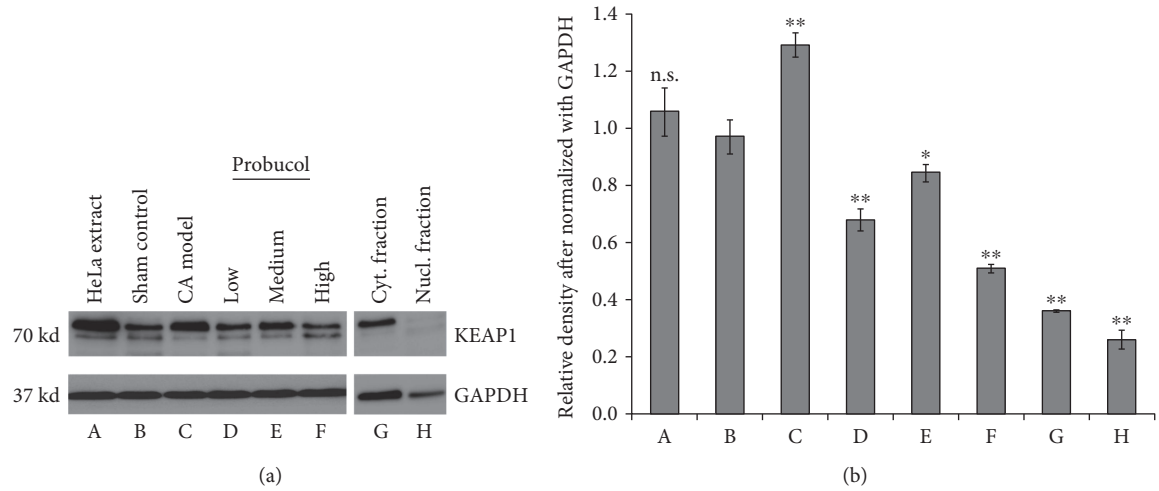

Figure 5: (a) Western blot analysis using anti-KEAP1 reveals that KEAP1 protein expression is elevated in myocardial tissue of CA model rat heart (C) comparing to sham control rat heart (B). The effect of probucol moderated the KEAP1 level induced by CA in a dose-dependent manner (D–F). Lane (A) shows KEAP1 by Western blotting with HeLa cell extract. KEAP1 protein is also analyzed in cytosolic (G) and nucleic (H) fractions of myocardial tissues of sham control to show the subcellular localization. (b). Relative density of the KEAP1 protein detected by Western blotting after normalizing with GAPDH. Data are expressed as mean ± SD with three measurements. One-way ANOVA was used to analyze the data against sham control. $^{*}P < 0.05$; $^{**}P < 0.001$; n.s.: not significant.

described in the literature [29] We also found that with the administration of probucol, the serum and myocardial MDA levels were reduced significantly comparing to the untreated CA model rats. At meantime, the activity of antioxidant enzymes CAT, GPx, GSH, and SOD increased significantly in the probucol treatment groups. These changes in oxidative stress parameters were in a dose-dependent manner, suggesting that probucol treatment significantly counteracted oxidative stress following ROSC. In addition, the results of this study showed that probucol treatment significantly improved HR, MAP, $+dP/dt_{max}$, and $-dP/dt_{max}$ 12 hours after ROSC. Also, LVESD and LVEDD decreased significantly, and LVEF and short-axis shortening increased significantly in the high-dose group, suggesting that probucol significantly improved hemodynamic parameters and heart function after ROSC. Taken together, the results suggested that probucol treatment significantly relieved CA-induced oxidative stress and ameliorated hemodynamic parameters and heart function after ROSC in CA-affected rats. This may be associated with the increase in ROSC rate and the prolongation of survival time in CA-affected rats after CPR.

NFE2L2 is an important transcription factor, and KEAP1 is an upstream regulator of NFE2L2. In organisms, the KEAP1-NFE2L2 system is closely associated with the antioxidant response [30]. Physiologically, NFE2L2 and KEAP1 form inactive complexes. Under oxidative stress, NFE2L2 becomes dissociated from KEAP1 and migrates into the cytonucleus and becomes an active antioxidative factor [31, 32]. The findings of this study indicated that KEAP1 expression was downregulated significantly and NFE2L2 expression was upregulated significantly, in the probucol treatment groups compared to the animal model group ($P < 0.05$ or $P < 0.01$), and especially so in the high-dose group ($P < 0.01$); such an effect followed a dose-dependent pattern. Probucol may have also regulated the expression of the KEAP1-NFE2L2 system, thereby relieving oxidative stress in CA-affected rats after CPR. There are many reports that previously analyzed the influence of promoter activity of downstream genes including HO-1 and NQO-1 during NFE2L2 activation [33–36]. We notice that further study of these downstream genes activities in our experimental setting would provide more mechanistic insights for the protective action of probucol treatment. We will explore these experiments in our future study to add strength to the notion that protection of probucol on CA occurs via KEAP1-NFE2L2 pathway system.

5. Conclusion

Treatment with probucol significantly increased ROSC rate and survival time. It also relieved CA-induced oxidative stress and ameliorated hemodynamic parameters and heart function in a dose-dependent manner in rats after CPR, possibly by regulating the expression of the KEAP1-NFE2L2 system.

Conflicts of Interest

The authors have no conflict of interest.

Acknowledgments

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Review Article

The Role of Nrf2 in Cardiovascular Function and Disease

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Free radicals, reactive oxygen/nitrogen species (ROS/RNS), hydrogen sulphide, and hydrogen peroxide play an important role in both intracellular and intercellular signaling; however, their production and quenching need to be closely regulated to prevent cellular damage. An imbalance, due to exogenous sources of free radicals and chronic upregulation of endogenous production, contributes to many pathological conditions including cardiovascular disease and also more general processes involved in aging. Nuclear factor erythroid 2-like 2 (NFE2L2; commonly known as Nrf2) is a transcription factor that plays a major role in the dynamic regulation of a network of antioxidant and cytoprotective genes, through binding to and activating expression of promoters containing the antioxidant response element (ARE). Nrf2 activity is regulated by many mechanisms, suggesting that tight control is necessary for normal cell function and both hypoactivation and hyperactivation of Nrf2 are indicated in playing a role in different aspects of cardiovascular disease. Targeted activation of Nrf2 or downstream genes may prove to be a useful avenue in developing therapeutics to reduce the impact of cardiovascular disease. We will review the current status of Nrf2 and related signaling in cardiovascular disease and its relevance to current and potential treatment strategies.

1. Introduction

The vascular endothelium modulates vascular structure, thrombolysis, vasoconstriction, and vasodilation and maintains internal homeostasis through synthesizing and releasing several active biomolecules [1]. A loss of function of the endothelium represents a key risk factor for cardiovascular disease (CVD) and initiates the development of atherosclerosis [2]. Endothelial dysfunction is associated with functional changes that diminish nitric oxide (NO) bioavailability and consequently leads to CVD [1]. Sustained failure to counteract the excessive production of reactive oxygen species (ROS) and dysregulation of the antioxidant defence system in the endothelium elicits cellular damage and dysfunction [2]. However, the original concept that all free radicals are damaging disease-causing entities have, over recent years, been replaced by an understanding of the important signaling role they play within and between cells. The production and control of free radicals need to be tightly regulated to prevent cytotoxicity, and the imbalance, caused by exogenous sources of free radicals with chronic upregulation and endogenous production, contributes to many pathological conditions and also to more general processes involved in aging [3–5]. There are multiple cellular defence strategies to prevent free radical toxicity, which are dynamically regulated to protect from oxidative insults and preserve cell function [6]. Nuclear factor erythroid 2-like 2 (NFE2L2; commonly known as Nrf2 [7]) has been identified as a major regulator of the oxidant/antioxidant balance.

The Nrf2 was first discovered in 1994 by Moi et al. during studies on regulation of the β-globin gene [7]. It was subsequently identified to be profoundly involved in the regulation of oxidant and antioxidant gene expression, through binding to the antioxidant response element (ARE) [8, 9]. Nrf2/ARE signaling is highly conserved in all species and controls a wide panel of genes that include cytoprotective and detoxifying phase II enzymes [10]. Nrf2 coordinates the cellular response to oxidative insults, preventing damage to cellular

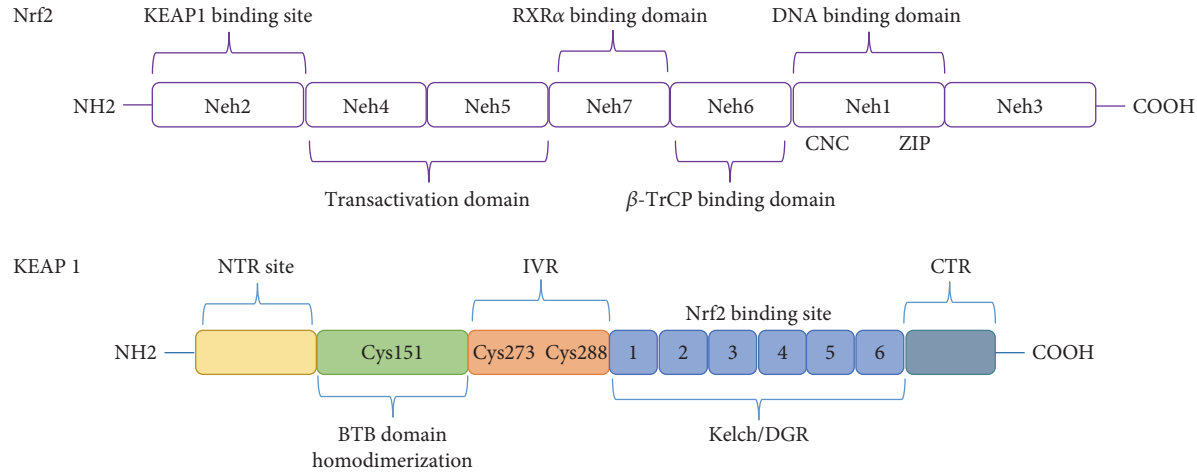

Figure 1: Nrf2 and KEAP1 structure. Nrf2 is a cap'n'collar-basic region leucine zipper (CNC-bZIP), and its human sequence contains 605 amino acids, divided into seven domains: Neh1 to Neh7. Neh1 contains a CNC-bZIP motif, allowing heterodimerization with Maf proteins and DNA binding [54]. The Neh2 domain contains the Keap1 binding site (DLG and ETGE motifs), necessary for its cytoplasmic retention and degradation [55]. The Neh3 domain is fundamental for Nrf2 transcriptional activation through binding with chromo-ATPase/helicase DNA-binding protein 6 (CHD6) [56]. Neh4 and Neh5 provide an interaction site for nuclear cofactor RAC3/AIB1/SRC-3 [57] and CREB-binding protein (CBP) [58] which enhances the Nrf2/ARE activation pathways, partially by promoting acetylation of Nrf2 [59]. Additionally, Nrf2 possesses a redox-sensitive nuclear exporting signal within the Neh5 transactivation domain able to regulate its cellular localization [60]. The serine-rich Neh6 domain contains two motifs (DSGIS and DSAPGS) involved in the negative regulation of Nrf2. Glycogen synthase kinase 3 (GSK-3) phosphorylates serine residues within Neh6 enabling the interaction with the β-transducin repeat-containing protein (β-TrCP) which acts as a substrate receptor for Skp1–Cul1–Rbx1/Roc1 ubiquitin ligase complex, leading to KEAP1-independent degradation [41]. Neh7 domain interacts with retinoid X receptor alpha (RXRα), responsible for Nrf2/ARE signaling inhibition [61]. Human Kelch-like ECH-associated protein 1 (KEAP1) is a 69 kD protein, containing 27 cysteine residues. It is a substrate adaptor for cullin (Cul3) which contains E3 ubiquitin ligase (E3). KEAP1 is composed of five domains starting from the N-terminal region, a BTB dimerization domain (Broad-Complex, Tramtrack, and Bric-à-brac) which contains the Cys151 residue, a cysteine-rich intervening region (IVR) domain with two cysteine domain residues Cys273 and Cys288, critical for stress sensing. A Kelch domain/double glycine repeat (DGR) domain possessing 6 Kelch repeats and ending with a C-terminal region [62]. KEAP1 needs a domain capable to homodimerize and interact with Cul3, forming the Nrf2 inhibitor complex (iNrf2), and this is the BTB domain [63]. The Cys151 in the same domain plays an important role on Nrf2 activation in response to oxidative stress [64]. Furthermore, the IVR domain is highly sensitive to oxidation and contains three cysteines, 273, 288, and 297 which regulate Nrf2 activation and repression [16, 65]. The DGR domain acts as an Nrf2 repressor; it contains six repetitive Kelch structures that specifically bind to the Neh2 domain on Nrf2 [15].

components sensitive to redox changes (i.e., proteins, lipids, and DNA).

2. Regulation of Nrf2 Activity

Nrf2 activity is highly regulated, suggesting that either hypoactivation or hyperactivation of Nrf2 may be detrimental to the cell, for example, unrestricted Nrf2 activity, elicited by knockout of Kelch-like ECH-associated protein 1 (KEAP1) in the mouse, results in postnatal lethality [11], while Nrf2 knockouts are viable but hypersensitive to oxidative stressors. The regulation of Nrf2 has been extensively reviewed elsewhere [12–14] but is briefly summarized here and in Figure 1 and Table 1. *Nrf2*-regulated gene expression is primarily controlled by KEAP1. In a situation without oxidative stimuli, Nrf2 is mostly sequestered in the cytosol through binding to the Kelch domain of KEAP1 [15]. KEAP1 acts as an adapter molecule for CUL-E3 ligase and mediates the ubiquitination and degradation of Nrf2 protein. Exposure of oxidative/electrophilic stress causes a modification of the cysteine groups on KEAP1 (particularly C151), relaxing the structure of KEAP1 causing dissociation of KEAP1 from CUL-E3 ligase [16–18]. It is unclear if Nrf2 protein dissociates from KEAP1 or if modification of C151 simply blocks further processing of Nrf2 [18]. De novo synthesized Nrf2, or protein released from KEAP1, is then free to translocate to the nucleus. In addition, p21, p62, and the tumor suppressor WTX also potentiate Nrf2 activation through sequestration of KEAP1 or binding to Nrf2 to prevent association with KEAP1 [19–21]. Upon entry into the nucleus, Nrf2 heterodimerizes with a number of transcription factors, including small Maf proteins (allowing formation of full basic zipper, summarized in Figure 1 and Table 1), and binds to the ARE (core sequence RTGACnnnGCA) to induce gene transcription [22, 23].

Dissociation of KEAP1 from the CUL-E3 ligase complex can be induced by a large range of compounds, including oxidized phospholipids [24], nitric oxide (NO), zinc, alkenals [25], and cigarette smoke, or fresh aqueous extracts of cigarette smoke [26–28]. However, not all forms of ROS appear to be able to modify KEAP-1/Nrf2 interactions, with data suggesting this is both cell type and context specific. Of particular relevance to CVD, laminar shear stress causes the activation of Nrf2 in endothelial cells [29], through lipid peroxide and COX2-derived 15-deoxy-12,14-prostaglandin J2 (15d-PGJ2) intermediates, enhanced by phosphoinositol

Table 1: List of proteins that bind to and modulate the activity of Nrf2.

Gene	Function	Reference
KEAP1	Retention in cytoplasm and degradation	[15]
CDH1/CTNNB1	Enhances KEAP1 interaction	[66]
CRF1	Ubiquitination and degradation	[67]
ATF4	Activation of gene expression	[68]
BRG1	Selective activation of gene expression	[69]
CBP	Activation of gene expression	[58]
CHD6	Activation of gene expression	[56]
JUN	Activation of gene expression	[9]
MAFF	Heterodimer activates gene expression	[70]
MAFG	Heterodimer activates gene expression	[71]
MAFK	Heterodimer activates gene expression	[71]
PMF1	Activation of gene expression	[72]
RAC3/AIB1/SRC-3	Activation of gene expression	[57]
PKC	Phosphorylation increases nuclear translocation	[73, 74]
HDAC1/2/3	Repression of gene expression	[75]
MYC	Repression of gene expression	[76]
PPARG	Repression of gene expression	[77]
RXRα	Repression of gene expression	[61]
FYN	Phosphorylation and nuclear export	[52]
SRC	Phosphorylation and nuclear export	[53]
YES	Phosphorylation and nuclear export	[53]

3-kinase/Akt signaling, but is surprisingly independent of endothelial nitric oxide synthase (eNOS) activity [30–33]. In addition, laminar shear stress increases the nuclear localization of Nrf2 via a KLF2-dependent mechanism [34]. Finally, tumor necrosis factor alpha (TNF-α) increases the activation of Nrf2 in human endothelial cells [28] and monocytes [35]. A number of naturally occurring compounds have been shown to release Nrf2 from KEAP1 [36], for example, sulforaphane [37], sulfuretin [38], 2-trifluoromethyl-2-methoxychalone [39], and isoliquiritigenin [40], suggesting that dietary modulation of ARE-dependent gene expression could play a potential role in modulating disease.

3. Additional Regulatory Systems

In addition to KEAP1-mediated sequestration and degradation of Nrf2 within the cytoplasm, there are a number of additional layers of regulation on Nrf2-dependent gene expression. Degradation of Nrf2 can also be induced by β-TrCP–Skp1–Cul1–Rbx1 E3 ubiquitin ligase complex [41, 42], triggered by phosphorylation of Nrf2 within the Neh2 domain. Subsequently, the E3 ligase complex ubiquitinates Nrf2 and causes its destruction by the proteasome. Mitra et al. also observed that the inhibition of P38 mitogen-activated protein kinase (MAPK) highly decreased Nrf2 nuclear translocation, with a corresponding reduction of Nrf2-dependent gene expression [43]. While the majority of KEAP1 is normally present in the cytoplasm, 10–15% has localized to the nucleus [44]; prothymosin-alpha (ProTα) binds KEAP1, shuttling it into the nucleus, where it can bind Nrf2 and promote its degradation [45]. Within the nucleus, B-zip proteins BACH1 and BACH2 can form dimers with Maf proteins through their BTB domain and compete for binding to the ARE, preventing Nrf2 binding and activation of transcription [46–48]. BACH1 is universally expressed, while BACH2 expression is predominantly limited to monocytes and in neural cells. Phosphorylation of BACH1 on Y486 provokes nuclear export of BACH1 increasing Nrf2-dependent gene expression [49, 50]. Nuclear export of Nrf2 is controlled through a GSK-3β-controlled phosphorylation cascade. GSK-3β phosphorylates Src family kinases (Src, YES, and Fyn), in turn phosphorylating Nrf2 on Y568 triggering nuclear export and degradation [51–53].

4. Nrf2 and Mitochondrial Dynamics in Cardiovascular Disease

Cardiovascular disease is the main cause of death worldwide [78], and it covers a wide array of disorders. The most common causes of CVD morbidity and mortality are stroke, ischemic heart disease (IHD), and congestive heart failure (CHF). Several risk profiles are involved in CVD where ROS is a central mediator and a common denominator, upregulated by multiple risk factors such as diabetes, inflammation, and smoking [79–81]. ROS can cause EC apoptosis and activate nuclear factor kappa-B (NF-κB), increasing adhesion molecules and cytokines that enhance monocyte adhesion [82, 83]. Oxidative stress is involved in mitochondrial dysfunction, which is related to bioenergetic defects and an alteration in mitochondrial dynamics. This provokes

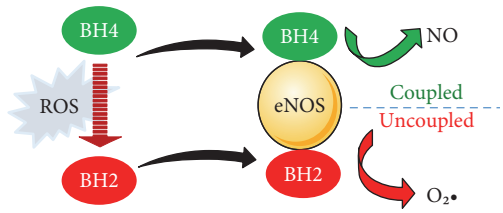

Figure 2: ROS-induced uncoupling of eNOS and the generation of O_2•. Excess ROS induce the conversion of BH4 to BH2 with subsequent eNOS uncoupling and synthesis of O_2• instead of NO. eNOS: endothelial nitric oxide synthase; ROS: reactive oxygen species; NO: nitric oxide; O_2•: superoxide; BH4: tetrahydrobiopterin; BH2: dihydrobiopterin.

transcription impairment and cell damage. Blockage of the mitochondrial electron transfer in complex III in diabetes leads to the release of electrons which reduce molecular oxygen to superoxide (O_2•) and increases intracellular ROS production [84]. Furthermore, ROS can activate membrane oxidases with a subsequent increase in the levels of asymmetric dimethylarginine that competes for the L-arginine transporters and active sites on eNOS [85]. Nrf2 modulates the activity of the mitochondrial respiration chain [86], with pharmacological activation of Nrf2 protecting against toxicity and maintaining mitochondrial homeostasis possibly via ablation of Akt2 signaling [87]. Liu and colleagues discovered acrolein, a component of cigarette combustion, inactivated the KEAP1/Nrf2 pathway, and decreased mitochondrial membrane potential [88], while Zou et al. demonstrated the ability of Nrf2 to prevent mitochondrial dysfunction, using hydroxytyrosol to activate Nrf2 [89].

5. Nrf2 in Endothelial Dysfunction

The vascular endothelium modulates vascular homeostasis through synthesizing and releasing several active biomolecules [1]. A loss of endothelium integrity represents a key risk factor for CVD, initiating atherosclerosis [2] and is associated with functional changes that diminish NO bioavailability and, consequently, lead to CVD [1]. Hypoxia, flow disturbances, and oxidative stress are important contributors to endothelial dysfunction [90]. Failure to counteract excessive production of ROS and modulation of the anti-oxidant defence system in the endothelium elicits cellular damage and dysfunction [2].

Normal vascular endothelial physiology is dependent on NO production via coupling of the eNOS heme group with L-arginine using tetrahydrobiopterin (BH4) as a cofactor [91]. Excess ROS induce the conversion of BH4 to 7,8-dihydrobiopterin (BH2) with subsequent eNOS uncoupling and synthesis of O_2• instead of NO [91] (Figure 2). O_2• can react with NO to produce the versatile oxidant peroxynitrite (ONOO−) [92]. The upregulation of iNOS and uncoupling of eNOS under hyperglycemic conditions are now well established [93, 94]. L-arginine is also a substrate for arginase [95] which is upregulated in the endothelium of coronary arterioles in hypertension and contributes to the impaired NO-mediated dilation [96]. In addition, ONOO− and hydrogen peroxide (H_2O_2) were reported to increase the activity/expression of arginase in endothelial cells [97], thus exacerbating the defects in myogenic tone. Therefore, ROS can trigger eNOS uncoupling through depletion of the substrate L-arginine. This notion has been supported by the study of Romero et al. [98] where increased arginase activity elicited L-arginine depletion and contributed to endothelial dysfunction in diabetes. ONOO− can also activate NADPH oxidases and influences further generation of ROS [99]. Additionally, blockage of the mitochondrial electron transfer in complex III in diabetes leads to the release of electrons, which reduce molecular oxygen to O_2• and increase intracellular ROS production [84]. Furthermore, ROS can activate membrane oxidases with a subsequent increase in the levels of asymmetric dimethylarginine that competes for the L-arginine transporters and active sites on eNOS [85].

Nrf2 in the endothelium can be activated via increased ROS production and PI3K-Akt signaling triggered by laminar shear stress [32]. In human arterial endothelial cells, Nrf2 activation resulted in increased intracellular HMOX1, GPx, GSH, GCLM, SRXN1, NQO1, PAR4, and OSGIN1 [27, 28, 100]. Adenoviral overexpression of Nrf2 in endothelial cells infected showed decreased expression of TNF-α, IL-1β, MCP1, and VCAM1, pointing to the anti-inflammatory potential of Nrf2 [28, 101]. When shear stress is disturbed at bifurcations, curved sections of arteries or distal to regions of stenosis, NO bioavailability decreases, O_2• generation increases [102], and Nrf2-activated genes are diminished, causing the endothelium to become predisposed to atherogenesis [103]. Our recent studies have demonstrated that free fatty acid- (FFA-) induced excessive ROS production diminished both the gene and protein expression of Nrf2, NQO1, and HO-1 in endothelial cells [104]. In addition, upregulation of Nrf2/ARE/HMOX1 signaling protected the human endothelial cells against TNF-α activation [105]. It could be that mitochondrial ROS may trigger a protective response via Nrf2 activation in endothelial cells. The study of Lo and Hannink [106] suggested that Nrf2–KEAP1 complex binds to the mitochondria through interaction with mitochondrial outer membrane protein PGAM5 and directly senses mitochondrial ROS production.

Another possibility through which Nrf2 can protect the endothelium against the cytotoxic ROS involves regulating the catalytic subunit of GCLC which reduces GSH biosynthesis [107]. In this context, impaired Nrf2–KEAP1–GCLC has been demonstrated in high glucose-induced retinal endothelial cells from diabetic donors [108]. In the human brain microvascular endothelial cells (HBVEC), GSH conferred protection against FFA-induced oxidative stress and apoptosis by activating the Akt pathway [109]. Human umbilical vein endothelial cells (HUVECs), human coronary artery endothelial cells (HCAECs), and endothelial progenitor cells exposed to cytotoxic ROS showed apoptosis and cell death accompanied by diminished nuclear localization and transcriptional activity of Nrf2 [2]. These findings highlight the crucial role of Nrf2 activation in protecting endothelial cells against oxidative stress-induced dysfunction.

6. Nrf2 in Atherosclerosis

Atherosclerosis is a focal inflammatory disease of the arterial system involving a number of different cell types. The focal nature of atherosclerosis highlights the involvement of local haemodynamics factors acting on the endothelium in the initiation and progression of atherosclerosis, which develops in regions that experience disturbed flow at bifurcations and curved sections of artery [110–113]. Straight sections of artery that experience normal laminar blood flow are relatively spared from disease through a coordinated modulation of gene expression, predominantly controlled by the transcription factors KLF2 and KLF4 and activation of Nrf2 [29, 32, 114–116]. By contrast, endothelial cells exposed to disturbed flow adopt a phenotype that amplifies endothelial dysfunction and increases permeability. While ROS are essential signaling molecules regulating vascular homeostasis, excessive ROS, elevated by many of the risk factors associated with the development of atherosclerosis, promote endothelial dysfunction and decrease NO availability. Thus, Nrf2-regulated antioxidant gene expression may play an atheroprotective role in endothelial cells.

Consistent with this hypothesis, the Nrf2-regulated gene, heme oxygenase 1 (HMOX1), demonstrates significant cytoprotective and anti-inflammatory effects that result in a reduction of atherosclerosis in mouse models [117], possibly through production of low levels of carbon monoxide. Hypercholesterolemic mice, deficient in both HMOX1 and ApoE ($HMOX1^{-/-}/ApoE^{-/-}$), demonstrated enhanced development of atherosclerosis compared to $ApoE^{-/-}$ single knockout mice [118]. HMOX1 expression in macrophages plays a protective role in atherosclerosis [119] with macrophages from $HMOX1^{-/-}$ mice displaying increased ROS generation, production of inflammatory cytokines, and increased foam cell formation when treated with oxLDL, attributable in part to increased expression of scavenger receptor A (SR-A). Smooth muscle cells from $HMOX1^{-/-}$ mice not only displayed increase neointimal formation but also enhanced cell death potentially due to greater susceptibility to oxidant stress [118]. Pharmacological modulation of HMOX1 expression also demonstrates a protective role of HMOX1 in atherogenesis [120, 121]. In addition to the anti-inflammatory effects of carbon monoxide, hydrogen sulphide also elicits an anti-inflammatory antiatherogenic effect [122]. Hydrogen sulfide activates the release of Nrf2 from KEAP1, increasing Nrf2-dependent gene expression [122].

Despite the antioxidant function of Nrf2 and the anti-atherogenic function of the key Nrf2 target gene HMOX1, the global knockout of Nrf2 ($Nrf2^{-/-}$) developed less rather than more atherosclerosis [123, 124]. Barajas et al. attributed this to an effect of Nrf2 in lipid metabolism, lowering plasma cholesterol and reducing foam cell formation [123], while Sussan et al. did not find a difference in serum cholesterol but attributed the effect to a reduction in scavenger receptor CD36 reducing foam cell formation [124]. The role of Nrf2 in NLRP3 inflammasome induction by cholesterol crystals within the atherosclerotic plaque may also be a contributing factor that explains the counterintuitive net detrimental effect of Nrf2 in hypercholesterolemic mouse models of atherosclerosis [125]. It might also explain why the expression of the Nrf2-regulated gene HMOX1 is highest in human plaques with the highest markers of plaque instability [121].

7. Nrf2 in Vascular Calcification

The presence of vascular calcification is often detected in atherosclerotic plaques and in patients with end-stage renal disease. Both of these pathologies have been targeted for prevention using pharmacological and genetic approaches by modulation of Nrf2 antioxidant pathways. For example, studies *in vitro* using rodent vascular smooth muscle cells show that dimethyfumarate or resveratrol could attenuate the deposition of a mineralised matrix and suggest protection against oxidative stress-induced mitochondrial damage, via activation of Nrf2 and SIRT1 signaling and downregulation of osteogenic transcription factors [126, 127]. In contrast, glucose-induced oxidative stress enhances the osteogenic differentiation and mineralisation of human embryonic stem (ES) cells, by the upregulation of runt-related transcription factor 2 (Runx2), Nrf2, and HMOX1, which was inhibited by Nrf2 knockdown [128] highlighting a context-specific regulation of the calcification process. Given the links between Nrf2 and bone homeostasis, it is not surprising to have an association between Nrf2 signaling and vascular calcification. Whether these initial *in vitro* studies can translate into the *in vivo* situation needs further study.

8. Nrf2 in Hypertension

Angiotensin II and associated renin-angiotensin system (RAS) are involved in the regulation of blood pressure, vasoconstriction, sodium intake, and potassium excretion [129]. Inappropriate activation of the RAS is the main cause of profound hypertension and cardiovascular morbidity. Angiotensin II increases the expression of NADPH oxidase and the generation of ROS potentially mediating some of the effects in renin-angiotensin-induced hypertension [130, 131]. It has been suggested that hypertension could be one of the causes of Nrf2 misregulation and not vice versa [132] through enhanced oxidative stress and vascular dysfunction in a hypertensive rat model [133]. This would suggest that the Nrf2 anti-oxidant defence system is insufficient to counteract the effects of oxidative stress, possibly due to elevated levels of Nrf2 repressors in hypertensive animals. Research is now moving from the adaptive and protective changes in the Nrf2 antioxidant response to focusing on the alternative mechanisms intrinsic to upstream and downstream molecules associated with a defective Nrf2 signaling system. Enhancing Nrf2 activity may have a therapeutic potential for a meliorating hypertension.

9. Nrf2 in Diabetic Cardiomyopathy

The heart is particularly vulnerable to oxidative damage compared to other organs, due to its low basal levels of antioxidant defences [134]. Diabetic cardiomyopathy (DCM) and other cardiovascular complications account for more than 80% of deaths among patients with diabetes [135].

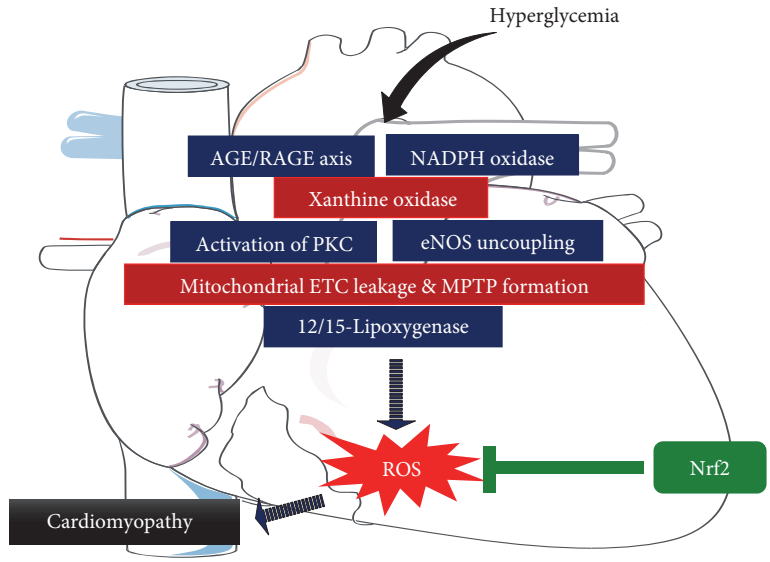

Figure 3: Hyperglycemia-induced ROS generation in the heart. A schematic model showing the potential pathways involved in cardiomyopathy and how Nrf2 could be targeted to reduce ROS and prevent the development of this pathology. AGEs: advanced glycation end products; NADPH: nicotinamide adenine dinucleotide phosphate; PKC: protein kinase C; eNOS: endothelial nitric oxide synthase; ETC: electron transport chain; MPTP: mitochondrial permeability transition pore.

DCM is characterized by impaired diastolic function, hypertrophy, apoptosis, and fibrosis of cardiomyocytes [136] and involves several mechanisms and pathogenic factors, with oxidative stress thought to be the common link [137–140]. Hyperglycemia generates excess ROS/RNS from activation of NADPH oxidases, PKC, leakage of the mitochondrial electron transport chain, eNOS uncoupling, AGE/RAGE signaling, xanthine oxidase, and 12/15-lipoxygenase (LOX) [141], impairing antioxidant defences in the diabetic heart [138, 140] (Figure 3).

Studies have established the importance of Nrf2/ARE signaling in the prevention of diabetic complications [142–144] and oxidative stress-induced cardiomyocyte injury [145, 146]. Significantly reduced Nrf2 expression has been observed in the left ventricle of diabetic patient heart by histological analysis [147], which has also been observed in a diabetic mouse model after 5 months [147]. These findings suggest adaptive overexpression of Nrf2 to combat early oxidative damage in diabetes, which is overcome by sustained ROS production and exhaustion of the antioxidant defences [148]. This concept is supported by our findings in palmitate-treated endothelial cells, where reduced Nrf2 expression and antioxidant defences are observed with surplus ROS [104].

Furthermore, it has been demonstrated that Nrf2 and its downstream target genes are downregulated in cardiomyocytes from diabetic (*db*/*db*) mice [146, 147], which may occur via extracellular signal-regulated protein kinase (ERK) 1/2 activity [149, 150]. Isoproterenol-stimulated contraction of primary cardiomyocytes from adult diabetic mice was also shown to be dependent on Nrf2 [151]. Hence, hyperglycemia-induced loss of Nrf2 function exacerbates oxidative stress and leads to severe myocardial damage [151]. Nrf2 knockout mice exhibit structural and functional abnormalities under conditions of pathological stress [152], and cardiomyocytes from Nrf2-knockout mice showed significantly increased apoptosis following incubation with high glucose [151]. These findings highlight the importance of the Nrf2 protective mechanisms, and thus, novel therapeutics to enhance Nrf2 could be beneficial in this scenario. The proteasome inhibitor MG-132, which increases Nrf2 signaling, was reported to decrease left ventricle hypertrophy by reducing inflammation and lowering the risk of cardiomyopathy [153]. In addition, in a mouse model of type I diabetes mellitus, Nrf2 activation by sulforaphane reduced heart weight and decreased diabetes-induced atrial natriuretic peptide (ANP) expression, thought to be related to induction of DCM [154]. Therefore, enhancing endogenous Nrf2 and subsequent antioxidant pathways in the heart is a potential strategy to prevent DCM [138, 155].

10. Nrf2 in the Aging Heart

Aging, a progressive decline of cellular functions, is related to the loss of homeostasis via a combination of epigenetic alterations and genetically programmed processes resulting in death [156, 157]. Heart capacity declines with age, with a concomitant increased CVD risk [158]. Herman's free radical theory proposes that the accumulation of damaged biomolecules by ROS/RNS plays a central role in aging [159–161]. In turn, this leads to activation of NF-κB [162], eliciting an inflammatory response via TNF-α, IL-6, and C reactive protein (CRP), reported to be associated with aging [163], and further stimulation of ROS production through activation of NADPH oxidase [164, 165] and NF-κB [166]. In support of this, elderly patients demonstrate an impaired endothelial-dependent dilation, associated with excess ROS, activated NADPH oxidase, and increased NF-κB [167].

Elevated ROS also increase the rate of apoptosis and necrosis in cardiomyocytes [168], resulting in functional and phenotypic changes, including decreased remodelling

Table 2: The effect of Nrf2 activation on CVD.

Activator	Animal model/cell line	Effects	Reference
Bardoxolone methyl derivative dh404	Male Akita mice at 26 weeks of age & human aortic endothelial cells (HAECs)	Attenuation of endothelial dysfunction Downregulation of inflammatory and prooxidant genes Reduction in systemic and vascular oxidative stress	[188]
	Streptozotocin- (STZ-) induced diabetic ApoE$^{-/-}$ mice	Prevention of atherosclerosis	[189]
Sulforaphane	Vascular smooth muscle cells (VSMCs)	Suppression of VSMC proliferation	[190]
	HUVECs	Protection against oxidized low-density lipoprotein- (oxLDL-) induced endothelial damage	[191]
	High-fat diet- (HFD-) induced type 2 diabetic mice	Prevention of aortic damage	[192]
	Low-dose STZ diabetic mice	Prevention of diabetic cardiomyopathy	[154]
	Multiple low dose STZ-induced type 1 diabetic mice	Prevents aortic oxidative damage, fibrosis, and inflammation	[193]
Miltirone	EA.hy926 endothelial cells	Protects against oxLDL-derived oxidative stress	[194]
Epigallocatechin-3-gallate	HUVECs	Protects against PM2.5-induced oxidative stress	[195]
Barleria lupulina alkyl catechols (4-ethylcatechol, 4-vinylcatechol, and 4-methylcatechol)	Human dermal microvascular endothelial cells	Improves organization of the cytoskeleton Organizes tight cell junctions Reduces inflammation and vascular leakage	[196]
Small molecule glycomimetics	HUVECs	Attenuates palmitate-induced oxidative stress and endothelial dysfunction. Increases NO production.	[104]
Rutin	HUVECs	Prevents hydrogen peroxide- (H_2O_2-) induced oxidative stress	[197]
1,25-Dihydroxycholecalciferol	HUVECs	Prevents leptin-induced oxidative stress and inflammation	[198]
Willow bark extract	HUVECs and *Caenorhabditis elegans*	Prevents ROS-induced cytotoxicity of HUVECs and death of *C. elegans*	[199]
Aged garlic extract	HUVECs	Enhances HO-1 and glutamate-cysteine ligase modifier subunit expression (GCLM)	[200]
Celastrol	HUVECs	Attenuates angiotensin II mediated endothelial damage	[201]
Paeotang	HUVECs	Prevents TNF-α-induced vascular inflammation	[202]
Cyanidin-3-O-glucoside	HUVECs	Ameliorates palmitate-induced insulin resistance and endothelial derived vasoactive factors	[203]
		Attenuates palmitate-induced inflammation	[204]
	EA.hy926 endothelial cells	Attenuates angiotensin II-induced oxidative stress and inflammation	[205]
Piceatannol	HUVECs	Attenuates homocysteine-induced endoplasmic reticulum stress and cell damage	[206]
Equol	ApoE$^{-/-}$ mice	Attenuates atherosclerosis and inhibits endoplasmic reticulum stress	[207]
	HUVECs	Abrogates apoptosis induced by t-BHP	
Sheep/goat whey protein	EA.hy926 endothelial cells	Increases antioxidant defences	[208]
Quercetin	HAECs	Inhibits LPS-induced adhesion molecule expression and ROS production	[209]

Table 2: Continued.

Activator	Animal model/cell line	Effects	Reference
Panax notoginseng saponins and Ginsenoside Rb1	HUVECs	Suppresses monocyte adhesion and inhibits ROS	[210]
Bortezomib	Human microvascular endothelial cells (HMECs)	Induces expression of HO-1	[211]
Sofalcone	HUVECs	Suppresses endothelial dysfunction	[212]
Salidroside	HUVECs	Suppresses ROS-induced damage	[213]
Caffeic acid	HUVECs	Attenuates high glucose-induced endothelial dysfunction	[214]
Myricitrin	H9c2 cardiomyocytes	Attenuates high glucose-induced apoptosis	[215]
	STZ-induced diabetic mice & AGE-induced H9c2 cardiomyocytes	Alleviates oxidative stress-induced inflammation, apoptosis, and cardiomyopathy	[216]
Andrographolide	EA.hy926 endothelial cells	Inhibits hypoxia-induced HIF-1α-driven endothelin 1 secretion	[217]
Tanshinone IIA	HUVECs	Inhibits cyclic strain-induced expression of interleukin 8	[218]
Lycopene	HUVECs	Inhibits cyclic strain-induced endothelin-1 expression and oxidative stress	[219]
Withaferin A	EA.hy926 endothelial cells & HUVECs	Induces HO-1 expression	[220]
Copper diethyldithiocarbamate	Bovine aortic endothelial cells	Inhibits proteasome and Nrf2 binding to Kelch-like ECH-associated protein 1	[221]
Clopidogrel	HAECs	Hinders TNF-α-induced VCAM-1 expression	[222]
Hericium erinaceus	EA.hy926 endothelial cells	Inhibits TNF-α-induced angiogenesis and ROS generation	[223]
Andrographolide	Primary cerebral endothelial cells	Prevents middle cerebral artery occlusion- (MCAO-) induced ischemic stroke	[224]
Butin	C57/BL6J diabetic mice	Prevents ischemia/reperfusion-induced myocardial injury	[225]
Aspalathin	H9c2 cardiomyocytes and diabetic db/db mice	Protects against hyperglycemia-induced oxidative damage and apoptosis	[146]
Broccoli sprout	Diabetic db/db mice	Prevents diabetic cardiomyopathy	[226]
Oleuropein	Spontaneously hypertensive rats	Attenuates oxidative stress and improves mitochondrial function in the hypothalamic paraventricular nucleus	[227]
Aralia taibaiensis	H9c2 cardiomyocytes	Protects against high glucose-induced oxidative stress and apoptosis	[228]
Compound C66	STZ-induced diabetic mice aorta	Prevents oxidative and nitrative stress, inflammation, apoptosis, cell proliferation, and fibrosis	[229]
Dimethyl fumarate	VSMCs	Attenuates vascular calcification	[127]
Gemigliptin	VSMCs	Prevents proliferation and migration of VSMCs	[230]
L6H9 (chalcone)	H9c2 cardiomyocytes	Prevents hyperglycemia-induced oxidative stress and inflammation	[231]
Magnesium lithospermate B	VSMCs	Prevents proliferation and migration of VSMCs	[232]
4-O-methylhonokiol	HFD-induced obese mice	Prevents cardiac pathogenesis	[233]

Table 2: Continued.

Activator	Animal model/cell line	Effects	Reference
Resveratrol	Coronary arterial endothelial cells	Protects against high glucose-induced endothelial protection	[234]
	VSMCs	Ameliorates vascular calcification	[126]
	db/db mice	Ameliorates vascular inflammation	[235]
	STZ-induced type 2 diabetic rats	Ameliorates vascular inflammation	[236]
MG132	OVE26 type 1 diabetic mice	Prevents aortic oxidative damage and inflammatory response	[237]
		Prevents cardiomyopathy	[238]

[169], cardiac hypertrophy [170], and increased systolic pressure [171, 172]. NADPH oxidase-2, its activator RAC1, and several profibrotic factors are elevated in hypertrophic hearts in aged rats [158], pointing to the important role of NADPH oxidase in aging-associated cardiomyocyte remodelling. Ischemia and reperfusion are characterized by increased accumulation of intracellular Ca^{2+}, altered substrate utilization, and elevated ROS production in the heart [173], which can damage ionic pumps and induce mitochondrial dysfunction via lipid peroxidation [174]. This damage can lead to necrotic cell death [175] and is exacerbated with aging [160, 176], as shown in mitochondria from aged rats [177].

Diminished activity of Nrf2 resulting in oxidative stress, apoptosis, and/or necrosis in the myocardium has been reported [178–180], thus predisposing the heart to disease [180]. Studies in mouse models have supported the notion that Nrf2 is involved in counteracting aging-associated cardiac effects via ARE signaling and expression of antioxidant enzymes. Bailey-Downs et al. [181] reported increased sensitivity of blood vessels to stress-induced damage along with impaired activity of Nrf2 in insulin-like growth factor 1 (*Igf1*) knockout mice, promoting an aging phenotype. Nrf2-knockout mice showed exaggerated cardiac hypertrophy, heart failure, increased mortality [152], and oxidative stress [182]. Aged rhesus macaques have shown increased ROS and decreased nuclear translocation of Nrf2 and protein expression of NQO1 and HO-1 in their carotid arteries [183]. Vascular smooth muscle cells (VSMCs) derived from old monkeys have exhibited diminished Nrf2 activation following incubation with high glucose as compared with those derived from younger monkeys [183]. Additionally, El Assar et al. [165] have reported a decreased expression of Nrf2-regulated antioxidants in aged vessels.

These data demonstrate clearly that decreasing levels of Nrf2 are age-dependent but may be reversed by exercising. Muthusamy et al. [184] demonstrated an increased nuclear translocation of Nrf2 in the hearts of mice following acute exercise training. They attributed their findings to the induction of an exercise-induced mild oxidative state. Endurance exercise training was reported to promote Nrf2 signaling and enhance antioxidant capacity in the hearts of 6-month-old mice [185], which might offset the reduced signaling observed in aged mice and men [171, 185–187].

11. Role of Nrf2 Activation in the Treatment of CVD

The role of activators of Nrf2 in attenuating oxidative stress-mediated cardiovascular disorders has been identified. In Table 2, we present a summary of the recently studied activators of Nrf2 and their beneficial effects in CVD.

12. Conclusions

The Nrf2 antioxidant system plays a significant role in cellular defence against free radical damage, while insufficiency of Nrf2-dependent gene expression is clearly implicated in multiple aspects and stages of CVD. Enhancing Nrf2 activity may be beneficial in diabetic cardiomyopathy, mitochondrial dysfunction, and reducing the effects of aging in the heart; however, the potential exacerbation of atherosclerosis by Nrf2-mediated inflammasome activation in plaque macrophages, along with the lethality of KEAP1 knockout mice, raises a cautionary note to pharmacological activation of Nrf2 as a therapeutic strategy. Selective upregulation of Nrf2 target genes such as HMOX1 may provide a more amenable therapeutic strategy. Modest activation of Nrf2 by dietary factors, such as sulforaphane, found in brassicas like broccoli, may highlight mild activation of Nrf2 as part of the protective role played in eating a healthy balanced diet, which may be sufficient to maximise the therapeutic benefit offered through the control of this gene expression network.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

Sandro Satta and Ayman M. Mahmoud contributed equally to this work.

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Review Article

Collaborative Power of Nrf2 and PPARγ Activators against Metabolic and Drug-Induced Oxidative Injury

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Mammalian cells have evolved a unique strategy to protect themselves against oxidative damage induced by reactive oxygen species (ROS). Especially, two transcription factors, nuclear factor erythroid 2p45-related factor 2 (Nrf2) and peroxisome proliferator-activated receptor γ (PPARγ), have been shown to play key roles in establishing this cellular antioxidative defense system. Recently, several researchers reported ameliorating effects of pharmacological activators for these Nrf2 and PPARγ pathways on the progression of various metabolic disorders and drug-induced organ injuries by oxidative stress. In this review, general features of Nrf2 and PPARγ pathways in the context of oxidative protection will be summarized first. Then, a number of successful applications of natural and synthetic Nrf2 and PPARγ activators to the alleviation of pathological and drug-related oxidative damage will be discussed later.

1. Reactive Oxygen Species and Human Diseases

Mammalian cells have evolved to utilize oxygen as a final electron acceptor to support their energy metabolism in the mitochondria. As a consequence, they need to deal with a group of unwanted oxygenated byproducts, which are generated during this oxygen-dependent metabolic process. In some cases, environmental stress such as UV or heat exposure also has been attributed to their production. Due to their detrimental nature, these oxygenated byproducts are collectively referred to as highly reactive oxygen species (ROS). Their typical examples include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen [1]. A number of cellular metabolic enzymes, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, and nitric oxide synthase (NOS), have been shown to be directly involved in ROS production [2]. Although a certain level of ROS is thought to be necessary for efficient signaling in various cellular pathways [3, 4], most of ROS are generally considered to be harmful due to their damaging effects on essential building blocks of cellular metabolism. For this reason, mammalian cells have developed multiple defense systems to work against this ROS-mediated oxidative stress. One of these antioxidative defense mechanisms is to create a highly reducing intracellular environment to neutralize ROS reactivity before their attack to cellular macromolecules [5].

A growing body of evidence strongly suggests an etiological role of oxidative stress-associated inflammation and cell death in the development of many human diseases [6–11]. Especially, oxidative damage has been intimately linked with the pathogenesis of several chronic metabolic disorders such diabetes, atherosclerosis, and hypercholesterolemia [12–14]. In addition, insufficient cellular protection against oxidative stress also has been ascribed as another contributing factor for developing various liver, kidney, brain, and skin diseases [15–17]. On top of this, oxidative stress was even demonstrated to play a major role in exhibiting many clinically relevant side effects of various pharmacological agents. Therefore, efficient reduction of oxidative stress through activation of multiple antioxidative defense systems was envisaged as a promising strategy to improve a wide range of ROS-induced pathological conditions. Recently, several research groups have published a series of encouraging data suggesting effectiveness of combined use of pharmacological activators for two critical antioxidative pathways. They

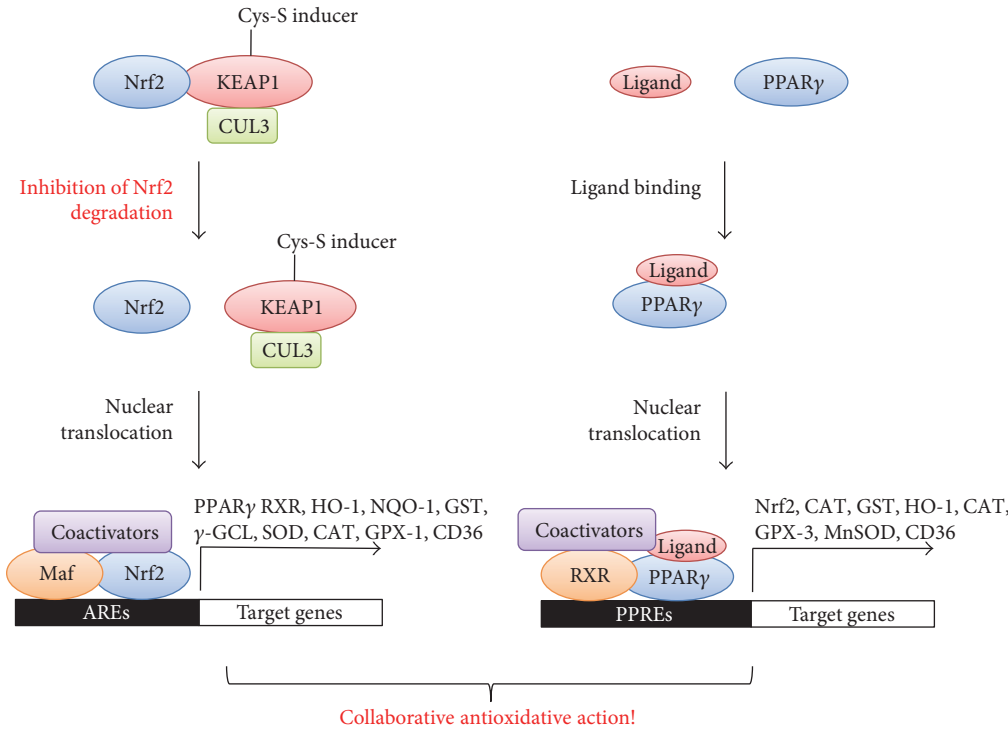

Figure 1: Crosstalk between Nrf2 and PPARγ pathways against oxidative stress. Abbreviations used within the figure are as follows. Nrf2; nuclear factor erythroid 2-related factor 2, KEAP1; Kelch-like ECH-associated protein 1, CUL3; cullin3, PPARγ; peroxisome proliferator-activated receptor γ, HO-1; heme oxygenase-1, NQO-1; NAD(P)H quinone oxidoreductase-1, GST; glutathione, γ-GCL; γ-glutamyl cysteine ligase, SOD; superoxide dismutase, CAT; catalase, GPX; glutathione peroxidase, AREs; anti-oxidant response elements, MnSOD; manganese superoxide dismutase, PPREs; PPAR response element, RXR; retinoid X receptor.

involve two nuclear transcription factors, which are nuclear factor erythroid 2p45-related factor 2 (Nrf2) and peroxisome proliferator-activated receptor γ (PPARγ). Stimulation of these two antioxidative pathways by various pharmacological agents turned out to be extremely beneficial for alleviating a variety of ROS-induced metabolic disorders and drug-induced injuries. In this review, general characteristics of Nrf2 and PPARγ pathways in the context of oxidative protection will be summarized first. Then, a number of successful applications of combined or separate use of Nrf2 and PPARγ activators for amelioration of pathological and drug-induced oxidative injuries will be discussed later.

2. Nrf2 Pathway against Oxidative Stress

Nrf2 is by far the best characterized transcription factor with an oxidant/electrophile-sensing capability [18]. It is a basic leucine zipper protein with six conserved Nrf2-ECH homology (Neh) domains [5]. Especially, ETGE and DLG motifs located in the second Neh2 domain were shown to play a critical role in its complex formation with another essential component of this pathway, Kelch-like ECH-associated protein 1 (KEAP1) [19]. This Nrf2/KEAP1 complex formation was demonstrated to be necessary for restraining the transcriptional activity of Nrf2 [20]. In regard to its sensing mechanism, KEAP1 acts as a main sensor molecule for oxidative stress in this pathway. It is an adaptor protein for cullin-3-based E3 ubiquitin ligase complex. Redox-sensitive twenty-five cysteine residues of KEAP1 in its linker region function as essential determinants for regulating its ubiquitin ligase activity [21]. Conjugation of a variety of ROS-inducing agents with these cysteine residues leads to inhibition of KEAP1-mediated ubiquitination [22], resulting in stabilization and nuclear translocation of Nrf2. Once transported inside the nucleus, Nrf2 associates with one of small Maf proteins and other coactivators to form a trimetric protein complex. Then, this complex binds to the antioxidant response elements (AREs) in the upstream promoter regions of many cytoprotective and detoxifying genes for their transcriptional activation (Figure 1). Typical examples of Nrf2-regulated genes include γ-glutamyl cysteine ligase (γ-GCL), NAD(P)H quinone oxidoreductase-1 (NQO-1), glutathione S-transferase (GST), heme oxygenase-1 (HO-1), uridine diphosphate (UDP) glucuronosyl transferase, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase-1 (GPX-1) [5, 23–28]. In addition to this KEAP1-dependent mechanism, Nrf2 has been reported to be regulated via a number of KEAP1-independent mechanisms. They include transcriptional activation of Nrf2 gene through aryl hydrocarbon receptor (AHR) and its nuclear translocator (ARNT) binding to xenobiotic response element (XRE) [29], transcriptional activation of Nrf2 target genes through association of NF-κB with ARE, post transcriptional regulation of Nrf2 mRNA with host microRNAs [30, 31], post translational modification of Nrf2

protein by phosphorylation [32, 33], acetylation [34, 35], and ubiquitination [36], and association of Nrf2 protein with novel binding partners [37, 38]. This seemingly complicated transcriptional, epigenetic, and posttranslational control of Nrf2 seems to be designed to fine-tune its antioxidative activity upon redox perturbation in order to minimize damaging effects of oxidative stress on cellular metabolism [39].

3. PPARγ Pathway against Oxidative Stress

PPARs are the members of a subfamily of the nuclear receptors and transcription factors. In general, they are involved in the regulation of a wide range of cellular processes such as differentiation, development, metabolism, and even oncogenesis [40–43]. Originally, peroxisome proliferators were found as genotoxic rodent carcinogens due to their proliferative effects on peroxisomes in rats [2, 44, 45]. Interestingly, their peroxisome proliferative activity turned out to be due to their oxidative DNA damage, which was caused by leakage of H_2O_2 from peroxisomes [44]. PPAR family genes comprise of three isoforms including PPARα, PPARβ/δ, and PPARγ [46]. All three subtypes of this PPAR subfamily were found to be highly expressed in mammalian tissues, which were necessary for energy homeostasis [47]. In regard to their signaling mechanisms, once ligand-bound PPARs enter the nucleus, they form a heterodimer with the retinoid X receptor (RXR). Then, they bind to specific PPAR response elements (PPREs) within the promoter region of PPAR-regulated genes [48–50]. Depending on isoforms of PPARs, this PPAR/RXR heterodimer recruits a large protein complex of coactivators to activate the transcription of different sets of PPAR target genes, ultimately leading to a unique physiological outcome (Figure 1) [45].

From the pharmacological point of view, PPARγ has been most extensively characterized as an antidiabetic target [45, 51]. For this reason, it is often called "a glitazone receptor." In general, PPARγ modulates fatty acid storage and glucose metabolism through stimulation of lipid uptake and adipogenesis by PPARγ-regulated gene expression in fat cells [51]. This was supported by an observation of very limited generation of adipose tissue in PPARγ knockout mice [52]. In addition, PPARγ also has been responsible for pathogenesis of several metabolic and vascular diseases including obesity, diabetes, and atherosclerosis [53–55]. Thanks to their regulatory roles in lipid and carbohydrate metabolism, PPARγ agonists have been widely used in the treatment of hyperlipidemia and hyperglycemia [56, 57]. Although PPARγ was initially regarded as a master regulator of transcription in adipogenesis [58], it was also shown to play additional roles in other biologically relevant processes such as infection and inflammation. In particular, many literatures identified PPARγ as a negative regulator of oxidative stress-induced inflammation under either infectious or pathological conditions [51, 59]. Detailed mechanistic studies also revealed that PPARγ was indeed able to suppress inflammation by transcriptional repression of many well-characterized proinflammatory transcription factors and enzymes such as nuclear factor kappa B (NF-κB), signal transducer and activator of trancription-6 (STAT-6), and activator protein 1 (AP-1), cyclooxygenase-2 (COX-2), and induced nitric oxide synthase (iNOS) [2, 48, 60–62]. Antioxidative function of PPARγ was also reported to be mediated by transcriptional activation of a number of several antioxidant genes such as HO-1, CAT, GPX-3, and manganese superoxide dismutase (MnSOD) through its direct association with PPREs of their promoter regions [48, 49, 63]. For this reason, PPARγ has emerged as a new target for anti-inflammatory and antioxidative pharmacotherapy in many diseases, which are adversely affected by oxidative stress and subsequent inflammation [48, 51, 59].

4. Crosstalk between Nrf2 and PPARγ Pathways against Oxidative Stress

Several studies have strongly suggested existence of reciprocal regulation of Nrf2 and PPARγ pathways to reinforce the expression of one another [48, 61, 64]. In this sense, Nrf2 and PPARγ pathways seem to be connected by a positive feedback loop, which maintains the expression of both transcription factors and their target antioxidant genes in a simultaneous manner. Then, what are known about molecular mechanisms for PPARγ regulation by Nrf2? Huang et al. provided insight into this question by identifying PPARγ as a direct target gene induced by Nrf2 transcriptional activation [64]. In line with this finding, several other researchers also reported direct binding of Nrf2 to newly identified AREs in the regions of the PPARγ promoter by using gel shift and coimmunoprecipitation assays (Figure 1) [48, 61, 64, 65]. In their studies, ARE sequences located at −784/−764 and −916 regions of the PPARγ promoter were found to be necessary for Nrf2-regulated PPARγ expression. As supporting evidence to this direct regulation of PPARγ by Nrf2 *in vivo*, PPARγ expression was also found to be markedly lower in Nrf2 knockout mice [64]. Other two studies also reported severely compromised expression of PPARγ in Nrf2 null mice and significantly reduced basal levels of PPARγ by Nrf2 deletion [48, 61]. Then, what is the biological significance of this positive regulation of PPARγ by Nrf2? It was found that Nrf2-regulated PPARγ expression was required for protection against acute lung injury in mice [65]. In this report, PPARγ induction was found to be suppressed in Nrf2-deficient mice in hyperoxia-susceptible manner [65]. This piece of evidence strongly suggests the requirement of positive induction of PAPRγ by Nrf2 for the amelioration of acute lung injury induced by hyperoxia. In addition, RXR, another critical component of PPARγ pathway, also turned out to be induced by activation of Nrf2 pathway by using chromatin immunoprecipitation and sequencing experiments [66]. These data further imply the presence of another layer of positive regulation of PPARγ pathway by Nrf2 (Figure 1).

Then, what is known about the opposite pattern of regulation, which is the PPARγ action on Nrf2 pathway? So far, several lines of evidence have raised the possibility of direct involvement of PPARγ in the activation of Nrf2 pathway. Chorley et al. found that PPARγ agonists were able to induce

transcription of a set of antioxidative defense genes such as GST, HO-1, and CD36 (Figure 1) [66]. Since these PPARγ-regulated genes belong to a group of Nrf2-regulated genes, this observation strongly suggests direct regulation of Nrf2 pathway by PPARγ. In support of this hypothesis, expression of Nrf2 was also shown to be reduced by knockdown of PPARγ in a mouse model [39]. Kvandova et al. even reported the presence of putative PPREs in the promoter regions of Nrf2 gene [2] (Figure 1). This finding further implies possibility of direct binding of PPARγ on Nrf2 promoter for positive regulation of Nrf2 pathway. On the other hand, collaborative action of both Nrf2 and PPARγ transcription factors on a single target gene also seems to be plausible since GST promoter was found to possess both ARE and PPRE sequences to allow for simultaneous stimulation of its transcription [2]. Therefore, concurrent activation of both Nrf2 and PPARγ pathways by different combinations of pharmacological agonists seems to be possible to achieve the maximum levels of antioxidative state for full protection against the harmful effects of ROS (Figure 1).

5. Pharmacological Targeting of Nrf2 and PPARγ Pathways

Many endeavors to pharmacologically manipulate Nrf2 and PPARγ pathways have been shown to be successful in different kinds of *in vitro* as well as *in vivo* disease models. In order to take full advantage of the collaborative action of these two critical antioxidant pathways for alleviation of ROS-induced damages in various metabolic diseases and drug-induced injury, many researchers have tried to apply several Nrf2 and PPARγ activators to various disease models. So far, several metabolic diseases including atherosclerosis, diabetes mellitus, and hepatic and renal diseases have been studied in order to test any beneficial effects of these Nrf2 and PPARγ activators on their disease progression. From now on, therapeutic efficacies and toxicities of various Nrf2 and PPARγ activators studied in these metabolic disorders and some drug-induced organ injuries will be summarized first (Table 1). In order to describe Nrf2 and PPARγ activators in a more systematic manner, they were categorized as Nrf2 activator, PPARγ activators, and dual Nrf2 and PPARγ activators based on their target specificities. Additionally, PPARγ activators were further classified as endogenous, synthetic, and natural PPARγ activators based on their origins of synthesis.

5.1. Nrf2 Activator

5.1.1. Bardoxolone Methyl. Bardoxolone methyl (BARD) is an orally available semisynthetic triterpenoid [67]. Its chemical structure is based on the scaffold of oleanolic acid, a naturally occurring pentacyclic triterpenoid. According to preclinical studies, BARD was shown to activate Nrf2 pathway for its antioxidant effect. It was also reported to inhibit NF-κB pathway for its anti-inflammatory effect [68]. Wu et al. found that BARD was able to ameliorate ischemic acute kidney injury (AKI) through increased expression of Nrf2, PPARγ, and HO-1 in the mouse model [69]. In this study, BARD was able to exert its positive effect on PPARγ pathway by enhancing the amount of PPARγ mRNA and protein [69]. In regard to its mechanism of action, they found that BARD was able to transcriptionally activate HO-1 gene during ischemic AKI via Nrf2-independent manner. This finding suggests that direct upregulation of HO-1 by PPARγ could be the main mechanism of action for the reduction of AKI by BARD. In spite of its impressive antioxidant activity, BARD failed to pass the third phase clinical trial for the treatment of chronic kidney disease due to a higher rate of heart-related adverse events, including heart failure, hospitalizations, and deaths [70].

5.1.2. Curcumin. Curcumin is a bright yellow plant-derived chemical used as a food additive and supplement. It is a well-known natural Nrf2 activator [71]. Olagnier et al. discovered that several Nrf2 activators were able to upregulate one of scavenger receptors, CD36, leading to the stimulation of phagocytosis of *Plasmodium falciparum*, a causative pathogen for malaria, on human monocyte-derived macrophages in inflammatory conditions [72]. In accordance with this finding, curcumin was also able to increase phagocytosis of *Plasmodium falciparum* through upregulation of CD36 surface expression on monocytes/macrophages [73]. In this study, seven putative AREs were identified in the promoter region of CD36 gene, which explained mode of the transcriptional activation of CD36 gene by curcumin. Inhibition of curcumin-induced Nrf2 protein expression by a general antioxidant molecule, N-acetyl cysteine treatment, resulted in the loss of upregulation of CD36 by curcumin. This further suggested direct involvement of ROS in the activation of Nrf2 pathway by curcumin [73]. Interestingly, curcumin was also able to increase the expression of PPARγ at transcriptional and translational level [73]. This implies that simultaneous activation of both Nrf2 and PPARγ pathways by curcumin may play a role in upregulation of CD36, which can lead to increased phagocytosis of *Plasmodium falciparum* by macrophages.

5.2. Endogenous PPARγ Activators

5.2.1. 15-Deoxy-Δ12, 14-Prostaglandin J2. 15-Deoxy-Δ12, 14-prostaglandin J2 (15d-PGJ2) is an electrophilic cyclopentene prostaglandin. It was shown to act as an endogenous ligand for PPARγ [74, 75]. Its highly reactive α, β-unsaturated carbonyl groups were shown to readily interact and make a covalent bonding with cysteine thiol groups in the ligand-binding domain of PPARγ [74]. 15d-PGJ2 was also demonstrated to be able to increase Nrf2 expression via a PPARγ-dependent manner [48]. Interestingly, cysteines of the linker region of KEAP1 were also shown to be engaged in direct binding of 15d-PGJ2 to KEAP1 [74]. In regard to mechanism for its antioxidative activity, 15d-PGJ2 was shown to protect neurons from homocysteic acid-induced oxidative death via Nrf2-dependent and PPARγ-independent mechanisms [75]. In this study, Nrf2 knockdown in astrocytes abrogated 15d-PGJ2's neuroprotective effect. Under this Nrf2 knockdown condition, 15d-PGJ2 was not able to facilitate induction of Nrf2 target genes. In contrast, knockdown of the

Table 1: Summary of Nrf2 and PPARγ pathway activators studied for their potential protective effects on various disease models. Nrf2 and PPARγ pathway activators are categorized based on their target specificities and origin of synthesis. Their compound names, target diseases, effects on experimental models, effect on Nrf2, PPARγ, and other relevant molecules, and their related references are listed accordingly. Abbreviations used within the table are as follows. Nrf2: nuclear factor erythroid 2p45-related factor 2; PPARγ: peroxisome proliferator-activated receptor γ; HO-1: heme oxygenase-1; CNS: central nerve system; ARE-luc: antioxidant response element-containing luciferase reporter; PI3K: phosphatide 3-kinase; PKC: protein kinase C; LPS: lipopolysaccharide; NF-κB: nuclear factor kappa B; COX-2: cyclooxygenase-2; MAPK: mitogen-activated protein kinase; iNOS: inducible nitric oxide synthase; GST-α: glutathione S-transferase-α; ABCA1: ATP-binding cassette transporter 1; MAPKAPK: mitogen-activated protein kinase-activated protein kinase; Bcl-xL: B-cell lymphoma-extra large; NQO-1: NAD(P)H quinone oxidoreductase-1; SOD: superoxide dismutase; GPX: glutathione peroxidase; GST: glutathione; TNF-α: tumor necrosis factor-α; MDA: malondialdehyde; CAT: catalase; NO: nitric oxide; SIRT1: silent information regulator 2 (Sir2) protein 1; γ-GCL: γ-glutamyl cysteine ligase.

Category	Compound name	Target disease	Effects on experimental models	Effect on Nrf2	Effect on PPARγ	Effect on others	Reference
Nrf2 activators	Bardoxolone methyl	Kidney disease	Amelioration of ischemic acute kidney injury in mice	Nrf2 ↑	PPARγ ↑	HO-1 ↑	Wu et al. [69]
	Curcumin	Malaria	Increased nonopsonic phagocytosis of *Plasmodium falciparum* in monocytes/macrophages and hepatoma cells	Nrf2 ↑	PPARγ ↑	CD36 ↑	Mimche et al. [73]
Endogenous PPARγ activators	15-Deoxy-D12, 14-prostaglandin J2	CNS disease	Protection against homocysteic acid-induced oxidative death in neurons	ARE-luc ↑	Independent	HO-1 ↑	Haskew-Layton et al. [75]
		Not specified	Attenuation of cell death in RAW264.7 mouse macrophages	Nrf2 ↑	Independent	HO-1 ↑	Gong et al. [76]
	Nitroalkene fatty acids	Not specified	Activation of Nrf2 and PPARγ pathways in human MCF7 breast cancer cells	Nrf2 ↑ (<1 μM)	PPARγ ↑ (>1 μM)	PI3K, PKC ↑	Bates et al. [78]
	Nitrated fatty acids	Respiratory disease	Decreased severity of LPS-induced acute lung injury in mice	Nrf2 ↑	PPARγ ↑	NF-κB ↓	Reddy et al. [80]
Synthetic PPARγ activators	Rosiglitazone	Diabetes	Protection against high glucose-induced toxicity in hepatocytes	Nrf2, ARE ↑	Independent	HO-1 ↑ PKC, COX-2 ↓	Wang et al. [67]
		Respiratory disease	Protection against paraquat-induced acute lung injury in rats	Nrf2 ↑	PPARγ ↑	NF-κB ↓	Liu et al. [83]
	Troglitazone and cyanidin	Liver disease	Protection against H_2O_2-induced cytotoxicity in human hepatoblastoma HepG2 and rat normal hepatocytes	Nrf2 and ARE ↑	PPARγ ↑	MAPK ↑	Shih et al. [84]
	Arylidene-thiazolidinedione	Diabetes	Blockage of LPS-induced inflammation and oxidative stress in RAW mouse macrophages	Independent	PPARγ ↑	CD36, HO-1 ↑ iNOS, COX-2 ↓	Faine et al. [82]
Natural PPARγ activators	Carotenoids	Cancer	Inhibition of proliferation of K562 myelogenous leukemia cells	Nrf2 ↑	PPARγ ↑	p21 ↑, cyclin D1 ↓	Zhang et al. [85]
	Monascin	Diabetes	Protection against methylglyoxal-induced toxicity in HepG2 cells and rats	Nrf2 ↑	PPARγ ↑	PKC ↓	Hsu et al. [88] Hsu et al. [87]
	Ankaflavin	Diabetes	Protection against methylglyoxal-induced toxicity in HepG2 cells and rats	Nrf2 ↑	PPARγ ↑	Glyoxalase, HO-1 ↑	Lee et al. [90] Hsu and Pan [89]

Table 1: Continued.

Category	Compound name	Target disease	Effects on experimental models	Effect on Nrf2	Effect on PPARγ	Effect on others	Reference
Dual Nrf2 and PPARγ activators	Genistein	Atherosclerosis	Attenuation of H_2O_2-induced endothelial cell injury in transformed human umbilical vein endothelial cells	Nrf2 ↑	PPARγ ↑	HO-1 ↑	Zhang et al. [92]
	Vitamin E	Atherosclerosis	Protection against hypercholesterolemia-induced atherosclerosis in rabbit aortae	Nrf2 ↑	PPARγ ↑	GST, ABCA1 ↑	Bozaykut et al. [94]
	Olmesartan	Kidney disease	Protection against oxidative and inhibition of inflammation in daunorubicin-induced nephrotoxicity in rats	Nrf2 ↑	PPARγ ↑	MAPKAPK, caspase-12, p47, p67 ↓ Bcl-xL, GST ↑	Gounder et al. [96]
	α-Methylene-γ-lactones	Kidney disease	Activation of Nrf2 and PPARγ pathways in mouse peritoneal macrophages from normal and PPARγ-knockout mice against	Nrf2 ↑	PPARγ ↑	Dectin-1, CD36, NQO-1, HO-1 ↑	Le Lamer et al. [97]
	18β-Glycyrrhetinic acid	Kidney disease	Protection against methotrexate-induced kidney injury in rats	Nrf2 ↑	Independent	GSH, SOD, GPX, GST, HO-1 ↑, TNF-α, MDA, NO ↓	Abd El-Twab et al. [100]
		Liver disease	Protection against cyclophosphamide-induced hepatotoxicity in rats	Nrf2 ↑	PPARγ ↑	MDA, NF-κB, iNOS ↓, GSH, GPX, SOD, CAT ↑	Mahmoud and Al Dera [102]
	(−)-Epigallocatechin-3-gallate	Kidney disease	Protection against crescentic glomerulonephritis induced by administration of rabbit anti-mouse glomerular basement membrane antibody in mice	Nrf2 ↑	PPARγ ↑	SIRT1, γ-GCL, GPX1, NQO-1↑, AKT, JNK, ERK, p38 ↓	Ye et al. [103]
	Mangiferin	Gastrointestinal disease	Protection against gastric ulcer in ischemia/reperfused rats	Nrf2 ↑	PPARγ ↑	HO-1 ↑, NF-κB ↓	Mahmoud-Awny et al. [104]
	3-O-Lauryl glyceryl ascorbate	Skin disease	Suppression of oxidative damage induced by H_2O_2 and UVB in normal human epidermal keratinocytes	Nrf2 ↑	PPARγ ↑	γ-GCS, HO-1, NQO-1, GSH ↑	Katsuyama et al. [105]
	Umbelliferone	Liver disease	Protection against cyclophosphamide-induced hepatotoxicity in rats	Nrf2 ↑	PPARγ ↑	HO-1 ↑	Mahmoud et al. [106]
	Graptopetalum paraguayense and resveratrol	Diabetes	Protection against carboxymethyllysine-induced pancreas dysfunction and hyperglycemia in mice	Nrf2 ↑	PPARγ ↑	PDX-1, GSH, GCL ↑	Lee et al. [109]
	Cyanidin-3-glucose and resveratrol	Gastrointestinal disease	Protection against cytokine-stimulated oxidative stress in human colon cancer cells	Nrf2 ↑	PPARγ ↑	HO-1, γ-GCS, GSH ↑	Serra et al. [110]

PPARγ did not alter the neuroprotective activity of 15d-PGJ2 [75]. Among many Nrf2-regulated genes, HO-1 turned out to play the most critical role in mediating the antioxidative effect of 15d-PGJ2 [75]. Gong et al. also reported protective activity of 15d-PGJ2 against oxidative stress in RAW264.7 mouse macrophages. In this study, they showed that attenuation of cell death by 15d-PGJ2 was due to its positive induction of the mouse HO-1 gene [76]. More specifically, they found that 15d-PGJ2-induced stabilization of Nrf2 was able to mediate transcriptional activation of the mouse HO-1 through Nrf2 binding on its enhancer region. However, this induction of mouse HO-1 expression by 15d-PGJ2 again turned out to be independent of PPARγ pathway [76].

5.2.2. Nitroalkene Fatty Acids. Nitroalkene fatty acids (NAs) are naturally occurring electrophilic derivatives of unsaturated fatty acids. NAs are formed via nitric oxide-dependent oxidative reactions [77]. Bates et al. found that NAs were able to form direct adduct with KEAP1, leading to the activation of Nrf2 pathway. In this report, they reported that NAs were able to display differential transactivation activities toward Nrf2 and PPARγ pathways in a dose-dependent manner [78]. Briefly, activation of PPARγ pathway occurred at nanomolar concentrations of NAs in MCF7 breast cancer cells. However, activation of Nrf2 pathway occurred at much higher concentrations of NAs ($\geq 3\ \mu$M) [78]. Based on these results, they concluded that direct activation of PPARγ transcription by NAs would dominate over their electrophilic activation of Nrf2 during antioxidant/protective responses [78]. Of note, both phosphatide 3-kinase (PI3K) and protein kinase C (PKC) activations were also shown to be required for transcriptional activation of Nrf2 and PPARγ pathways by NAs in this study [78].

5.2.3. Nitrated Fatty Acids. Endogenous nitrated fatty acids (NFAs) are produced by nonenzymatic reaction of nitric oxide or its inorganic reaction products with naturally present unsaturated fatty acids [79]. NFAs can act as activating ligands for all three PPARs, particularly with the greatest potency as PPARγ agonists [80]. Reddy et al. found that a nitro-oleic acid, one of the most potent NFAs, was able to diminish severity of lipopolysaccharide- (LPS-) induced acute lung injury in mice [80]. In regard to its mechanism of action, they found that its protective effect against LPS-induced inflammation was mediated by increased transcriptional activity of PPARγ. They also showed that this upregulation of PPARγ by a nitro-oleic acid led to subsequent induction of Nrf2 and decreased transcription of the proinflammatory gene, NF-κB [80].

5.3. Synthetic PPARγ Activators. Thiazolidinedione (TZDs) drugs are cognate ligands for PPARγ. They are frequently used for the treatment of type 2 diabetes [45, 49]. TZDs drugs are known to facilitate insulin-mediated adipocyte differentiation by counteracting the negative effects of inflammatory cytokines [81]. In general, TZDs drug treatment was shown to decrease ROS production in vascular smooth muscle cells [2]. Effects of three kinds of synthetic PPARγ activators on oxidative stress-induced disease models have been examined so far [50, 82–84]. They include rosiglitazone (RSG), troglitazone (TG) in combination with cyanidin, and arylidene-thiazolidinedione. Here, their activities against oxidative stress and mechanisms of action for these antioxidative activities will be discussed briefly.

5.3.1. Rosiglitazone. RSG is a member of the TDZs family and a ligand for the PPARγ. Wang et al. found that RSG was able to protect hepatocytes from high glucose-induced toxicity via both PPARγ-dependent and PPARγ-independent manners [50]. In this study, they found that RSG increased the expression of Nrf2 and HO-1 in a PPARγ-dependent manner, leading to the elimination of excessive ROS [50]. In addition, they also found that the inhibitory effect of RSG on ROS generation was related with PKC inactivation. In line with this positive role of RSG in reduction of oxidative stress, Liu et al. also reported that RSG was able to inhibit paraquat- (PQ-) induced acute lung injury in rats [83]. In this study, they found that protection of rats against PQ-induced acute lung injury by RSG was mediated by activating both Nrf2 and PPARγ pathways. They also showed that inhibition of NF-κB activation by RSG was required for the alleviation of PQ-induced acute lung injury [83].

5.3.2. Troglitazone with Cyanidin. Cyanidin is a natural organic pigment found in many red berries. Shih et al. reported that cyanidin in combination with TG was able to prevent H_2O_2-induced cytotoxicity in human hepatoblastoma HepG2 and rat normal hepatocyte cells [84]. In this study, they found that antioxidative activities of cyaniding and TG were mediated through activation of mitogen-activated protein kinase (MAPK) and Nrf2 pathways [84]. They also reported that cotreatment of cyanidin and TG was able to transcriptionally upregulate expression of antioxidant and detoxifying genes through activation of ARE-mediated Nrf2 pathway [84]. Based on these results, they suggested simultaneous administration of cyanidin and PPARγ agonists to reverse the metabolic dysfunction-related oxidative damage [84].

5.3.3. Arylidene-Thiazolidinedione. Fair amount of efforts has been devoted to the modification of chemical structures of TZDs in order to reduce their endogenous side effects such as water retention, weight gain, and eyesight problems. Faine et al. found that one of their chemically modified TZDs, the arylidene-thiazolidinedione 5-(4-methanesulfonyl-benzylidene)-3-(4-nitrobenzyl)-thiazolidine-2,4-dione (SF23), possessed a weaker affinity for PPARγ [82]. However, SF23 turned out to have impressive anti-inflammatory and antioxidant properties, which were evidenced by efficient blockage of LPS-induced inflammation and oxidative stress in RAW 267.4 macrophages [82]. SF23 was also able to enhance the mRNA expression of CD36 and suppress the mRNA expression of both iNOS and COX-2. They also reported that SF23 was able to display better antioxidant effects on the LPS-stimulated macrophages than RSG. Interestingly, this antioxidant activity of SF23 was shown to be exerted via an Nrf2-independent manner [82].

5.4. Natural PPARγ Activators

5.4.1. Carotenoids. Carotenoids are organic plant pigments with a tetraterpenoid structure. Zhang et al. found that carotenoids were able to inhibit proliferation of K562 cancer cells through induction of cell apoptosis and blockage of cycle progression [85]. Especially, this carotenoid-induced cell cycle arrest was shown to be mediated by increased expression of a cell cycle blocker, p21, and decreased expression of cyclin D1. This antiproliferative effect of carotenoids was shown to be dependent on upregulation of both Nrf2 and PPARγ expression [85]. Based on these results, they concluded that Nrf2 and PPARγ pathways could be activated in order to induce the growth inhibitory effects on cancer cells [85].

5.4.2. Monascin. Monascin is a natural compound obtained from Monascus-fermented products. Beisswenger found that monascin was able to attenuate the hyperglycemic toxicity induced by methylglyoxal (MG). MG is a major precursor of advanced glycation end products, which were well known for their diabetes-inducing activities through impairment of an insulin transcription factor, pancreatic and duodenal homeobox-1 (PDX-1) [86]. The protective activity of monascin against MG-induced diabetes was shown to be mediated through positive modulation of both Nrf2 and PPARγ pathways [87]. In this report, Hsu et al. identified monascin as novel natural Nrf2 and PPARγ agonists by using Nrf2 and PPARγ promoter reporter assays in HepG2 cells. Activation of Nrf2 pathway by monascin also resulted in downregulation of hyperinsulinemia in an oral glucose tolerance test [87]. In their related studies, they also reported that cotreatment of monascin with another Nrf2 activator, allyl isothiocyanate, was able to attenuate MG-Induced PPARγ phosphorylation and degradation through inhibition of the oxidative stress via a PKC-dependent manner [88].

5.4.3. Ankaflavin. Ankaflavin (AK) is a natural pigment isolated from Monascus-fermented products. It was found to possess the PPARγ agonist activity [89]. Lee et al. reported that AK was able to upregulate Nrf2 pathway to attenuate MG-induced diabetes *in vivo* [90]. Although AK failed to alter hepatic Nrf2 mRNA or protein expression, it significantly increased Nrf2 phosphorylation at serine 40. This led to increased transcriptional activation of HO-1 gene. They also found that protective effects of AK against diabetes were mediated by the upregulation of Nrf2 pathway, resulting in induction of glyoxalase and HO-1 [89, 90]. In addition, AK also was able to increase Maf-A and PDX-1 expression through activation of PPARγ pathway. They suggested that this could be one potential mechanism for elevating pancreatic insulin synthesis and improving hyperglycemia by AK in MG-treated rats [89].

5.5. Dual Nrf2 and PPARγ Activators

5.5.1. Genistein. Genistein is a primary isoflavone from soybeans [91]. Zhang et al. found that genistein was able to induce activation of both Nrf2 and PPARγ pathways and that this led to attenuation of H_2O_2-induced cell injury in transformed human umbilical vein endothelial cells [92]. In this report, dual activation of Nrf2 and PPARγ pathways by genistein was demonstrated by enhanced promoter activity of both Nrf2 and PPARγ reporters by genistein [92]. In regard to its mechanism of action, induction of HO-1 by genistein seemed to mediate its protective effect against oxidative stress [92].

5.5.2. Vitamin E. Vitamin E is a group of compounds including both tocopherols and tocotrienols. Their antioxidant activities have been extensively characterized by many researchers [93]. Bozaykut et al. reported that vitamin E was able to afford protection against hypercholesterolemia-induced atherosclerosis in the rabbit aorta model. In this study, they found that vitamin E was able to show this protective effect through decreased expression of matrix metalloproteinase-1 (MMP-1) and increased expression of PPARγ, GST-α, and ATP-binding cassette transporter 1 (ABCA1) in the aortae of cholesterol-fed rabbits [94]. Protein expression of Nrf2 was also increased in both the cholesterol-fed and the vitamin E-supplemented groups. Vitamin E appeared to afford this protection through activation of both Nrf2 and PPARγ pathways, resulting in induction of several antioxidant genes [94].

5.5.3. Olmesartan. Daunorubicin is a chemotherapeutic medication used to treat various kinds of cancer. Oxidative injury has been suspected to play a major role for daunorubicin in inducing chronic nephrotoxicity [95]. Gounder et al. found that olmesartan, an angiotensin II receptor antagonist, which was used for the treatment of high blood pressure, was able to protect against this daunorubicin-induced nephrotoxicity in rats [96]. In this study, they found that olmesartan treatment downregulated phosphorylation of several key signaling molecules such as mitogen-activated protein kinase-activated protein kinase (MAPKAPK), caspase-12, p47, and p67. Olmesartan was also able to upregulate renal expression of PPARγ, B-cell lymphoma-extra large (Bcl-xL), GPX, and Nrf2 [96]. Based on these results, they concluded that positive regulation of both Nrf2 and PPARγ pathways seemed to mediate protective effects of olmesartan against daunorubicin-induced nephrotoxicity.

5.5.4. α-Methylene-γ-Lactones. Protolichesterinic acid is a lichen paraconic acid with an α, β-unsaturated lactone moiety. Le Lamer et al. found that protolichesterinic acid derivatives, α-methylene-γ-lactones, were able to induce expression of Nrf2 target genes such as NQO-1 and HO-1 and PPARγ target genes such as Dectin-1 and CD36 in macrophages. Based on these results, they concluded that α-methylene-γ-lactones were potent dual activators of both Nrf2 and PPARγ pathways [97]. In regard to more detailed mechanism of action for activation of PPARγ pathway by α-methylene-γ-lactones, they suggested that α-methylene-γ-lactones may act as covalent ligands through a Michael addition with a cysteine residue in the PPARγ ligand-binding domain [97].

5.5.5. 18β-Glycyrrhetinic Acid. Methotrexate (MTX) is a dihydrofolate reductase inhibitor used for several human

malignancies and autoimmune disorders. Due to its prooxidant and nonspecific action, MTX has been reported to induce a variety of adverse effects [98, 99]. 18β-Glycyrrhetinic acid (18β-GA) is one of the active ingredients of *Glycyrrhiza glabra* (Liquorice). Abd El-Twab et al. reported that 18b-GA supplementation was able to significantly upregulate the mRNA abundance of both Nrf2 and HO-1 in the kidney of MTX-treated rats [100]. 18b-GA administration was also able to downregulate levels of circulating kidney function markers, tumor necrosis factor-α (TNF-α), kidney lipid peroxidation, and nitric oxide. This protective activity of 18b-GA against MTX-induced kidney injury appeared to depend solely on activation of Nrf2 with no participation of PPARγ pathway [100].

Cyclophosphamide (CP) is a chemotherapeutic agent used to suppress the immune system and cancer. CP-induced ROS generation and oxidative stress have been implicated in its hepatotoxic effects [101]. Mahmoud and Al Dera found that 18β-GA acid was able to exert protective effects against CP-induced hepatotoxicity. They also showed that this hepatoprotective activity of 18β-GA was mediated through activation of both Nrf2 and PPARγ pathways and suppression of NF-κB pathway [102]. More specifically, 18β-GA decreased expression levels of malondialdehyde (MDA), NF-κB, and iNOS and increased expression levels of GSH, GPX, SOD, and CAT [102].

5.5.6. (−)-Epigallocatechin-3-Gallate. (−)-Epigallocatechin-3-gallate (EGCG) is a well-known green tea polyphenolic compound with an antioxidant activity. Ye et al. found that EGCG was able to ameliorate crescentic glomerulonephritis through activation of Nrf2 pathway [103]. In this study, they induced crescentic glomerulonephritis by administration of a rabbit anti-mouse glomerular basement membrane antibody. Under this condition, EGCG-treated mice showed significant reduction in phosphorylation levels of several signaling molecules such as AKT, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38. EGCG administration also induced a marked increase in the levels of Nrf2, GCL, GPX-1, NQO-1, PPARγ, and silent information regulator 2 (Sir2) protein 1 (SIRT1) in the kidney tissue [103]. All these transcriptional changes induced by activation of both Nrf2 and PPARγ pathways seemed to contribute to amelioration of crescentic glomerulonephritis induced by a glomerular basement membrane antibody.

5.5.7. Mangiferin. Mangiferin is a naturally occurring glucosylxanthone xanthonoid from *Mangifera indica*. Mahmoud-Awny et al. found that mangiferin was able to mitigate gastric ulcer in ischemia/reperfused rats. They also found that mangiferin was able to exert its gastroprotective effect via inducing the expression of Nrf2, HO-1, and PPARγ along with downregulating that of NF-κB [104]. The effect of mangiferin, especially at the high dose, exceeded that was mediated by omeprazole, a proton pump inhibitor [104].

5.5.8. 3-O-Laurylglyceryl Ascorbate. Ascorbic acid is a water-soluble vitamin with an antioxidant activity. A newly synthesized amphipathic derivative of ascorbic acid, 3-O-laurylglyceryl ascorbate, was shown to activate both Nrf2 and PPAR-γ pathways [105]. Specifically, 3-O-laurylglyceryl ascorbate was shown to be able to upregulate the expression of mRNAs encoding PPAR-γ and Nrf2 and their target genes including γ-GCS, HO-1, and NQO-1 [105]. Downregulation of Nrf2 mRNA level in siPPARγ-treated cells further supported the reciprocal positive modulation of Nrf2 and PPARγ pathways. In addition, the effects of 3-O-laurylglyceryl ascorbate on PPARγ and Nrf2 mRNA levels were reduced by PPARγ knock down in normal human epidermal keratinocytes [105]. This suggested that PPARγ played a major role for 3-O-laurylglyceryl ascorbate in inducing transcription of antioxidant genes.

5.5.9. Umbelliferone. Umbelliferone is a natural product of the coumarin family used in sunscreens. Mahmoud et al. reported that umbelliferone was able to confer a protective effect against hepatotoxicity induced by cyclophosphamide (CP), which is an anticancer and immunosuppressive drug [106]. This hepatoprotective activity of umbelliferone was shown to be mediated by upregulation of Nrf2 and PPARγ pathways. In this report, CP-treated rats showed significant downregulation of Nrf2, HO-1, and PPARγ. However, this effect was markedly reversed by umbelliferone treatment [106]. Activation of PPARγ also appeared to inhibit the fibrogenic response to hepatic injury and protect against CP-induced inflammation [106].

5.5.10. Graptopetalum paraguayense and Resveratrol. As previously mentioned, advanced glycation end products were generated by nonenzymatic reactions between carbohydrates and proteins and found to cause pancreatic damage and oxidative stress in hyperglycemic patients [107, 108]. Lee et al. used carboxymethyllysine (CML) to induce pancreas dysfunction and hyperglycemia through formation of advanced glycation end products. Using this model, they found that cotreatment of *Graptopetalum paraguayense* (GP) and resveratrol was able to ameliorate CML-induced pancreas damage and hyperglycemia. Especially, resveratrol and ethanol extracts of GP increased insulin synthesis via upregulation of pancreatic PPARγ and PDX-1. Resveratrol and ethanol extracts of GP also strongly activated Nrf2 pathway including GSH and γ-GCL to attenuate oxidative stress and improve insulin sensitivity [109].

5.5.11. Cyanidin-3-Glucose and Resveratrol. Cyanidin-3-glucose (C3G) is a natural plant pigment with an anthocyanin structure. Serra et al. found that cotreatment of C3G and resveratrol was able to induce Nrf2 activation leading to increased HO-1 and γ-GCL mRNA expression in human colon cancer cells [110]. Resveratrol was also able to increase nuclear levels of PPARγ in cytokine-stimulated cells. Based on these results, they suggested the use of polyphenols as nutraceuticals to lessen intestinal inflammation in patients with inflammatory bowel disease [110].

6. Concluding Remarks

In this paper, we have reviewed roles of oxidative stress in the development of human diseases, two major antioxidant

signaling cascades such as Nrf2 and PPARγ pathways, their potential crosstalk against oxidative stress, and pharmacological targeting of these two pathways by various Nrf2 and PPARγ activators. Since a growing body of evidence strongly suggests existence of the intimate relationship between oxidative stress and the development of various metabolic disorders and drug-induced organ injuries, discovery of the best combination of Nrf2 and PPARγ activators to achieve the maximal protection against this oxidative stress will be greatly beneficial for alleviating burden of numerous patients suffering from many oxidative stress-induced diseases and side effects of anticancer drugs.

Conflicts of Interest

The author declares that there are no competing interests regarding the publication of this paper.

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Review Article

Modulatory Mechanism of Polyphenols and Nrf2 Signaling Pathway in LPS Challenged Pregnancy Disorders

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Early embryonic loss and adverse birth outcomes are the major reproductive disorders that affect both human and animals. The LPS induces inflammation by interacting with robust cellular mechanism which was considered as a plethora of numerous reproductive disorders such as fetal resorption, preterm birth, teratogenicity, intrauterine growth restriction, abortion, neural tube defects, fetal demise, and skeletal development retardation. LPS-triggered overproduction of free radicals leads to oxidative stress which mediates inflammation via stimulation of NF-κB and PPARγ transcription factors. Flavonoids, which exist in copious amounts in nature, possess a wide array of functions; their supplementation during pregnancy activates Nrf2 signaling pathway which encounters pregnancy disorders. It was further presumed that the development of strong antioxidant uterine environment during gestation can alleviate diseases which appear at adult stages. The purpose of this review is to focus on modulatory properties of flavonoids on oxidative stress-mediated pregnancy insult and abnormal outcomes and role of Nrf2 activation in pregnancy disorders. These findings would be helpful for providing new insights in ameliorating oxidative stress-induced pregnancy disorders.

1. LPS Overview and Its Drawbacks

Early pregnancy failure is a main obstacle that leads to significant effects on pregnancy outcomes in humans and animals [1]. Approximately 15–20% clinical pregnancies experience abortion [2], and about 30–50% conception resulted in early embryonic loss in mammals [3]. Moreover, assisted reproductive techniques enhance pregnancy rate in infertile women without avoiding early embryonic loss [4]. Humans get constant exposure of LPS at minimum levels in gastrointestinal inflammatory diseases [5]. Lipopolysaccharide (LPS) is derived from G-negative bacteria; maternal exposure to pregnant rodents causes placental inflammation contributing in embryonic resorption, fetal growth restriction (FGR), preeclampsia fetal brain injury, and miscarriages which develops by alternation in cytokine productions [6, 7]. These cytokines were released by trophoblastic, decidual, and chorioamnionitic cells and other cell types [8]. In humans, LPS infection provokes fetal loss and preterm labor [9] and is thought to be regulated by LPS-induced ROS-mediated

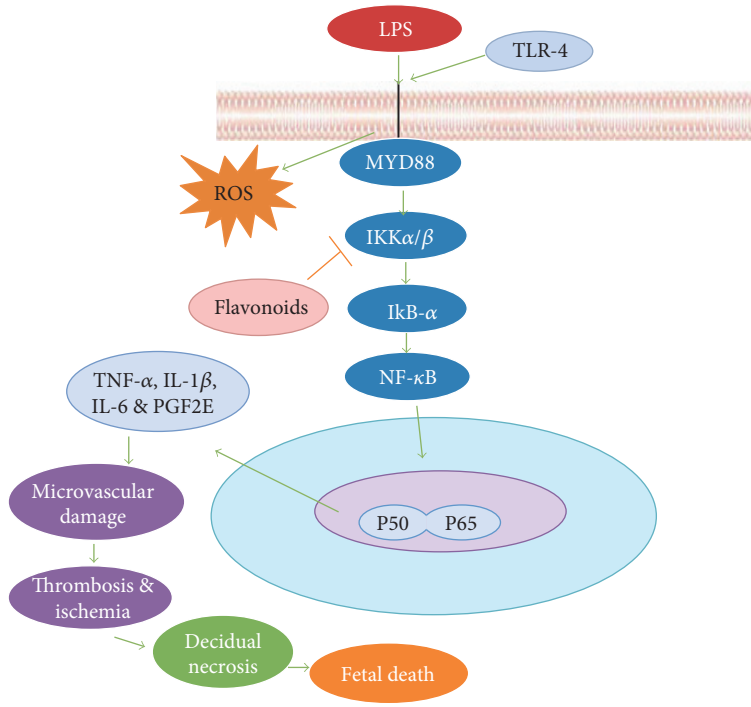

Figure 1: LPS-induced abortion by regulating strong cellular network. After induction of LPS, binding protein interacts with Toll-like receptor 4 (TLR4) and activates downstream adaptor proteins MYD-88, which subsequently stimulate IKK complex, resulting in ubiquitination and phosphorylation of IkBα proteins that translocate NF-κB into the nucleus for production of several proinflammatory cytokines such as TNF-α, IL-β, IL-6, and PGF2E which causes microvascular damage leading to thrombosis, ischemia, necrosis of decidual cells, and finally abortion. On the other hand, flavonoids prevent abortion by inhibition of IKK complex proteins and bring NF-κB into its inactivated form in cytoplasm. These beneficial effects of flavonoids are mediated by activation of PI3K/Akt pathway; hence, it prevents development of free radicals by supplementation of flavonoids during pregnancy.

teratogenesis [10]. In addition, basal amount of ROS is necessary in early embryonic growth and metabolism; excessive generation of uterine ROS is detrimental to oxidative DNA damage of the embryo [11, 12]. In pregnant mice, LPS exposure at late gestation leads to preterm delivery and fetal demise [13, 14], and in later gestational stages, maternal LPS infection causes intrauterine fetal growth restriction [15]. The signaling molecule, nitric oxide (NO), displays an essential role in implantation, decidualization, vasodilation, myometrial relaxation, and overactivation possibly induced by free radical-mediated pathology. Enhanced production of LPS-induced nitric oxide has been reported in embryonic resorption and abortion [16]. LPS-triggered abortion mechanism has been depicted in Figure 1. Nrf2 proteins display a key role in the elimination of oxidative stress through Nrf2-ARE signaling pathway [17, 18] as reported in preeclampsia conditions [19]. Nrf2 is very sensitive to maternal immune status and is responsible for fetal growth and survival through maintaining fetus desirable placental environment; later, Nrf2 protein expression of the placenta was decreased following delivery [20] suggesting its important function in fetal survival. Thus, any inappropriate function could lead to inducing numerous pregnancy disorders. The flavonoids of the polyphenol group are well-recognized natural compounds, which elicit strong antioxidant and anti-inflammatory activities that would be helpful in the elimination of LPS-potentiated pregnancy disorders. The polyphenols such as curcumin possess strong anti-inflammatory activity through influencing diverse pathways to modulate cellular functions. It can also decrease inflammation by inhibiting NF-κB pathway via inactivation of IKK complexes [21, 22]. A study reported that curcumin polyphenols suppress methylglyoxal-induced apoptosis in mouse ESC-B5 cells and blastocysts by inhibiting reactive oxygen species (ROS) [23]. The anti-inflammatory strategy would be helpful in alleviating pregnancy-related complications [24]. This review emphasizes LPS-mediated pregnancy disorders and adverse birth outcomes, modulatory activities of polyphenols, and the role of Nrf2 signaling pathway. We have given detailed description below on the previous reports of polyphenol supplementations such as epigallocatechin gallate, curcumin, baicalin, and tricin which attenuate LPS-induced reproductive disorders, while genistein and quercetin develop strong antioxidant pregnant uterine environment that encounters disease in extrauterine life. These findings would be helpful in improving animal productions.

1.1. Disruption Pregnant Uterine Environment by Inflammatory Cytokines. The LPS binds with Toll-like receptor 4 (TLR4) with the facilitation of cluster of differentiation 14 (CD14) on cell surface of macrophages and monocytes.

Upon activation of TLR4, it disseminates LPS signals to myeloid differentiation factor (MYD88) adaptor molecules; thus, its stimulation is known to be regulated by several signaling molecules including NF-κB proteins [25]. NF-κB exerts an important role in the development of inflammation while its activation occurs by degradation and phosphorylation of IκB kinases such as IKKα and IKKβ which results in the translocation of NF-κB into the nucleus where it induces the formation of inflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin-beta (IL-1β), interleukin-6 (IL-6), and interleukin-8 (IL-8), and inducible inflammatory enzymes, nitric oxide (NO) and reactive oxygen species (ROS) [26, 27]. As mentioned above, that LPS persuades inflammation which triggered various pregnancy disorders in mid and late gestation [28]. The inflammatory mediators, such as TNF-α, interrupt placental blood supply and its function [29] resulting in fetal injury [30]. Studies on mice report that inflammation mediated by TNF-α and interferon-gamma (IFNγ) in macrophages and uterine natural killer (uNK) cells causes vascular injury and placental ischemia in uterine endothelial cells [31]. It was further noted that LPS mediates IFNγ and TNF-α through activation of Toll-like receptors and is responsible for activation of cytokine-induced abortion [32] by possibly downregulating expression of cyclooxygenase-2 enzyme (COX-2) protein that encounters fibrinogen-like protein-2 (Fgl2) in the fetomaternal site [33]. The abortogenic effect varies according to the nature of LPS, source, time length, and dose regimen [32]. The interleukin-10 (IL-10) is an anti-inflammatory cytokine which minimizes pregnancy-related inflammation through regulation of TNF-α and other cytokines and chemokines [34]. The growing body of texture revealed that maternal LPS induced uterine inflammation by cytokines through transplacental transmission that enhances the risk of brain diseases at the adult stage of life [35].

The peroxisome proliferator-activated receptor (PPAR) is a nuclear protein stimulated by multiple ways such as activations of prostaglandins (PGs) and leukotrienes (LTs). After activation, it stimulates transcription factors and mediates various cellular functions including cell differentiation, apoptosis, lipid metabolism, peroxisome proliferation, and inflammation response. In pregnancy, PPAR signals regulate trophoblast invasion and differentiation [36], placentation [37], and maternal metabolism [38]. The improper regulation of PPAR receptors causes complications including preeclampsia (PE), intrauterine growth restriction (IUGR), and preterm birth [39]. *In vitro* studies on knockout mice propose that stimulation of PPAR suppresses proinflammatory cytokines and distinguishes immune cells from anti-inflammatory phenotypes [40]. Naturally occurring compounds polyphenols exert ability to stimulate PPAR nuclear receptors and exert fruitful impact on pregnancy. It has been noted that PPARγ was considered as a potential target for therapeutic intervention against preeclampsia [41]. Limited research on PPAR signals in pregnancy disorders have been observed; therefore, more studies are needed to explore further insights.

2. Positive Effects of Cytokines in Pregnancy Development

Naturally, the immune system protects uterine environment from invading pathogens to full-term birth [42]. Excessive levels of endometrial cytokines, prostaglandins, and leukocytes are released during inflammatory condition [43]. The endometrial mediated cytokine and chemokine productions give directions to the blastocyst to connect with endometrial walls. When invasion and lysis of trophoblast exist, conversion from epithelial cells to stromal cells repairs endometrial tissue which replaces cells in the placenta. This structure is mediated by Th1 cellular responses where an ample amount of proinflammatory molecules such as IL-6, LIF, IL-8, and TNF-α was contributed [44] and these also recruit immune cells towards the decidua. In human and mouse, a huge population of decidual leukocytes has been witnessed at the site of implantation. Of note, these cells are comprised of 65–70% uterine natural killer (uNK) cells and 10–20%, based on macrophages and dendritic cells (DCs) [45]. The macrophages and DCs localize in the decidua during the entire pregnancy and exhibit a key role at maternal-fetal interface [46]. The macrophages and DCs have the capability to secrete a variety of anti-inflammatory molecules (IL-4, IL-10, and IL-13) and enzymes, which are mainly involved in structural modification and angiogenesis [47]. Moreover, it was documented that macrophages mediate trophoblast invasion and might exert main function in eliminating debris which comes from trophoblastic apoptosis at different phases of gestation [44]. The presence of DCs in maternal tissue during implantation has been observed [48], and it was further illustrated that DCs have the ability to alter Th1 proinflammatory cytokines to Th2 anti-inflammatory cytokines at latter stages of gestation [49]. Near to parturition, anti-inflammatory response converted into pro-inflammatory response in order to induce uterine contractions initiates to parturition [50]. Overall, observation indicates the key functions of anti- to pro-inflammatory cytokine response during the entire pregnancy. Of note, limited evidences of inflammatory response have been documented before implantation of the embryo.

3. Interruption in Redox Balance

In normal homeostasis, ROS are neutralized by antioxidant defense *in vivo*. This balance is encountered by overpowering of ROS production and incompetency of antioxidant system to eliminate them. Growing evidences show that early exposure of oxidative stress in pregnancy might have long-term complications [51]. The antioxidant defense against locally produced NO by inducible nitric oxide synthase enzyme downregulates NO signals in the placenta which are crucially important for normal vascular development. In the first trimester of pregnancy, fetal growth was subjected to hypoxia [52], while in the prenatal period, it was documented that the fetus is highly vulnerable to oxidative damage whereas antioxidant supplementation during pregnancy ameliorates reproductive disorders such as implantation failure and fetal anomalies [53]. It has been reported that enhanced sodium dismutase-1 (SOD1) in mice suppresses fetal anomalies and

protects against diabetes-related embryopathy [54]. In pregnancy, having intrauterine growth restriction (IUGR), preeclampsia (PE), and gestational diabetes mellitus (GDM) has been recorded to have higher chances of fetal hypoxia (markers of oxidative stress). Moreover, a deficient supply of oxygen has been observed in pathogenesis of IUGR and PE conditions [55]; on the other hand, preterm delivery arises from ischemia-reperfusion injury which decreased body weight.

4. LPS-Driven Inflammatory Pathways

LPS activates inflammation through multifaceted mechanism [56, 57] Maternal LPS triggers embryonic resorption through strong cellular network which is responsible for increased excessive placental TNF-α, IL-1β, and IL-6 expressions that subsequently reduced phosphorylated Akt protein (serine/threonine-specific protein kinase) thereby causing decreased number of live pups, fetal weight, and placental weight [6, 58, 59]. Moreover, LPS also stimulates both transcription factors such as MAP kinases (MAPKs) and nuclear factor-κB (NF-κB) [60]. Prevalence of uterine inflammation is a major outcome of infection and idiopathic preterm birth [61] caused by alleviation of cytokine activity before preterm labor, cervix and fetal membranes by neutrophils and macrophages [62]. Several studies reported that proinflammatory cytokines such as IL-1β, IL-6, and TNF-α may activate contraction-associated proteins (CAPs) comprising oxytocin receptor (OTR), connexin 43 (CX43), prostaglandin H synthase- (PGHS-) 2, and prostaglandin receptors, in the myometrium, which exert uterotonic factors such as PGs that induce subsequently labor and inflammatory signals, suggesting a potential target in attenuating preterm birth [63]. In addition, normal labor in mouse associates with subsequent stimulation of NF-κB and AP-1 within the uterus, whereas LPS-induced preterm labor (PTL) in two mouse models has been reported to have activated NF-κB and Jun N-terminal kinase (JNK) transcription factors [64].

5. Pregnancy-Related Disorders and Adverse Birth Outcomes

5.1. Effects of LPS on Decidual Cells. The vast literature has been published on decidual cells, which focuses on pregnancy recognition, fetal growth, and survival. Decidual cells are the maternal tissue which acts under the influence of progesterone and testosterone in circulation in order to maintain growth following implantation of blastocyst with the endometrium. Later on, decidual and trophoblastic cells form the placenta of maternal portion [65]. Crosstalk between LPS and Toll-like receptor 4 (TLR4) resulted in harmful effects on pregnancy through releasing a variety of inflammatory cytokines in murine models [66]. Certain cytokines such as IL-4, IL-6, and IL-10 elicit beneficial effects on pregnancy [67]. Wang et al. [68] demonstrated that baicalin treatment at 4 μg/mL to uterine decidual cells which was cultured with LPS on day 6 of pregnancy. Meanwhile, in *in vivo* experiment, LPS was inducted at day 6 of pregnancy and subjected on oral doses of baicalin at day 7 and day 8 of pregnancy. The results documented that baicalin prevents damage to decidual cells and reduces TNF-α activity, hence producing fruitful effects on pregnant mice.

5.2. Maternal LPS-Mediated Teratogenicity. Some studies have found that LPS induces teratogenicity by overriding of free radicals. The subcut induction of LPS causes fetal malformation such as anencephaly and eye deformities [69] and developmental toxicity regulated by maternal side [70]. Uprising of tumor necrosis factor-alpha (TNF-α) in fetal liver and brain-induced fetal death occurs through either maternal circulation or amniotic fluid which mediated LPS induction [71]. In addition, LPS also induced lipid peroxidation and GSH depletion in maternal liver and placenta and increases expression of HO-1 in fetal liver that was counteracted by radical trapping agent N-tertiary-butylnitrone (PBN), a compound used for spin trapping. It has been well characterized that ROS are unstable reactive species which could not be eliminated successfully during organogenesis process and transfer from maternal to fetal tissues, irrespective of avoiding antioxidant defense. Hence, lacking of GSH proclaimed to develop ROS within fetal tissues. ROS developments in fetal tissues are not well clarified [72] though TNF-α can cross maternal serum and amniotic fluid to fetuses [71].

5.3. Oxidative Stress and Preterm Birth. Premature birth frequently occurs prior to normal delivery, when antioxidants could not be so active to alleviate oxidative stress resulting in preterm birth. It develops due to hindrance in uteroplacental transfer of nutrients which keeps newborns more sensitive against increasing ROS insults [73]. The MnSOD mRNA seems to be present in fetal membranes after labor and show its existence in chorioamnionitis [74]. It has been revealed that inflammation might be involved in placental antioxidant system which depends upon the concept development. Recently, studies rectified that [75, 76] cytokines are the main regulators for premature birth; hence, expression of NF-κB induced cytokines as a novel target for alternative therapeutic options. NF-κB is recognized in the induction of proinflammatory genes and mediates the expression of adhesion molecule, chemotactic factors, and acute phase proteins. The activation of NF-κB signaling pathway may enhance synthesis of proinflammatory cytokines that induce infected preterm birth [77, 78]. The current study has shown that polyphenols particularly curcumin exert beneficial effects on inhibition of NF-κB-linked pregnant tissue-infected premature birth in mice, suppress TNF-α and IL-8, and mitigate oxidative insult in mothers and fetuses [79].

5.4. Preeclampsia and Oxidative Stress. Preeclampsia seems to be reported after 20 weeks of gestation in humans [80]. Some literatures build up strong relations between maternal inflammation and oxidative stress. Researchers stated that increased maternal inflammation through a variety of signaling pathways and presence of oxidative stress might be the possible factors for inducing preeclampsia condition [81, 82]. In preeclampsia, reactive oxygen species initiates apoptosis of syncytiotrophoblast in placentation mechanism

Table 1: Some enlisted Nrf2 gene regulation in maternofetal tissues.

Origin	Regulation of Nrf2 protein/gene	Functional significance	References
Human umbilical endothelial cells	NQO1, GCLM, Nrf2, GSK3β	GDM ↑ oxidative stress and ↓ Nrf2 activity and overexpression of antioxidant expression	[112]
Rat	Nrf2, HO-1, SOD2	Hydroxytyrosol (HT) and moderate Restraint stress (GD14-20) ↑ Nrf2-dependent gene expression	[113]
Rat liver	GSTP, Nrf2	Maternal perfluorooctane sulfonate ↑ methylation of Nrf2-dependent GSTP gene promoter	[114]
$Nrf2^{-/-}$ and WT mice	Nrf2, GSTA3, MGST1, GSTA4 Gpx2, AKR1B1, AKR1B10, NQO1	Postnatal hyperoxia ↑ Nrf2-dependent gene expression, abolished in $Nrf2^{-/-}$ mice	[115]
Mouse embryos	Nrf2, SOD1, SOD2, SOD3, CAT, Trx, Gpx1, Gpx2, Gpx3, GR	Maternal ethanol or D3T exposure ↑ Nrf2-dependent gene expression	[116]
Mouse embryos	GSH, NQO1, HO-1, GCLC, GST, Prx1 Trx1, Trx2	Maternal D3T administration ↑ Nrf2-dependent gene and ↓ H2O2-induced Trx1 and Trx2 oxidation	[117]

AKR1B1: aldo-keto reductase family-1 member B1 (aldose reductase); AKR1B10: aldo-keto reductase family-10 member B10 (aldose reductase); CAT: Catalase); GCLC: glutamate-cysteine ligase catalytic subunit enzyme; GCLM: glutamate-cysteine ligase regulatory subunit enzyme; GDM: gestational diabetes mellitus; GR: glutathione reductase; GSK3β: glycogen synthase kinase 3 beta; GSH: glutathione peroxidase; GSTA3: glutathione S-transferase alpha-4; GSTA4: glutathione S-transferase alpha-4; Gpx1, 2, and 3: glutathione peroxidase 1, 2, and 3; GST: glutathione S-transferase; GSTP: glutathione reductase; GPO: glutathione peroxidase; HO-1: heme oxygenase; MGST1: microsomal glutathione S-transferase 1; NQO1: (NAD(P)H:quinone dehydrogenase 1; Nrf2: NF-E2-related factor 2; Prx1: peroxiredoxin 1; SOD1, 2, and 3: sodium dismutase 1, 2, and 3; Trx1 and 2: thioredoxin-1 and 2).

and affects anterior remodeling [83]; hence, it mediates inflammation. In addition, oxidative stress has been presumed to stimulate maternal endothelial cells as an inducer of preeclampsia condition [84].

5.5. Oxidative Stress-Induced IUGR Complications. Liu et al. [85] revealed that LPS induced intrauterine growth restriction in late gestation mice. It is stated that fetal IUGR is more susceptible in late gestation to increased risk of metabolic disorders such as insulin resistance, diabetes mellitus, obesity, hypertension, and cardiovascular diseases in model animals [86, 87]. Moreover, maternal protein restriction during pregnancy triggers fetal IUGR after prompt growth and alters gene expression in adipose tissue which is more prone to obesity in adult mice [88]. Numerous literatures established links of IUGR with oxidative stress. In IUGR pregnancies, oxidative stress markers such as MDA and protein oxidation of mother and fetus erythrocytes confirmed the strong relations [89]. In addition, oxidative/antioxidant markers were elevated in IUGR pregnancies, suggesting that neonates with IUGR elicited low level of antioxidant defense lipid peroxidation [90].

6. Significant Impact of Nrf2 Pathway on Pregnancy

Nrf2 is a leucine-based zipped transcription factor which displays a key role against oxidative stress by induction of phase II antioxidant enzymes [91]. Activation of Nrf2 is crucial for ameliorating oxidative stress-mediated cellular damage via protection through 20S proteasome or [92] by p62-dependent autophagy [93]. Normally, Nrf2 is located in Kelch-like ECH-associated protein-1 (Keap1) [94]. Keap1 functions as sensors for oxidative stress [95]; upon activation, Nrf2 binds with Maf recognition/antioxidant response element and electrophilic response element (ARE/EpRE) in promoter target genes encompassing NAD(P)H:quinone oxireductase 1 (NQO1) [96], heme oxygenase1 (HO-1) [97], glutamate cysteine ligase(GCL) [98], and the light chain of the amino acid cystine-glutamate exchanger (xCT) [99] involved in glutathione biosynthesis. Notably, more than hundred genes have been identified; many of them are redox-sensitive transcription factors [100].

Numerous reports were described the protective effects of Nrf2 on the embryo against adverse effects of oxidative stress in utero (Table 1). Nrf2 knockout mice were considered as indicators of placental oxidative stress which suppress fetal growth [101]. Nrf2-deficient mice are vulnerable to methamphetamine-induced fetal DNA insult and neurological deficits [102], whereas polyphenols such as hydroxytyrosol-induced Nrf2 stimulation ameliorate oxidative stress-mediated effects in cognitive function and neurogenesis in offspring [103]. Activation of Nrf2 has exhibited to reduce Et-OH-induced neural crest apoptotic cells in a fetus [104], and trophoblastic triggered apoptosis by inflammation [105]. At the same time, aforementioned studies indicated that Nrf2 exerts protective effects towards oxidative insult during early-pregnancy development (i.e., neutral crest formation), while some other studies documented significant effects of Nrf2 in redox mechanism in later-developmental phases. The in utero Keap1/Nrf2 signals have been demonstrated in response to amniotic fluid through increased expressions of genes contributed in epidermal development [106]. The Nrf2 is very sensitive to the maternal immune system to mediate the function of fetal membranes to birth. Importantly, Nrf2 protein expression was decreased in fetal membranes during pregnancy due to amniotic infection. The pitfalls in Nrf2 regulation can facilitate preterm delivery; knockdown of Nrf2 in amniotic cells causes upregulations of proinflammatory cytokines which causes rupturing fetal membranes. Moreover, a beneficial effect of Nrf2 on antioxidant mechanism

is more obvious in alleviating adverse developmental outcomes. In neural crest cells, where excessive glucose declines, CuZnSOD, MnSOD, catalase, GPx1, Nrf2, and Pax3 expressions induce vulnerability to these cells which leads to oxidative damage [107]. Importantly, overexpression of catalase enhances Nrf2 and its downstream HO-1 expression, thus showing a protecting role in obesity-induced diabetes fetal renal damage [108]. The Nrf2 expression is also decreased in IUGR placenta [109]. In preeclampsia pregnancies, the role of Nrf2 has been reported to be somehow controversial, whereas reduced expression of Nrf2 was noted in placental oxidative stress-induced preeclampsia [110]. Inappropriate regulation of Nrf2-based HO-1 expression mediates soluble fms-like typrsine kinase-1 (sFlt-1) [111]. Increased level of sFtl-1 has been recorded in the pathogenesis of PE and development of maternal hypertensive condition during pregnancy. Overall, Nrf2 function in normal pregnancy is incomplete although it is providing protection during uterofetal life against a variety of stressors.

Cheng et al. [112] have demonstrated that protein levels were significantly affected during redox status of GDM due to overproduction of superoxide radicals, protein oxidation, DNA damage, and reduced GSH synthesis. Moreover, in GDM cells, lipid peroxidation did not show Nrf2 genes and protein levels to its targeted genes NAD(P)H:quinone oxidoreductase 1 (NQO1), Bach1, cystine/glutamate transporter, and glutamate cysteine ligase. Lipid peroxidation triggered GSH and NQO1 activity which was revoked by Nrf2 in normal cells, and overexpression of Nrf2 in GDM cells partly restored NQO1 induction. Improper functions of Nrf2 in fetal endothelium increased the risk of inducing T2DM and CVD diseases to offspring. Zheng et al. [113] revealed the alternation in spontaneous activity and impair in learning and memory levels in prenatal stress male and female offspring. The stress was found to be due to downregulating of neuronal proteins and glucocorticoid levels. Similarly, alteration in protein oxidation, SOD, and mitochondrial activity was also declined, whereas hydroxytyrosol (HT) enhanced FOXO1 and FOXO3, Nrf2, and HO1 proteins and restored mitochondrial functions. It indicates that HT is a potential maternal nutritive compound that provides protection towards neurogenesis and cognitive offspring. In a study documented by Wan et al. [114], exhibiting the overexpressions of GSTP was contributed with transcription factors Keap1-Nrf2/MafK. Therefore, early induced alternations in cytosines within GSTP gene were referred as a biomarker of hepatic PFOS, whereas the direct role of PFOS-induced hepatotoxicity needs to be further elucidated. In another findings demonstrated by [115], it was shown that hyperoxia induced alveolar growth in neonatal lung by induction of p21/p53 pathways, a potential risk for developing bronchopulmonary dysplasia (BPD) in preterm infants. Results indicate that activation of Nrf2 pathway promoted antioxidant response genes which were declined by hyperoxia. Dong et al. [116] reported that exposure of maternal ethanol induces fetal alcohol syndrome that enhanced expression of Nrf2 and Nrf2-ARE protein levels in mouse embryos. Hence, it increases the response of proteins and antioxidant enzymes. In addition, dithiole-3-thione (D3T) treatment minimizes ethanol-mediated reactive oxygen species productions and inhibits apoptosis in mouse embryos. The results reported that simulation of Nrf2 was involved in releasing antioxidant response against exposure of ethanol embryos. In other investigation, it was documented that H_2O_2 decreased glutathione peroxidase (GSH), thioredoxin-1 (Trx1), and mitochondrial thioredoxin-2 (Trx2) in a whole cultured embryo with 10 μM dithiole-3-thione (D3T). D3T enhanced Nrf2 responsive genes. These findings showed that stimulation of Nrf2 provides protection against chemically mediated oxidative stress by maintaining intracellular redox mechanism, thereby stabilizing normal embryo development [117].

7. Dietary Supplementation of Polyphenols during Pregnancy

Flavonoids, the compounds of polyphenols, have received worldwide recognition due to their enormous existence in nature, and more than 10000 diverse molecular components have been identified so far [118]. Foods, vegetables, fruits, and herbs are rich sources of flavonoids [119]. It has come into central position due to presenting several functions encompassing antioxidant, anti-inflammatory, and antiabortogenic properties [120, 121]. LPS mediates inflammation through numerous series of cellular events that subsequently stimulates NF-κB pathway which encodes genes for inducing inflammation such as iNOS, NO, and COXs that synthesize prostaglandins and cytokines. Moreover, Toll-like receptors are responsible for the production of reactive oxygen species [122, 123]. As described above, LPS mediates pregnancy disorders and adverse birth outcomes through the regulation of proinflammatory cytokines such as TNF-α and IL-8 in maternal sera, amniotic fluid, fetal liver and fetal brain [124] and induced fetal IUGR, fetal resorption, and preterm delivery which was reversed by TNF-α inhibitor and chemokine inhibitor, respectively. Flavonoids suppress chemokine production comprising TNF-α, IL-1β, and monocyte chemoattractant protein-1 [125]; some protective effects of polyphenols are illustrated in Table 2.

The uptake of enriched polyphenol food has been documented to enhance plasma antioxidant status in humans [126] and reduce incidences of oxidative insult *in vitro* and *in vivo* studies in a human placenta and trophoblasts, respectively [127]. The flavonoids ameliorate oxidative stress-mediated inflammatory response by suppression of inflammatory mediators (reactive oxygen species (ROS) and nitric oxide (NO)), decreased inflammatory enzymes such as cyclooxygenases (COXs) and inducible nitric oxide synthase (iNOS) modulating NF-κB and activating protein-1 (AP-1) signals [26, 128] decreasing cytokine expressions, and activation of phase II enzymes glutathione S-transferase (GST) [129]. Supplementation of polyphenols has shown beneficial effects on pregnancy and was referred as therapeutic intervention to encounter pregnancy disorders and adverse birth outcomes [130]. Lack of antioxidant defense creates hindrances in homeostasis due to the exceeding amount of ROS, while their supplementation may show protective effects [130].

Table 2: Beneficial effects of polyphenols in LPS-induced pregnancy disorders.

LPS doses	Gestation stages (days)	Pregnancy disorders	Flavonoids/protective effects	References
LPS 0.2 mL/0.2 μg/mouse	4–7	Abortion	Quercetin indicates antiabortive effects through influence on CD4+/CD8+ T lymphocytes and IFN and IL-4	[136]
LPS 0.1 μg per mouse	6.7	Fetal resorption	Polyphenolic compounds of Radix Scutellariae and Rhizoma Atractylodis (baicalein, wogonin, oroxylin, baicalin, wogonoside, oroxylin A-7-glucuronide reduced fetal resorption and including IL-10 Pharmacological effects and pharmacokinetic properties of Radix Scutellariae and its bioactive flavones	[137, 138]
LPS, 0.1 mL/10 g *in vitro*/*in vivo*	6 6 & 7	Injury of decidual cells	Baicalin, 4 μg/mL *in vitro* and *in vivo* at different doses prevents decidual cell injury by inhibition of TNF-α	[68]
LPS at 0.2 ml, murine model	7	Abortion and reabsorption	Bao Tai Wu You, Tai Shan Pan Shi, or Bai Zhu San at 0.5 ml oral medication for 7 days ameliorates INF-γ and increases IL-10 and IL-4 thus showing beneficial effects	[139]

CD4 and 8: cluster of differentiation 4 and 8; IFN: interferon; IL-4: interleukine-4; IL-10: interleukine-10; INF-γ: interferon gamma; TNF-α: tumor necrosis factor-alpha.

Vanhees et al. [131] exhibited that exposure to intrauterine flavonoids such as quercetin and genistein at lower level inhibited oxidative stress and DNA damage in the liver of adult mice that subsequently develops antioxidant environment through regulation of Nrf2 signaling pathway. It indicates that dietary antioxidant supplementation during gestation develops long lasting antioxidant defense that eliminate oxidative stress at adulthood, where oxidative stress was assumed to be involved in chronic diseases. Importantly, LPS-mediated inflammation plays a key role in embryonic resorption, fetal growth restriction, and preeclampsia [132]. The polyphenols like curcumin ameliorate abnormal pregnancy outcomes by suppressed LPS-triggered inflammation in mice. The anti-inflammation activity of curcumin was achieved by upregulation of phosphorylated Akt pathway which was decreased by LPS induction [59]. Tricin, a polyphenol-derived compound, encountered inflammation by activation of Akt signals and cellular proliferation. This anti-inflammatory effect of Akt pathway was obtained by inhibition of IKK protein activity which brings NF-κB back into the cytoplasm in its normal physiological position [133]. Several compounds like the flavonoid group of polyphenols induced stimulation of Nrf2 signals. This evidence was proved by [134] who revealed the neuroprotective properties of polyphenols by activation of Nrf2/HO-1, thereby exerting therapeutic insights of polyphenolic compounds. Another example of epigallocatechin gallate (EGCG) treatment enhanced nuclear accumulation, anti-oxidant response element (ARE) binding with Nrf2.These results indicated that ECGC regulated Nrf2-mediated expression of few antioxidant enzymes particularly stimulation of Akt and ERK1/2 signaling; hence, it supports antioxidant system in attenuating oxidative stress [135]. Some polyphenols and their chemical structure are depicted in Figure 2. Literature has shown a less number of studies on antioxidant supplementation such as flavonoids during pregnancy, as it was known as strong antioxidative compounds proven from other evidence, whereas some report exhibited ambiguous results that might be due to timing of supplementation, improper dose regimen, and lack of antioxidant-targeted compounds. More research should be warranted to explore methods for minimizing uterine oxidative stress and mimic ROS-mediated diseases from mothers to newborns.

8. Concluding Remarks and Future Perspectives

We have clearly defined that stimulation of Toll-like receptors by LPS-induced generation of free radicals and their excessive amount leads to fetal resorption, preterm birth, teratogenicity, intrauterine growth restriction, abortion, neural tube defects, fetal demise, and skeletal development retardation. Moreover, oxidative stress also activates NF-κB, PPAR-γ, AP-1, and JNK pathways which accounts for pathological conditions in aforementioned pregnancy disorders. In addition, NF-κB is responsible for transcription of several proinflammatory cytokines which are known to induce pregnancy disorders and adverse outcomes such as TNF-α, IL-1β, IL-6, and PGF2E. Importantly, stimulation of Nrf2 signals plays a crucial role in ameliorating pregnancy insults. It was further counted that oxidative stress is the major contributing factor, whereas polyphenols are the novel compounds for treating oxidative stress-related disorders. Limited studies have been documented on polyphenol supplementation during pregnancy and its outcomes. It was presumed that intrauterine fetal life decides the future of a wide array of complications which appear at later stages of life. Nutrition and antioxidant supplement are the main players for fetal reprogramming. Any impairment in this system might have disturbance in extrauterine life. Studies reported that strong maternal uterine antioxidant environment could prevent pregnancy disorders and abnormal birth outcomes and could also prevent other complications later in life which might initiate from embryonic stage. More molecular evidences are required for antioxidant/inflammatory events from

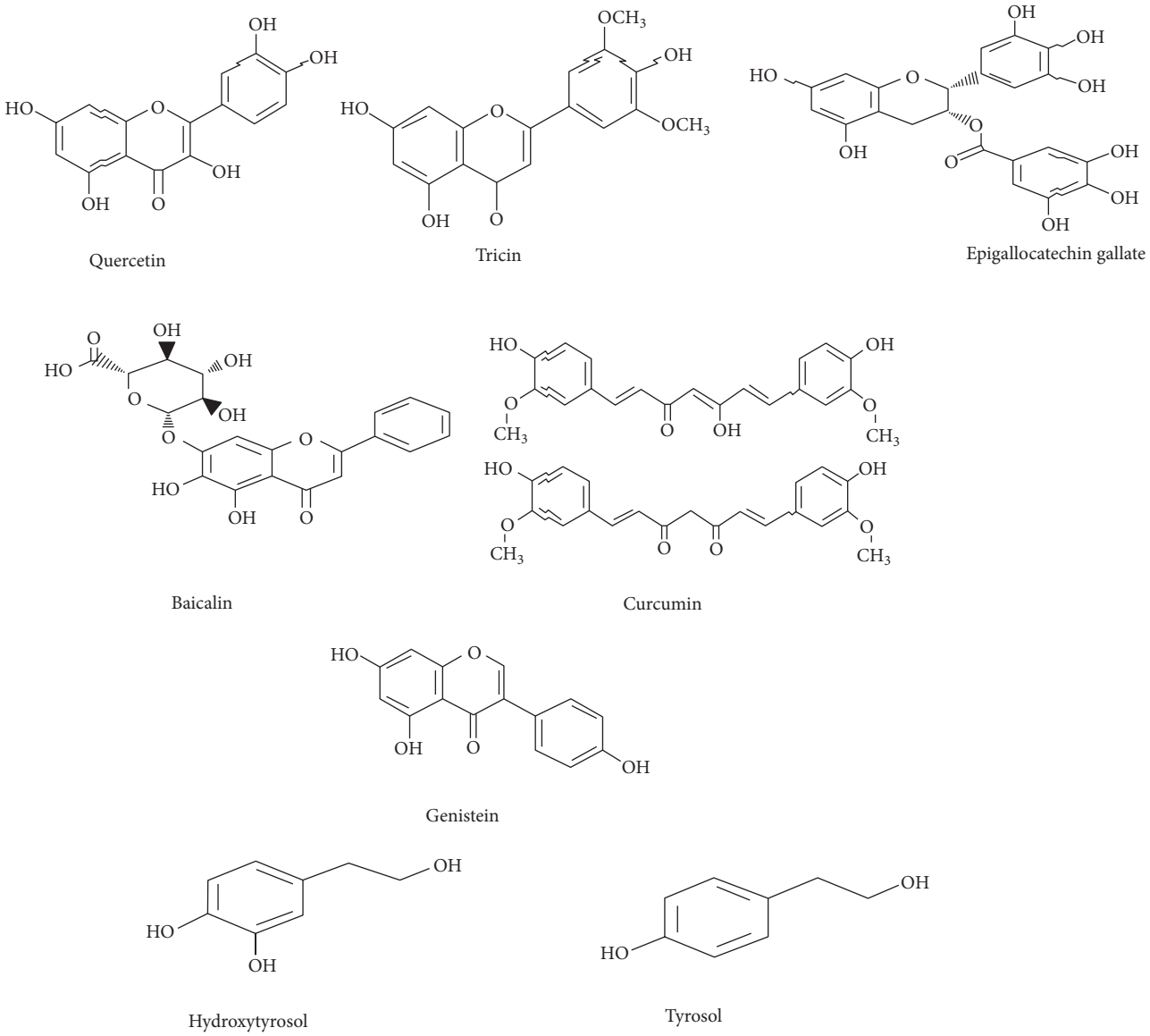

Figure 2: Some polyphenol compounds and their chemical structures.

fertilization to parturition during pregnancy. We assume that these findings would be helpful in understanding oxidative stress-induced pregnancy insults and might give new road-map to researchers for therapeutic intervention which could subsequently improve human and animal fertility.

Abbreviations

Akt:	Serine/threonine-specific protein kinase
AP-1:	Activated protein kinase-1
CAPs:	Contraction-associated proteins
CD14:	Cluster of differentiation 14
COX-2:	Cyclooxygenases-2
CX43:	Connexin 43
EGCG:	Epigallocatechin-3-gallate
ERK1/2:	Extracellular signal-regulated kinase 1/2
ESC-B5:	Embryonic stem cell B5
FGR:	Fetal growth rate
FOXO1 and 3:	Forkhead box protein O 1 and 3
GCL:	Glutamate cysteine ligase
GDM:	Gestational diabetes mellitus
GST:	Glutathione S-transferase
HO-1:	Heme oxygenase1
IFNγ:	Interferon-γ
iNOS:	Inducible nitric oxide synthase
IUGR:	Intrauterine growth restriction
JNK:	Jun N-terminal kinase transcription factors
LPS:	Lipopolysaccharides
MAPKs:	Mitogen-activated protein kinases
MDA:	Malondialdehyde
MYD88:	Myeloid differentiation factor
NF-κB:	Nuclear factor kappa B
Nrf2:	NF-E2-related factor 2
OTR:	Oxytocin receptor

NO: Nitric oxide
PE: Preeclampsia
PGs: Prostaglandins
PGHS: Prostaglandin H synthase
PPARγ: Peroxisome proliferator-activated receptor gamma
ROS: Reactive oxygen species
sFlt-1: Soluble fms-like typrsine kinase-1
SOD1: Sodium dismutase-1
TNF-α: Tumor necrosis factor-α
uNK: Uterine natural killer
xCT: Cystine-glutamate exchanger.

Conflicts of Interest

The authors declare not any conflict of interest.

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Research Article

Expression of the *NRF2* Target Gene *NQO1* Is Enhanced in Mononuclear Cells in Human Chronic Kidney Disease

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Reduced nuclear factor erythroid 2-related factor 2 (*NRF2*) pathway activity was reported in models of chronic kidney disease (CKD). Pharmacological activation of *NRF2* is supposed to improve renal function, but data concerning the *NRF2* activity in human CKD are lacking. We investigated the *NRF2* target NAD(P)H:quinone oxidoreductase 1 (*NQO1*) as a readout parameter for *NRF2* activity in monocytes of CKD patients ($n = 63$) compared to those of healthy controls ($n = 16$). The *NQO1* gene expression was quantified using real-time PCR and the protein content by in-cell Western assays. We found a 3-4-fold increase in *NQO1* gene expression in CKD 1–5 ($n = 29$; 3.5 for *NQO1*/ribosomal protein L41; $p < 0.001$). This was accompanied by a 1.1-fold increase in *NQO1* protein ($p = 0.06$). Cardiovascular disease prevalence was higher in CKD 1–5 patients with higher compared to those with lower *NQO1* gene expression ($p = 0.02$). In advanced uremia, in dialysis patients ($n = 34$), *NQO1* gene expression was less robustly upregulated than that in CKD 1–5, while *NQO1* protein was not upregulated. We conclude that in mononuclear cells of CKD patients, the *NRF2* pathway is activated by coexisting pathogenic mechanisms, but in advanced uremia, the effectiveness of this upregulation is reduced. Both processes could interfere with pharmacological *NRF2* activation.

1. Introduction

The transcription factor *NRF2* is a master transcriptional regulator of cellular response to oxidative and electrophilic stress. It activates a multitude of cellular defense processes through induction of its target genes, including drug-metabolizing and antioxidant enzyme genes [1]. Oxidative or electrophilic stress can activate the *NRF2* pathway by an interaction with the cytoplasmic complex between Kelch-like ECH-associated protein 1 (*KEAP1*) and *NRF2*. As a result, *NRF2* can escape ubiquitination and proteasomal degradation, accumulate, and translocate to the nucleus. It forms heterodimers with small Maf proteins, binds to antioxidant/electrophilic response elements, and thereby finally induces target gene expression [1, 2].

Cytosolic *NQO1* is a conserved target gene of *NRF2* and can serve to monitor the activity of the *NRF2* pathway [2]. During its clinical development, the *NRF2* activator bardoxolone methyl was investigated in a phase 1 clinical trial, in patients with advanced malignancies. In this trial, the *NQO1* gene expression levels in peripheral blood mononuclear cells (PBMCs) were shown to be indicative of *NRF2* pathway activation by the substance [3].

In animal models of renal diseases, impaired activity of the *NRF2* pathway and downregulation of the *NQO1* gene expression are major findings, both in renal and in nonrenal cells [2, 4, 5]. Furthermore, it was shown in animal models that activation of the *NRF2* pathway and increased gene transcription, including those of *NQO1*, attenuated kidney injury [6–8]. Therefore, there is a strong interest

in the therapeutic potential of *NRF2* activators in kidney disease [9]. A phase 2b clinical trial with the *NRF2* activator bardoxolone methyl in CKD stages 3b and 4 demonstrated an improvement in estimated glomerular filtration rate (eGFR) but suggested adverse effects, for example, on liver tissue [10]. A phase 3 clinical trial in CKD stage 4 patients (BEACON) with the same substance also showed an increased eGFR but had to be terminated because of a higher rate of cardiovascular events in the treatment group [11]. Currently, one more phase 2 clinical trial with bardoxolone methyl in CKD patients is recruiting participants (NCT02316821). In the publication of the so far largest clinical trial of bardoxolone methyl, the authors refer to the impairment of *NRF2* activity-dependent gene transcription that was reported in animal models of CKD as one rational for the clinical application of the substance in CKD [11]. Accordingly, in our study, we investigated the gene expression of *NQO1* as a parameter of *NRF2*-dependent gene transcription in human CKD. We present a systematic analysis of this drug target on the level of gene expression and protein content in CKD patients with and without renal replacement therapy. The BEACON trial was terminated because of a higher rate of cardiovascular events in the intervention group [11]. A post hoc analysis by the manufacturer of the substance identified prior hospitalization for heart failure as a risk factor for heart failure experienced during bardoxolone treatment [12]. Therefore, we investigated a relation between the *NQO1* gene expression as a measure for *NRF2* pathway activity and cardiovascular disease (CVD) prevalence in patients with non-dialysis-dependent CKD.

Since systemic *NRF2* activators target the *NRF2* pathway systemically and nonrenal adverse events were reported, both nonrenal and renal cells of CKD patients require investigation.

We therefore quantified the *NQO1* gene expression as a readout parameter for *NRF2* pathway activity in monocytes of patients with CKD with and without dialysis therapy and compared it to those of healthy control subjects. In parallel, *NQO1* protein content was quantified in cells of the same subjects.

2. Subjects and Methods

2.1. Study Subjects. We prospectively enrolled 63 consecutive patients with CKD from the outpatient clinics of the Department of Nephrology, Odense University Hospital, and 16 healthy control subjects. Among the group of CKD patients, 34 were undergoing dialysis therapy (hemodialysis, $n = 33$; peritoneal dialysis, $n = 1$). The study was approved by the regional ethics committee and written informed consent was obtained. Hemodialysis patients were dialyzed with biocompatible membranes, three times per week, for 4-4.5 hours. Healthy control subjects were free from chronic or acute disease. Study participants were 30 years or older. CKD patients had verified CKD according to the 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease, and patients with dialysis therapy had been in dialysis treatment for at least 3 months [13]. Exclusion criteria included pregnancy or breastfeeding, acute illness leading to hospital admission, and functioning renal allograft.

2.2. Collection of Clinical Data and Blood Samples. Clinical data were obtained by physical examination, medical history taking, and electronic medical records. This included the record of age, sex, height, weight, smoking status, medical history, and use of medications. Venous blood samples were drawn in the morning from study participants. Samples from hemodialysis patients were obtained before the start of hemodialysis sessions. Plasma and serum were separated and stored at −80°C. The eGFR was calculated using the 2009 CKD-EPI creatinine equation, and CKD stages were determined based on eGFR categories according to the KDIGO 2012 guideline [13].

2.3. Isolation of Circulating Monocytes. We isolated PBMCs by density gradient centrifugation (Histopaque-1077, Sigma-Aldrich, Denmark). Monocytes then were isolated from PBMCs using anti-CD14-coated superparamagnetic polystyrene beads (Fisher Scientific, Denmark) according to the manufacturer's protocol.

2.4. RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR (RT-qPCR). Total RNA was isolated from monocytes using an RNeasy Mini kit (Qiagen, Denmark) according to the protocol described by the manufacturer. cDNA was synthesized from 200 ng of total RNA by reverse transcription using a Transcriptor First-Strand cDNA Synthesis Kit (Roche, Denmark). The amplification of genes was performed by quantitative real-time PCR using SYBR Green (FastStart Essential DNA Green Master, Roche, Denmark). The PCR conditions using a LightCycler96 Instrument (Roche, Denmark) were as follows: 95°C for 600 s and 50 cycles of 95°C for 10 s, 64°C (for *NQO1*, beta-actin (*ACTB*), and ribosomal protein L41 (*RPL41*)) or 60°C (for TATA-box binding protein (*TBP*)) for 10 s, and 72°C for 10 s. The primers used were in Table 1.

To ensure a high reliability of the gene expression results in our study, we tested a group of available reference genes in the cellular material of our study. We then employed the "NormFinder" algorithm (http://moma.dk/normfinder-software; [14]). The reference genes with the highest expression stability in our sample material were *RPL41*, *ACTB*, and *TBP* with stability values (SE) of 0.12 (0.06), 0.18 (0.06), and 0.22 (0.05), respectively. We therefore used these three genes for the relative quantification of *NQO1* gene expression. The ratio was calculated as follows: $\text{ratio} = 2^{(\text{Cq reference gene})}/2^{(\text{Cq } NQO1)}$.

PCR products were size-fractionated on agarose gels for product length control.

In the PCRs, water controls, no-template controls, and no-RT controls were included. A melting curve analysis was performed for each sample to ensure product homogeneity. All measurements were performed in duplicate.

2.5. SDS-PAGE and Western Blotting. Protein was extracted from monocytes using an extraction reagent for mammalian

Table 1

Name of gene product	Forward primer Reverse primer	Expected PCR product length (bp)
NQO1 NM_000903.2	5′-CTGCCATCATGCCTGACTAA-3′ 5′-TGCAGATGTACGGTGTGGAT-3′	216
ACTB NM_001101.3	5′-GGACTTCGAGCAAGAGATGG-3′ 5′-AGCACTGTGTTGGCGTACAG-3′	234
RPL41 NM_021104.1	5′-AAGATGAGGCAGAGGTCC-3′ 5′-TCCAGAATGTCACAGGTCCA-3′	248
TBP NM_003194.4	5′-TGCACAGGAGCCAAGAGTGAA-3′ 5′-CACATCACAGCTCCCCACCA-3′	132

cells including a protease inhibitor cocktail (cOmplete Lysis-M, pH 7.6, Roche, Denmark). Proteins were separated by 10% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (10% RunBlue SDS gel, Expedeon, UK) at 120 V for 45 minutes and transferred to polyvinylidendifluoride membranes at 100 V for 75 minutes (Immun-Blot LF PVDF, Bio-Rad, USA). Membranes were blocked with blocking buffer (Superblock, Thermo Fisher Scientific, USA) for 1 hour at room temperature and incubated with the primary antibodies rabbit anti-human *NQO1* (ab34173, Abcam, UK) or rabbit anti-human *ACTB* (sc-130656, Santa Cruz Biotechnology, Germany). After washing with tris(hydroxymethyl)aminomethane-buffered saline, the membranes were incubated with the fluorescence-labeled secondary antibody $F(ab')_2$-goat anti-rabbit IgG (H+L) Alexa Fluor 488 (Fisher Scientific, Denmark). Imaging was performed using a Carestream Imager 4000pro (Fisher Scientific, Denmark) at 535 nm emission with an excitation wavelength of 470 nm.

2.6. Quantitative In-Cell Western Assay. To quantify the *NQO1* protein content in circulating monocytes, in-cell Western assays were performed as recently described by our group [15, 16]. For that purpose, monocytes were fixed with formaldehyde and permeabilized using Triton X-100 in 96-well plates. Cells were blocked using 1% bovine serum albumin overnight at 4°C, then incubated with the primary antibodies rabbit anti-human *NQO1* (ab34173, Abcam, UK) or rabbit anti-human *ACTB* (sc-130656, Santa Cruz Biotechnology, Germany). After washing, cells were incubated with the fluorescence-labeled secondary antibody $F(ab')_2$-goat anti-rabbit IgG (H+L) Alexa Fluor 488 (Fisher Scientific, Denmark). All measurements were performed in triplicate, and the *NQO1* protein content was analyzed relative to the *ACTB* protein content as an internal reference. Imaging was performed using an EnSpire Multimode Plate Reader (PerkinElmer, Denmark) at 520 nm emission with an excitation wavelength of 490 nm.

3. Statistics

Continuous variables are given as median and interquartile range, and categorical variables are given as counts and percentages. Groups were compared using Kruskal-Wallis test with Dunn's posttest or Mann–Whitney test. Differences in categorical variables between groups were analyzed by X^2 test (GraphPad prism software, version 5.0, GraphPad Software, San Diego, CA). All statistical tests were two-sided and a two-sided value of $p < 0.05$ was considered statistically significant.

4. Results

Population characteristics of study subjects are shown in Table 2.

The *NQO1* gene expression was significantly higher in monocytes from patients with CKD 1–5 (Kruskal-Wallis, $p = 0.004$ for *NQO1*/*ACTB*; $p < 0.001$ for *NQO1*/*RPL41*; and $p < 0.001$ for *NQO1*/*TBP*; Figures 1(a) and 1 (b)). Compared to healthy controls, patients with CKD 1–5 showed a 3-4-fold increase in the *NQO1* gene expression (3.1 for *NQO1*/*ACTB*, 3.5 for *NQO1*/*RPL41*, and 4.2 for *NQO1*/*TBP*). In CKD 5 patients undergoing dialysis therapy (CKD 5D), median *NQO1* gene expression was numerically higher than that in healthy controls but this was significant only for the *NQO1*/*TBP* ratio (a 2.6-fold increase for *NQO1*/*TBP*, $p < 0.05$, Figure 1(b)). The relation between the *NQO1* gene expression level and CKD stage followed approximately a bell shape. This is shown in the Supplementary Figure 1 available online at https://doi.org/10.1155/2017/9091879 representing the distribution of the *NQO1* gene expression at the different CKD stages including advanced CKD with hemodialysis therapy (Kruskal-Wallis, $p = 0.005$).

We also quantified the *NQO1* protein content in cells of the identical blood samples used for the gene expression analyses described above. First, we proved that in the cell material used for our study, the antibodies effectively detected *NQO1* and *ACTB* proteins. Immunoblot analyses showed staining of protein bands at the expected sizes (Figure 2(a)). Figure 2(b) shows a representative example of an in-cell Western assay used for the relative quantification of the NQO1 protein content in monocytes. Measurements were performed in triplicate for each subject. Figure 2(c) depicts the summary data for all in-cell Western analyses. The *NOQ1* protein content showed a 1.1-fold increase in CKD 1–5 patients compared to healthy controls (Mann–Whitney, $p = 0.06$). In CKD 5D, the *NQO1* protein content was not different from control subjects (Mann–Whitney, $p = 0.72$).

Furthermore, since cardiovascular events led to the early termination of a large *NRF2* activator trial in

Table 2: Clinical and demographical population characteristics.

	Healthy ($n = 16$)	CKD 1–5 ($n = 29$)	CKD 5D ($n = 34$)
Age, years	38 (33–48)	68 (58–77)	67 (55–76)
Men, *n* (%)	9 (56)	19 (66)	23 (68)
BMI, kg/m^2	24 (22–26)	30 (24–37)[a]	24 (22–28)[b]
Smoking, *n* (%)	2 (13)	3 (10)	6 (18)
Kidney disease, underlying cause, *n* (%)	None		
Glomerulonephritis		8 (28)	5 (15)
Hypertensive nephropathy		3 (10)	5 (15)
Interstitial nephritis		2 (7)	4 (12)
Diabetic nephropathy		1 (3)	2 (6)
Hereditary kidney disease		1 (3)	2 (6)
Other/unknown		14 (48)	16 (47)
Comorbidities, *n* (%)	None		
Hypertension		28 (97)	26 (76)
Diabetes		8 (28)	8 (24)
CVD			
Myocardial infarction, coronary artery disease		6 (21)	10 (29)
Heart failure		8 (28)	16 (47)
Cerebrovascular disease		4 (14)	6 (18)
Peripheral artery disease		1 (3)	9 (26)
Medications	None		
AT receptor antagonist, ACE inhibitor		22 (76)	16 (47)
Beta blocker		9 (31)	15 (44)
Calcium channel inhibitors		13 (45)	9 (26)
Platelet aggregation inhibitor		6 (21)	6 (18)
Diuretic		19 (66)	11 (32)
Erythropoietin analog		6 (21)	29 (85)
Coumarin derivatives		5 (17)	6 (18)
eGFR, mL/min/1.73m^2	n.d.	28 (17–43)	n.a.
Time on dialysis, months	n.a.	n.a.	22 (11–49)
CRP, mg/L	n.d.	5.3 (2.3–10.8)[c]	3.0 (1.4–10.5)[d]
Albumin, g/L	n.d.	38 (35–41)[e]	38 (36–40)

Values are given as median (25%–75% percentile) or number (percentage). BMI: body mass index; AT: angiotensin; ACE: angiotensin converting enzyme; CRP: C-reactive protein; n.d.: not done; n.a.: not applicable. [a]$n = 27$; [b]$n = 33$; [c]$n = 28$; [d]$n = 33$; [e]$n = 28$.

non-hemodialysis-dependent CKD, we investigated the prevalence of CVD in our study. For the analysis of the *NQO1* expression and prevalence of CVD in non-hemodialysis-dependent CKD stages 1–5, we grouped the patients according to their *NQO1* gene expression below or above the median and compared the frequency of CVD between them. When we analyzed the overall prevalence of CVD (myocardial infarction/coronary artery disease, heart failure, cerebrovascular disease, and peripheral artery disease), it was 2 out of 14 in the patient group below median gene expression whereas in the group above median *NQO1* gene expression, the prevalence of CVD was 8 out of 14 (X^2 test, $p = 0.02$). The only common underlying single disease type in our patient group was cardiac insufficiency (1 out of 14 in the below median group versus 7 out of 14 in the above median group; X^2 test, $p = 0.18$).

5. Discussion

This study showed for mononuclear cells of CKD patients (i) increased gene expression of the *NRF2* target *NQO1* in CKD 1–5, (ii) compared to CKD 1–5 a less robust increase in the *NQO1* gene expression in CKD 5D, (iii) slightly increased *NQO1* protein content in CKD 1–5, and (iv) higher prevalence of CVD among CKD 1–5 patients with a higher *NQO1* gene expression.

In our study, we investigated monocytes from peripheral blood of CKD patients. The monocyte-macrophage lineage is of special importance in CKD. On the one hand, they are involved in kidney injury and fibrosis [17, 18]. On the other hand, monocytes and monocyte-derived macrophages are involved in the pathogenesis of atherosclerosis and vascular disease in CKD [19–22]. The latter is important with respect to our finding of higher CVD

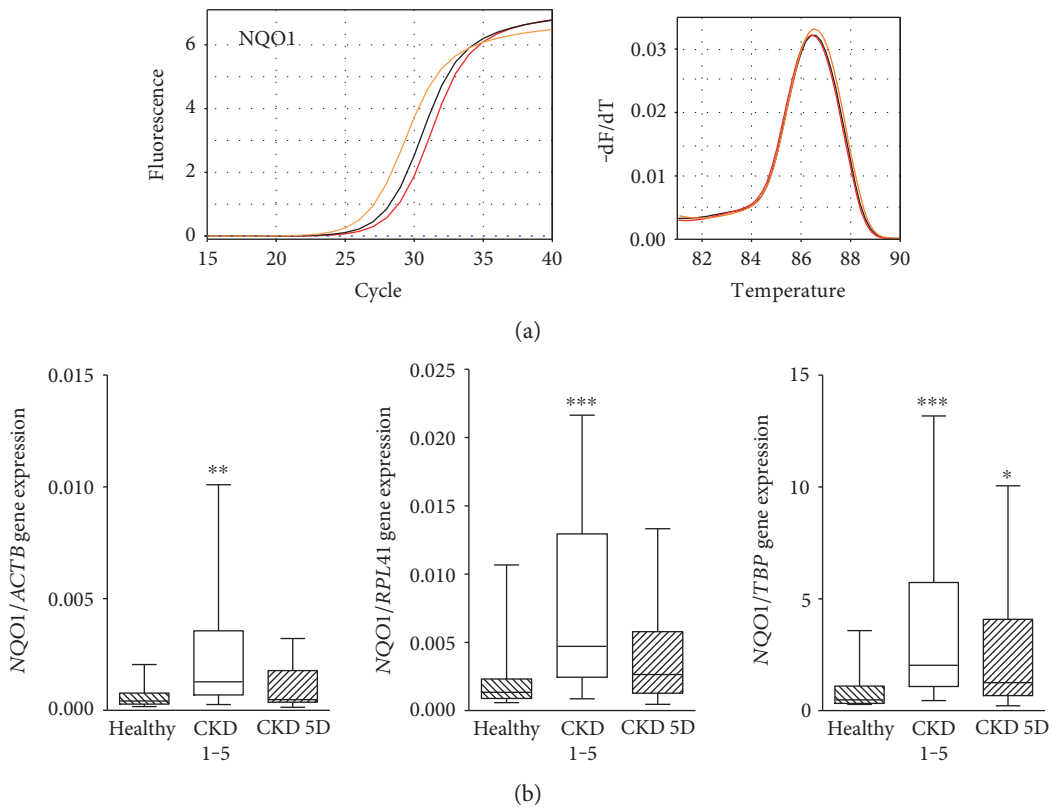

Figure 1: *NQO1* gene expression. (a) Amplification curves and melting curves for *NQO1* in monocytes from a patient with CKD 4 (CKD, orange), a hemodialysis patient (CKD 5D, black), and a healthy subject (red). (b) Box and whisker plots (whiskers, minimum to maximum) showing summary data of the *NQO1* gene expression in healthy subjects ($n = 16$), CKD 1–5 patients ($n = 29$), and CKD 5D patients ($n = 34$) normalized to *ACTB*, *RPL41*, and *TBP*. Comparison by Dunn's posttest. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

prevalence in CKD patients with an *NQO1* gene expression above the median in monocytes.

Why did we choose *NQO1* and in particular the *NQO1* gene expression as a readout parameter for *NRF2* pathway activity and activation in kidney disease? Several lines of evidence support our approach. First, *NQO1* belongs to the conserved *NRF2* target genes, and in a recent integrated transcriptomic and proteomic analysis of *NRF2* function, *NQO1* was confirmed as a robust marker for *NRF2* activity [2, 23]. Second, reduced expression of *NQO1* was shown in several kidney disease models, also in nonrenal cells, and upregulation of *NQO1* was repeatedly shown in *NRF2*-dependent kidney protection, again including nonrenal cells [4–8, 24]. Third, it has been shown that the *NQO1* expression is stimulated by the *NRF2* activators CDDO (1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazolide), dh404 (2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid-9,11-dihydro-trifluoroethyl amide), and bardoxolone methyl and that this effect is abrogated in $NRF2^{-/-}$ mice [5, 8, 23]. Notably, bardoxolone methyl stimulates the *NQO1* gene expression also in PBMCs [3].

Some consideration should be given to the effect size of the *NQO1* increase in our study. With respect to the gene expression of *NQO1*, we confirmed a 3-4-fold increase in *NQO1* mRNA in CKD 1–5 patients compared to healthy control subjects using three different housekeeping genes for the relative quantification. A 5.6-fold increase in *NQO1* mRNA was reported to be present in PBMCs from cancer patients after 3 weeks of treatment with bardoxolone methyl [3]. In T cell-specific *KEAP1*-deficient mice, T cells showed an 8-fold relative change in *NQO1* mRNA [7]. In a mouse model of ischemia-reperfusion injury- (IRI-) induced kidney damage, the maximal response of *NQO1* mRNA to IRI was an ~1.8-fold increase in wild-type mice and a 5-fold increase in *KEAP1*-knockdown mice [8]. We therefore conclude that the difference in *NQO1* mRNA level that we observed in our study is of relevant magnitude and points to an activation of the *NRF2* pathway in CKD patients per se.

On the protein level, the *NQO1* protein content in CKD 1–5 patients was not significantly increased to 1.1-fold compared to that in control subjects in our study. Similarly, the treatment of mice with bardoxolone methyl resulted in an ~1.3-fold increase in *NQO1* protein level [23]. Treatment of 5/6 nephrectomy rats with dh404 resulted in an ~3.3-fold increase in *NQO1* protein in the colon [5]. However, the increase in the *NQO1* protein content that we observed in CKD 1–5 needs further investigation.

Effects on gene expression and protein content in CKD are mediated by a multitude of mechanisms. The observed increase in the *NQO1* gene expression in our study might be a response to NRF2-stimulating conditions prevalent in patients with CKD, like oxidative stress or

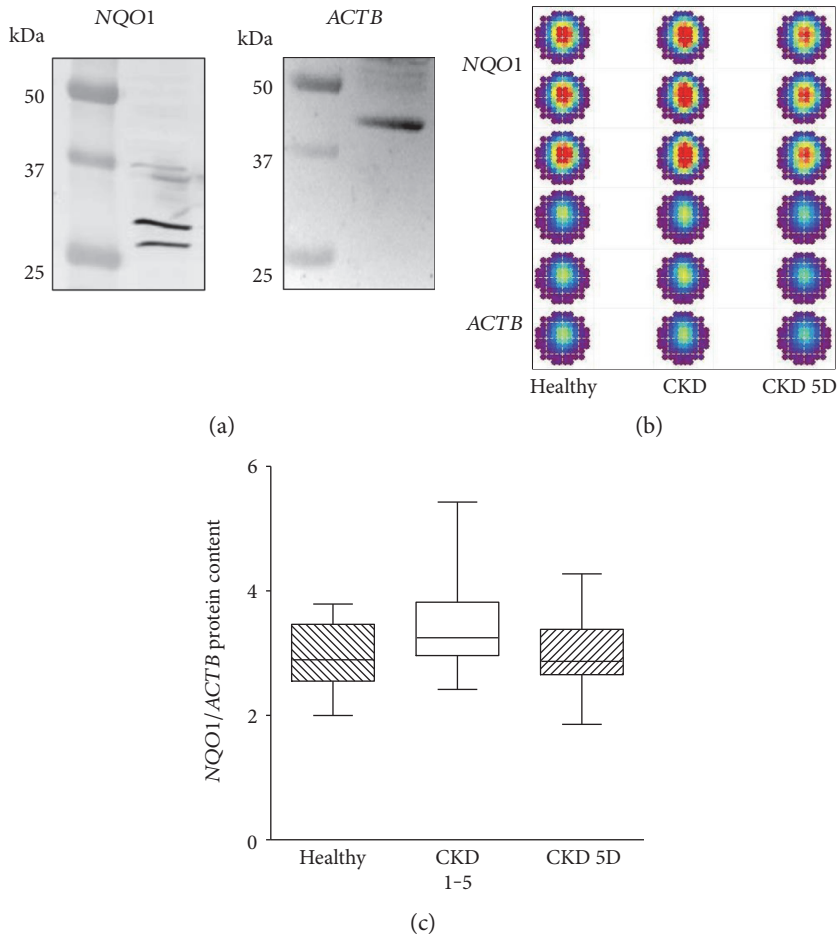

Figure 2: *NQO1* protein content. (a) To show the effective detection of *NQO1* and *ACTB* protein by antibodies in the cell material used in our study, we performed immunoblot analyses with cells obtained from healthy control subjects. Immunoblots of *NQO1* (expected size 26/27 kDa and 31 kDa) and *ACTB* (expected size 42 kDa) in monocytes are shown. (b) Pseudocolored fluorescence intensities of in-cell Western assays for the quantification of the *NQO1* protein content relative to the *ACTB* protein content in monocytes from a healthy subject, a patient with CKD, and a patient with CKD 5D. Measurements were always performed in triplicate. (c) Box and whisker plots (whiskers, minimum to maximum) showing summary data of *NQO1* protein in healthy subjects ($n = 13$), CKD 1–5 patients ($n = 23$), and CKD 5D patients ($n = 29$) normalized to *ACTB*. $p = 0.07$ by Kruskal-Wallis test.

lipopolysaccharide-induced inflammation [25, 26]. Patients with coexisting CVD, which is also associated with oxidative stress, might be especially affected [27]. This is supported by the higher CVD prevalence in CKD 1–5 patients with a higher *NQO1* gene expression that we observed. In addition, low concentrations of the uremic toxin methylglyoxal were shown to increase the *NQO1* mRNA concentration [28].

We also observed that the increase in the *NQO1* gene expression in CKD 5D patients was less pronounced compared to that in CKD 1–5 patients. This is in line with a depressive effect of high concentrations of uremic toxins on the *NRF2* pathway. This effect might be more pronounced in this patient group with advanced uremia. For example, the uremic toxin indoxyl sulfate was shown to downregulate *NRF2* mRNA and *NRF2* protein content [24].

The protein content of *NQO1* appeared slightly upregulated in CKD 1–5 patients and not upregulated in long-term uremic CKD 5D patients. Such discrepancy between gene expression and protein content was already demonstrated by our group for another cytosolic antioxidant protein that also is under the transcriptional regulation of an antioxidant response element—superoxide dismutase type 1 [15].

Our study adds important information to the discussion about the putative use of *NRF2* activators in CKD. *NRF2* activators serve to upregulate the expression of *NRF2* target genes like *NQO1*. However, our study showed that the *NQO1* gene expression was already significantly upregulated in monocytes, which points to a relevant stimulation of the *NRF2* pathway in these cells in CKD patients. Therefore, as the systemic application of *NRF2* activators affects different cell and tissue types, the *NRF2* pathway in kidney disease needs to be investigated in different human cells and tissues. This is of relevance as the overactivation of a potent transcription factor like *NRF2* was suggested as potentially deleterious for the cardiovascular system [27]. Therefore, a preexisting activation of the *NRF2* system in CKD patients especially with CVD, as suggested by our results, could be seen in line with the increased rate of cardiovascular events in the BEACON trial [11]. Moreover, an additional

depressive effect of CKD on antioxidant enzymes, especially in advanced uremic conditions, could reduce effectiveness of *NRF2* activation.

Taken together, we showed a relevant upregulation in gene expression of the *NRF2* target *NQO1* in patients with CKD 1–5 together with a slight increase in the *NQO1* protein content in monocytes from these patients. Moreover, we found that in more pronounced uremia (CKD 5D), the *NQO1* gene expression was less upregulated than that in CKD 1–5 and *NQO1* protein content was not increased. We conclude that in CKD patients, *NRF2* activation is modulated through influence on both gene expression and protein content of *NRF2* targets in a complex way.

Conflicts of Interest

None are declared.

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Research Article

Commiphora molmol Modulates Glutamate-Nitric Oxide-cGMP and Nrf2/ARE/HO-1 Pathways and Attenuates Oxidative Stress and Hematological Alterations in Hyperammonemic Rats

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Hyperammonemia is a serious complication of liver disease and may lead to encephalopathy and death. This study investigated the effects of *Commiphora molmol* resin on oxidative stress, inflammation, and hematological alterations in ammonium chloride-(NH_4Cl-) induced hyperammonemic rats, with an emphasis on the glutamate-NO-cGMP and Nrf2/ARE/HO-1 signaling pathways. Rats received NH_4Cl and *C. molmol* for 8 weeks. NH_4Cl-induced rats showed significant increase in blood ammonia, liver function markers, and tumor necrosis factor-alpha (TNF-α). Concurrent supplementation of *C. molmol* significantly decreased circulating ammonia, liver function markers, and TNF-α in hyperammonemic rats. *C. molmol* suppressed lipid peroxidation and nitric oxide and enhanced the antioxidant defenses in the liver, kidney, and cerebrum of hyperammonemic rats. *C. molmol* significantly upregulated Nrf2 and HO-1 and decreased glutamine and nitric oxide synthase, soluble guanylate cyclase, and Na^+/K^+-ATPase expression in the cerebrum of NH_4Cl-induced hyperammonemic rats. Hyperammonemia was also associated with hematological and coagulation system alterations. These alterations were reversed by *C. molmol*. Our findings demonstrated that *C. molmol* attenuates ammonia-induced liver injury, oxidative stress, inflammation, and hematological alterations. This study points to the modulatory effect of *C. molmol* on glutamate-NO-cGMP and Nrf2/ARE/HO-1 pathways in hyperammonemia. Therefore, *C. molmol* might be a promising protective agent against hyperammonemia.

1. Introduction

Hepatic encephalopathy (HE) is a serious complication of both acute and chronic liver diseases [1, 2]. HE has been estimated to occur in 10–50% of patients with transjugular intrahepatic portosystemic shunt and 30–45% of patients with cirrhosis, whereas minimal HE affects 20–60% of patients with liver disease [3]. Although the pathological mechanism of HE is not fully understood, ammonia is known to play a key role in HE [4]. Ammonia is a known neurotoxin and induces harmful effects to the central nervous system [5]. Blood ammonia level is strongly correlated with the

increased risk of HE and is therefore used as a diagnostic marker for encephalopathy [6]. Ammonia is normally detoxified in the liver via urea cycle, and if does not proceed properly, as in cases of liver failure or congenital defect of the urea cycle enzymes, ammonia increases and lead to HE [7].

The brain removes ammonia through glutamine synthesis driven by glutamine synthetase (GS). Therefore, high ammonia levels can increase glutamine synthesis and cause swelling of astrocytes and brain edema [8]. In addition, hyperammonemia can alter the mitochondrial function and neurotransmission and induce oxidative/nitrosative stress [9–11]. Hyperammonemia has been demonstrated to increase the activity of nitric oxide synthase (NOS) and subsequently nitric oxide (NO) production in the brain [12]. High levels of NO were associated with HE, hyperammonemic syndromes, and other disorders without significant neuronal damage [13, 14]. Moreover, ammonia-induced oxidative stress occurs due to increased production of reactive oxygen species (ROS) and subsequent damage of proteins, lipids, and DNA [15]. Previous studies have demonstrated increased ROS production and oxidative stress in hyperammonemia [10, 11, 16]. Hence, counteracting oxidative/nitrosative stress may represent a protective strategy against hyperammonemia-induced brain injury. In this context, several in vitro and in vivo studies have demonstrated the role of nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway as a contributor to the cellular responses to neuronal injury [17–20]. Through binding to the promoter sequence ARE, Nrf2 controls the expression of antioxidant, defensive, and detoxification genes to remove ROS and reactive nitrogen species (RNS) [21]. However, its role in hyperammonemia is still not fully understood.

Excess ammonia reduces glutamate uptake and increases extracellular glutamate levels [22], leading to activation of the N-methyl-D-aspartic acid (NMDA) glutamate receptor in the brain cortex [23]. Consequently, intracellular calcium (Ca^{2+}) increases followed by increased NO production and activation of soluble guanylate cyclase (sGC) and subsequently increases cyclic guanosine monophosphate (cGMP) production [24]. Excess ammonia also increases the activity of Na^+/K^+-ATPase in the brain [25]; however, the underlying mechanism remains unclear. Increased activity of the brain Na^+/K^+-ATPase in hyperammonemia has been demonstrated in multiple previous studies [16, 26].

Current treatments used to reduce ammonia levels are of limited value, and therefore new psychopharmacological agents acting on cellular molecular targets involved in brain neurological alterations are required [27]. Medicinal plants and their derived bioactive phytochemicals have been gaining recognition in the treatment of neurological diseases. *Commiphora molmol* (family *Burseraceae*) is a shrub resembling tropical tree grows in dry forest and produces a resinous exudate called myrrh or oleo-gum resin [28]. Myrrh has been used traditionally for several centuries for the treatment of various diseases and has showed multiple beneficial effects, including antibacterial [29], hypoglycemic [30], anti-inflammatory [31], antioxidant [32], and hepatoprotective efficacies [33]. To the best of our knowledge, nothing has yet been reported on the possible protective effect of *C. molmol* resin against hyperammonemia. Therefore, the present study aimed to investigate the effects of *C. molmol* resin extract against ammonium chloride- (NH_4Cl-) induced hyperammonemia in rats, pointing to the role of oxidative stress and inflammation, and the glutamate-NO-cGMP and Nrf2/ARE signaling pathways.

2. Materials and Methods

2.1. Preparation of C. molmol Extract and Assay of Radical Scavenging Activity. C. molmol resin was purchased from a local herbalist (Harraz Medicinal Plants Co., Cairo, Egypt) and was ground into fine powder. The resin powder was soaked in 90% ethanol for 24 h, filtered, and concentrated using a rotary evaporator at a temperature not exceeding 45°C.

The scavenging activity of *C. molmol* extract against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was assayed according to the method of Kamel et al. [34] using vitamin C as antioxidant reference.

2.2. Experimental Animals and Treatments. Eight-week-old male albino Wistar rats (*Rattus norvegicus*) purchased from the Institute of Ophthalmology (Giza, Egypt) were used in the present investigation. The animals were housed in standard cages at 23 ± 2°C with a 12 h dark/light cycle. All animal procedures were approved by the Institutional Animal Ethics Committee of Beni-Suef University (Egypt).

The experimental rats were divided randomly into 4 groups as following:

Group I (control): rats received intraperitoneal (i.p.) injection of 0.9% sodium chloride (NaCl) (3 times/week) and orally administered 0.5% carboxymethyl cellulose (CMC) daily for 8 weeks.

Group II (*C. molmol*): rats received 0.9% NaCl (3 times/week) and orally administered 125 mg/kg body weight *C. molmol* extract [35] suspended in 0.5% CMC daily for 8 weeks.

Group III (NH_4Cl): rats received 100 mg/kg NH_4Cl (Sisco Research Laboratories, Mumbai, India) dissolved in 0.9% NaCl (i.p., 3 times/week) [10] and orally administered 0.5% CMC daily for 8 weeks.

Group IV (NH_4Cl + *C. molmol*): rats received 100 mg/kg NH_4Cl (i.p., 3 times/week) and orally administered 125 mg/kg body weight *C. molmol* extract suspended in 0.5% CMC daily for 8 weeks.

The doses were adjusted in accordance with changes in the body weight.

2.3. Samples Collection and Preparation. By the end of 8 weeks, the animals were fasted overnight and were then sacrificed, and samples were collected for analysis. Whole blood was collected for the assay of ammonia and hematological parameters. Citrated blood samples were used to assay prothrombin time (PT) and activated partial thromboplastin time (aPTT). Other blood samples were left to coagulate for serum preparation.

The liver, kidney, and cerebrum were immediately excised, washed in cold phosphate-buffered saline (PBS),

and weighed. Samples from the liver, kidney, and cerebrum were homogenized in cold PBS, centrifuged, and kept frozen for the determination of lipid peroxidation, NO, and antioxidants. Homogenized cerebrum samples were also used to assay glutamine and Na^+/K^+-ATPase. Other samples from the cerebrum were collected and kept at −80°C for RNA isolation and Western blot analysis.

2.4. Biochemical Assays

2.4.1. Determination of Ammonia, Urea, Liver Function Markers, and TNF-α. Blood ammonia was estimated using reagent kit purchased from Spinreact (Spain), according to the method of da Fonseca-Wollheim [36].

Circulating levels of ALT and AST were determined following the method of Schumann and Klauke [37] whereas ALP was assayed according to the method of Wenger et al. [38]. The assay kits were purchased from Spinreact (Spain).

Serum TNF-α levels were estimated using specific ELISA kits (R&D Systems, USA), according to the manufacturer's instructions.

2.4.2. Assay of Oxidative Stress and Antioxidant Defenses. Samples from the liver, kidney, and cerebrum were used to assays lipid peroxidation following the method of Preuss et al. [39] and NO using Griess reagent. Reduced glutathione (GSH) [40] and activity of superoxide dismutase (SOD) [41], catalase (CAT) [42], and glutathione peroxidase (GPx) [43] were also determined in the liver, kidney, and cerebrum homogenates.

2.4.3. Assay of Glutamine Level and Na^+/K^+-ATPase Activity. Glutamine concentration in the cerebrum of control and experimental rats was determined according to the method of Lund [44]. Na^+/K^+-ATPase activity was determined spectrophotometrically through determination of the inorganic phosphate (Pi) liberated from ATP [45]. The concentration of Pi was estimated using reagent kit purchased from Spinreact (Spain), according to the method of Fiske and Subbarow [46].

2.5. Hematological Assays. Erythrocytes, hemoglobin content, platelets, total leukocytes, PT, and aPTT were determined using an automated analyzer.

2.6. Gene Expression Analysis. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine the effect of *C. molmol* extract on the expression of NOS1, sGC, and Na^+/K^+-ATPase in the cerebrum of rats. Briefly, total RNA was isolated from the frozen cerebrum samples using TRIzol reagent (Invitrogen, USA). The isolated RNA was quantified and its integrity was checked using formaldehyde-agarose gel electrophoresis. Two μg RNA was used to synthesize cDNA by AMV reverse transcriptase. cDNA was then amplified by Green Master Mix (Fermentas, USA) [47] and the primer set listed in Table 1. The amplified PCR products were loaded into agarose gel and visualized using UV transilluminator. The obtained gel images were scanned and analyzed by ImageJ (version 1.32j, NIH, USA) using β-actin as housekeeping gene.

2.7. Western Blotting. To test the effect of *C. molmol* on Nrf2 and HO-1 expression in the cerebrum, Western blotting was used as we previously reported [48]. In brief, samples from the cerebrum were homogenized in RIPA buffer with proteinase inhibitors and centrifuged, and protein concentration was determined in the homogenates using Bradford reagent [49]. To determine Nrf2, nuclear proteins were extracted using a commercial kit purchased from Beyotime (China). The samples were electrophoresed on SDS/PAGE, transferred to PVDF membranes, blocked, and incubated with primary antibodies for Nrf2, lamin B, HO-1, and β-actin (Santa Cruz Biotechnology, USA). After washing, the membranes were incubated with the secondary antibodies, washed, and then developed using enhanced chemiluminescence kit (Bio-Rad, USA). The intensity of bands was determined and quantified using ImageJ (version 1.32j, NIH, USA).

2.8. Statistical Analysis. Results were analyzed by means of one-way ANOVA followed by Tukey's post hoc analysis using GraphPad Prism 5 (La Jolla, CA, USA). The data were presented as means ± standard error of the mean (SEM), and a P value <0.05 was considered to be statistically significant.

3. Results

3.1. Effect of C. molmol on Body Weight Changes in Hyperammonemic Rats. Initial body weight showed nonsignificant ($P > 0.05$) changes between all experimental groups. Rats received NH_4Cl administration for 8 weeks which showed a significant ($P < 0.05$) decrease in body weight when compared with the control rats (Figure 1). Concurrent administration of *C. molmol* significantly ($P < 0.05$) improved body weight in NH_4Cl-induced hyperammonemic rats, while exerting nonsignificant effect when administered to control rats.

3.2. Effect of C. molmol on Blood Ammonia and Liver Function Markers in Hyperammonemic Rats. Hyperammonemic rats showed a significant ($P < 0.001$) increase in blood ammonia when compared with the control group of rats. Oral administration of *C. molmol* extract to hyperammonemic rats significantly ($P < 0.001$) ameliorated circulating levels of ammonia when compared with the hyperammonemic group (Table 2).

NH_4Cl-induced hyperammonemia in rats produced a significant ($P < 0.001$) increase in circulating levels of the liver function markers, ALT, AST, and ALP. Treatment of the hyperammonemic rats with *C. molmol* significantly ($P < 0.001$) improved the circulating levels of ALT, AST, and ALP (Table 2). Rats received *C. molmol* alone exhibited nonsignificant changes in blood ammonia, ALT, AST, and ALP when compared with the control rats.

3.3. C. molmol Decreases Circulating TNF-α Levels in Hyperammonemic Rats. Serum levels of the proinflammatory cytokine TNF-α showed a significant ($P < 0.001$) increase in NH_4Cl-induced hyperammonemic rats when compared with the control group (Table 2). Oral supplementation of *C. molmol* resin extract significantly ($P < 0.01$) decreased the

Table 1: Primers used for RT-PCR.

Gene	GenBank Accession number	Sequence (5′-3′)
NOS1	XM_017598257	F: GGCCCTTTTAATGAGGGTTGC R: TCTGTGCTAAGTAGCCGCTC
sGC	M57405	F: TCACCCCCATACCCTTCTGT R: GGTAGACTCTGTTGCGGCTT
Na^+/K^+-ATPase (*Atp1a1*)	NM_012504	F: TGGCATCCGAAGTGCTACAG R: CCAGATCACCAACGACGACA
β-Actin (*Actb*)	NM_031144	F: CCGCGAGTACAACCTTCTTG R: CAGTTGGTGACAATGCCGTG

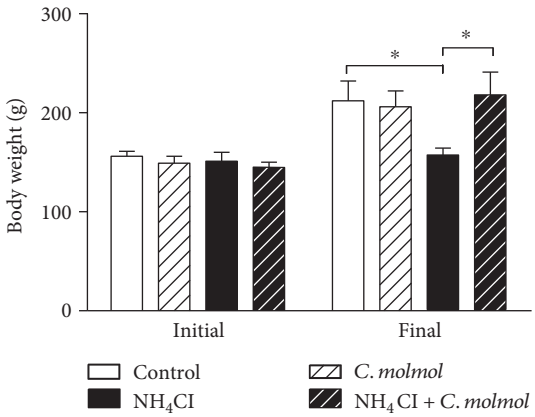

Figure 1: Effect of *C. molmol* resin extract on body weight changes in control and NH_4Cl-induced hyperammonemic rats. Data are expressed as mean ± SEM ($N = 6$). $^*P < 0.05$.

circulating levels of TNF-α in hyperammonemic rats, with no effect on normal rats.

3.4. Effect of C. molmol on Lipid Peroxidation, NO, and Antioxidant Defenses in the Liver of Hyperammonemic Rats. Hyperammonemic rats showed a significant ($P < 0.001$) increase in lipid peroxidation (Figure 2(a)) and NO levels (Figure 2(b)) in the liver of rats when compared with the control group. Treatment of the hyperammonemic rats with *C. molmol* markedly decreased liver lipid peroxidation ($P < 0.001$) and NO ($P < 0.001$) levels.

On the other hand, hyperammonemic rats exhibited a significant ($P < 0.01$) decrease in liver GSH content, an effect that was significantly ($P < 0.05$) prevented by *C. molmol* (Figure 2(c)). Similarly, hyperammonemia was associated with significant decline in the activity of SOD ($P < 0.01$; Figure 2(d)), CAT ($P < 0.01$; Figure 2(e)), and GPx ($P < 0.001$; Figure 2(f)) in the liver of rats. Oral administration of *C. molmol* significantly alleviated the activity of SOD ($P < 0.05$), CAT ($P < 0.01$), and GPx ($P < 0.001$) in the liver of hyperammonemic rats.

Oral administration of *C. molmol* to normal rats did not affect liver lipid peroxidation, NO, and antioxidant defenses.

3.5. Effect of C. molmol on Lipid Peroxidation, NO, and Antioxidant Defenses in the Kidney of Hyperammonemic Rats. Lipid peroxidation and NO levels showed a significant ($P < 0.001$) increase in the kidney of hyperammonemic rats when compared with the control group. Treatment of the hyperammonemic rats with *C. molmol* significantly ($P < 0.001$) reduced both lipid peroxidation (Figure 3(a)) and NO (Figure 3(b)) in the kidney. *C. molmol* administration to normal rats exerted nonsignificant ($P > 0.05$) effect on kidney lipid peroxidation and NO.

GSH levels showed a significant ($P < 0.01$) decrease in the kidney of hyperammonemic rats when compared with the control rats, an effect that was markedly ($P < 0.01$) prevented by *C. molmol* extract as depicted in Figure 3(c). Similarly, hyperammonemic rats exhibited significantly declined activity of kidney SOD ($P < 0.001$, Figure 3(d)), CAT ($P < 0.01$, Figure 3(e)), and GPx ($P < 0.001$, Figure 3(f)) when compared with the control group. *C. molmol* administration significantly improved the activity of SOD ($P < 0.05$), CAT ($P < 0.05$), and GPx ($P < 0.01$) in kidneys of hyperammonemic rats, with no effect on normal rats.

3.6. Effect of C. molmol on Lipid Peroxidation, NO, and Antioxidant Defenses in the Cerebrum of Hyperammonemic Rats. NH_4Cl administration induced a significant ($P < 0.001$) increase in the levels of lipid peroxidation (Figure 4(a)) and NO (Figure 4(b)) in the cerebrum of rats when compared with the control group. In addition, hyperammonemic rats exhibited marked decrease in the cerebral GSH levels ($P < 0.01$; Figure 4(c)), and the activity of SOD ($P < 0.01$; Figure 4(d)), CAT ($P < 0.01$; Figure 4(e)), and GPx ($P < 0.001$; Figure 4(f)). Treatment of the hyperammonemic rats with *C. molmol* significantly decreased lipid peroxidation ($P < 0.001$) and NO ($P < 0.01$) and significantly improved GSH ($P < 0.01$), SOD ($P < 0.05$), CAT ($P < 0.05$), and GPx ($P < 0.01$) in the cerebrum. Oral supplementation of *C. molmol* did not affect lipid peroxidation, NO, and antioxidant defenses in the cerebrum of normal rats.

3.7. C. molmol Upregulates the Nrf2/ARE/HO-1 Pathway in the Cerebrum of Hyperammonemic Rats. To investigate the effect of *C. molmol* resin extract on the Nrf2/ARE/HO-1 pathway in hyperammonemic rats, the protein expression of Nrf2 and HO-1 was determined in the cerebrum using Western blotting assay.

NH_4Cl-induced hyperammonemia in rats induced a significant ($P < 0.001$) downregulation of cerebral Nrf2 expression when compared with the control group of rats (Figure 5(a)). Concurrent administration of *C. molmol* resin

Table 2: Effect of *C. molmol* on ammonia, liver function marker enzymes, and TNF-α in control and hyperammonemic rats.

	Control	*C. molmol*	NH_4Cl	NH_4Cl + *C. molmol*
Ammonia (μmol/dL)	75.30 ± 6.23	72.69 ± 4.89	$418.20 \pm 18.48^{***}$	$136.25 \pm 8.79^{\#\#\#}$
ALT (U/L)	28.78 ± 4.25	26.50 ± 2.48	$64.81 \pm 6.23^{***}$	$36.12 \pm 3.14^{\#\#\#}$
AST (U/L)	53.45 ± 4.26	48.16 ± 3.89	$132.71 \pm 8.49^{***}$	$68.51 \pm 6.77^{\#\#\#}$
ALP (U/L)	82.69 ± 6.13	84.26 ± 5.23	$179.18 \pm 10.26^{***}$	$95.50 \pm 7.56^{\#\#\#}$
TNF-α (pg/mL)	32.60 ± 2.42	35.78 ± 3.88	$78.59 \pm 5.53^{***}$	$48.36 \pm 4.33^{\#\#}$

Data are expressed as mean ± SEM ($N = 6$). $^{***}P < 0.001$ versus control, and $\#\#P < 0.01$ and $\#\#\#P < 0.001$ versus NH_4Cl.

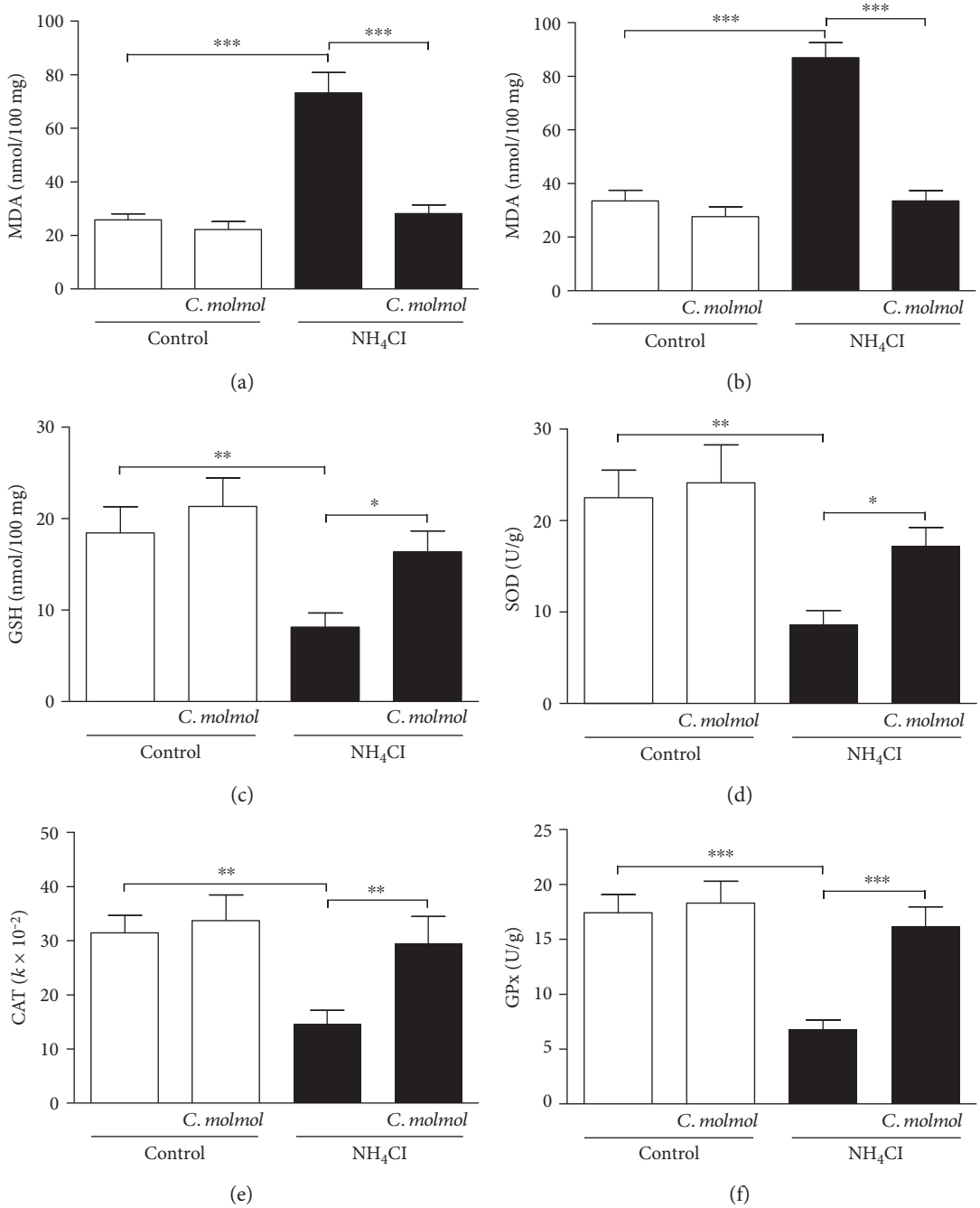

Figure 2: Effect of *C. molmol* resin extract on (a) lipid peroxidation, (b) nitric oxide, (c) GSH, (d) SOD, (e) CAT, and (f) GPx in the liver of NH_4Cl-induced hyperammonemic rats. Data are expressed as mean ± SEM ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. MDA, malondialdehyde; NO, nitric oxide; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

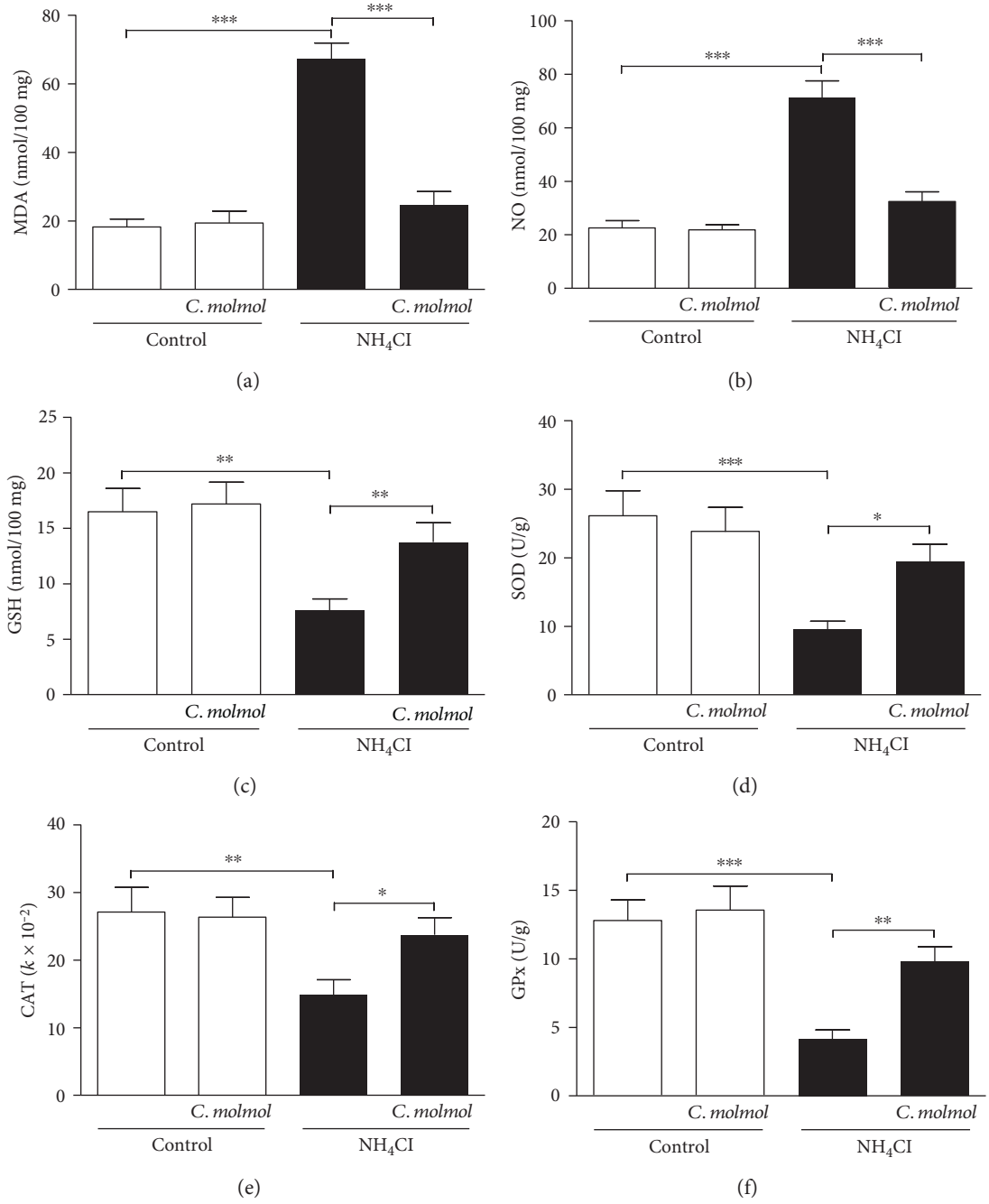

Figure 3: Effect of *C. molmol* resin extract on (a) lipid peroxidation, (b) nitric oxide, (c) GSH, (d) SOD, (e) CAT, and (f) GPx in the kidney of NH_4Cl-induced hyperammonemic rats. Data are expressed as mean ± SEM ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. MDA, malondialdehyde; NO, nitric oxide; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

extract significantly ($P < 0.001$) increased Nrf2 expression in the cerebrum of hyperammonemic rats.

Similarly, HO-1 expression showed a significant ($P < 0.001$) downregulation in the cerebrum of NH_4Cl-induced hyperammonemic rats when compared with the control rats as depicted in Figure 5(b). *C. molmol* resin supplementation significantly ($P < 0.001$) ameliorated the expression of HO-1 in the cerebrum of hyperammonemic rats. Oral supplementation of *C. molmol* did not induce significant changes in cerebral Nrf2 and HO-1 expression.

3.8. C. molmol Prevents Hyperammonemia-Associated Hematological Alterations in Rats. To evaluate the effect of *C. molmol* extract on hyperammonemia-associated hematological alteration, RBCs, Hb, WBCs, and platelets were determined in the control and hyperammonemic rats.

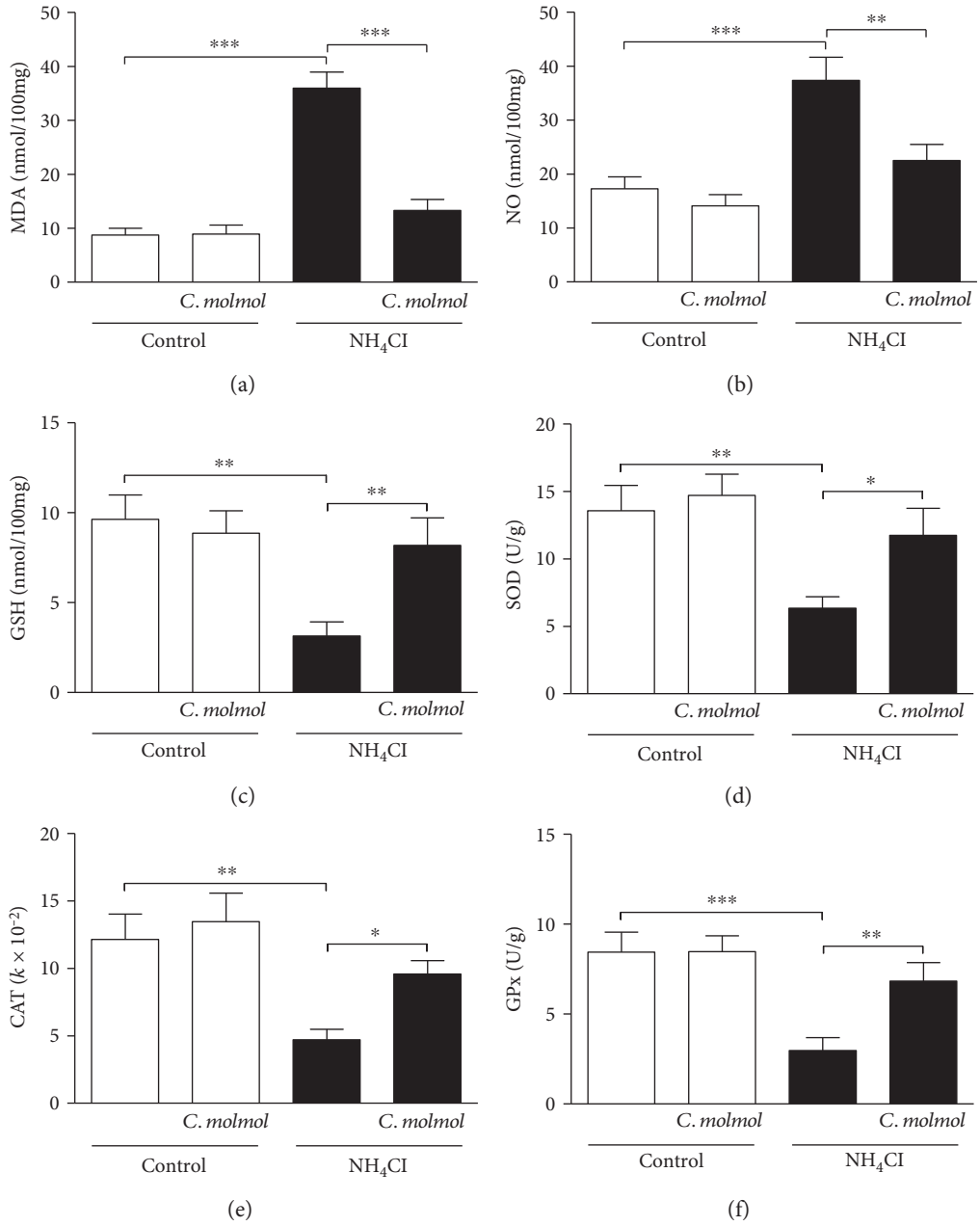

Figure 4: Effect of *C. molmol* resin extract on (a) lipid peroxidation, (b) nitric oxide, (c) GSH, (d) SOD, (e) CAT, and (f) GPx in the cerebrum of NH_4Cl-induced hyperammonemic rats. Data are expressed as mean ± SEM ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. MDA, malondialdehyde; NO, nitric oxide; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

Hyperammonemic rats exhibited a significant ($P < 0.01$) decrease in the number of erythrocytes (Figure 6(a)) and in the Hb content (Figure 6(b)) when compared with the control rats. On the other hand, NH_4Cl-induced hyperammonemia was associated with significant ($P < 0.001$) leukocytosis (Figure 6(c)). *C. molmol* administration markedly prevented hyperammonemia-induced anemia ($P < 0.05$) and leukocytosis ($P < 0.001$).

Thrombocytopenia was a characteristic feature for hyperammonemia where the NH_4Cl-induced hyperammonemic rats showed significant ($P < 0.05$) decrease in the number of thrombocytes as compared to the control group (Figure 6(d)). Oral supplementation of *C. molmol* extract to NH_4Cl-induced hyperammonemic rats did not affect significantly the thrombocytes count.

To examine hyperammonemia-induced changes in the coagulation system and the effect of *C. molmol*, we determined PT and aPTT. Hyperammonemic rats exhibited a significant ($P < 0.01$) prolongation of PT (Figure 6(e)) and aPTT (Figure 6(f)), an effect that was markedly ($P < 0.01$) reversed by *C. molmol*.

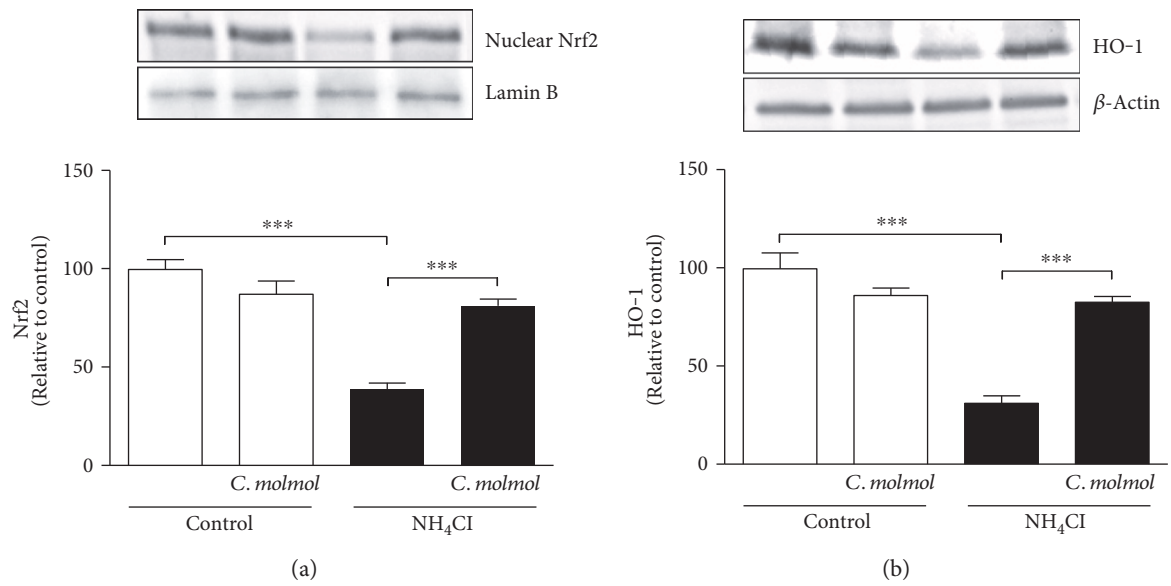

Figure 5: Effect of *C. molmol* resin extract on (a) Nrf2 and (b) HO-1 protein expression in the cerebrum of NH_4Cl-induced hyperammonemic rats. Data are expressed as mean ± SEM ($N = 6$); $^{***}P < 0.001$. Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, hemoxygenase-1.

Of note, *C. molmol* did not induce any significant changes on hematological parameters of normal rats.

3.9. C. molmol Downregulates NOS1, sGC, and Na^+/K^+-ATPase and Decreases Glutamine in the Cerebrum of Hyperammonemic Rats. NOS1 mRNA expression showed a significant ($P < 0.001$) increase in the cerebrum of NH_4Cl-induced hyperammonemic rats when compared with the control group (Figure 7(a)). Concurrent supplementation of *C. molmol* significantly ($P < 0.001$) improved the expression of NOS1 mRNA in the cerebrum of hyperammonemic rats.

Hyperammonemic rats exhibited a significant ($P < 0.001$) upregulation in the expression of sGC mRNA, an effect that was significantly ($P < 0.001$) ameliorated by *C. molmol* (Figure 7(b)). Glutamine levels as well showed a significant ($P < 0.001$) increase in the cerebrum of NH_4Cl-induced hyperammonemic rats when compared with the control rats (Figure 7(c)). Concurrent administration of *C. molmol* significantly ($P < 0.001$) decreased cerebral glutamine levels in NH_4Cl-induced hyperammonemic rats.

NH_4Cl-induced hyperammonemia induced a significant ($P < 0.001$) increase in both the expression and activity Na^+/K^+-ATPase in the cerebrum of rats. Oral supplementation of *C. molmol* to hyperammonemic rats significantly improved the expression ($P < 0.01$) and activity ($P < 0.001$) of the cerebral Na^+/K^+-ATPase as represented in Figures 7(d) and 7(e), respectively.

Oral supplementation of *C. molmol* did not affect the gene expression of NOS1, sGC, Na^+/K^+-ATPase, or glutamine levels in the cerebrum of normal rats.

4. Discussion

C. molmol has showed multiple therapeutic effects; however, nothing has yet been reported on its protective effect against hyperammonemia. The present study shows for the first time the protective effect of *C. molmol* resin extract against excess ammonia-induced alterations, pointing to the role of Nrf2/HO-1 pathway.

An initial objective of this study was to investigate the protective activity of *C. molmol* resin extract against NH_4Cl-induced liver injury. The liver plays a central role in detoxification of both endogenous and exogenous toxins. This detoxification capacity is hampered upon liver injury, and the body is exposed to the harmful effects of toxicants. Hyperammonemia occurs as a consequence of liver failure [7]. In the present investigation, increased circulating levels of ammonia indicate liver damage induced by ammonia intoxication in rats as we previously reported [10, 11]. Excess ammonia-induced liver injury was confirmed by increased circulating liver-specific marker enzymes ALT, AST, and ALP. In NH_4Cl-induced animal model, hyperammonemia occurs as a consequence of liver damage induced by injection of NH_4Cl. Hyperammonemia induces liver damage which may contribute to or exacerbate hyperammonemia and other alterations resulting from liver damage. We have previously reported increased circulating levels of ALT, AST, and ALP in NH_4Cl-induced hyperammonemic rats [10]. Elevated liver marker enzymes in serum is an indicator for the assessment of hepatocellular damage [50]. *C. molmol* resin extract significantly ameliorated body weight and decreased blood ammonia levels and circulating levels of ALT, AST, and ALP in hyperammonemic rats. These findings suggest hepatoprotective and membrane-stabilizing potentials of *C. molmol*. Accordingly, *C. molmol* resin protected the liver against ethanol-induced hepatotoxicity in rats and decreased the circulating levels of ALT, AST, and ALP [51]. In addition, treatment with *C. molmol* resin extract decreased circulating ALT and AST in carbon tetrachloride- (CCl_4-) [52] and D-GalN/LPS-induced [53] liver injury in rats and in a rodent model of chemically induced hepatocarcinogenesis [54]. The declined body weight in NH_4Cl-induced hyperammonemic rats could

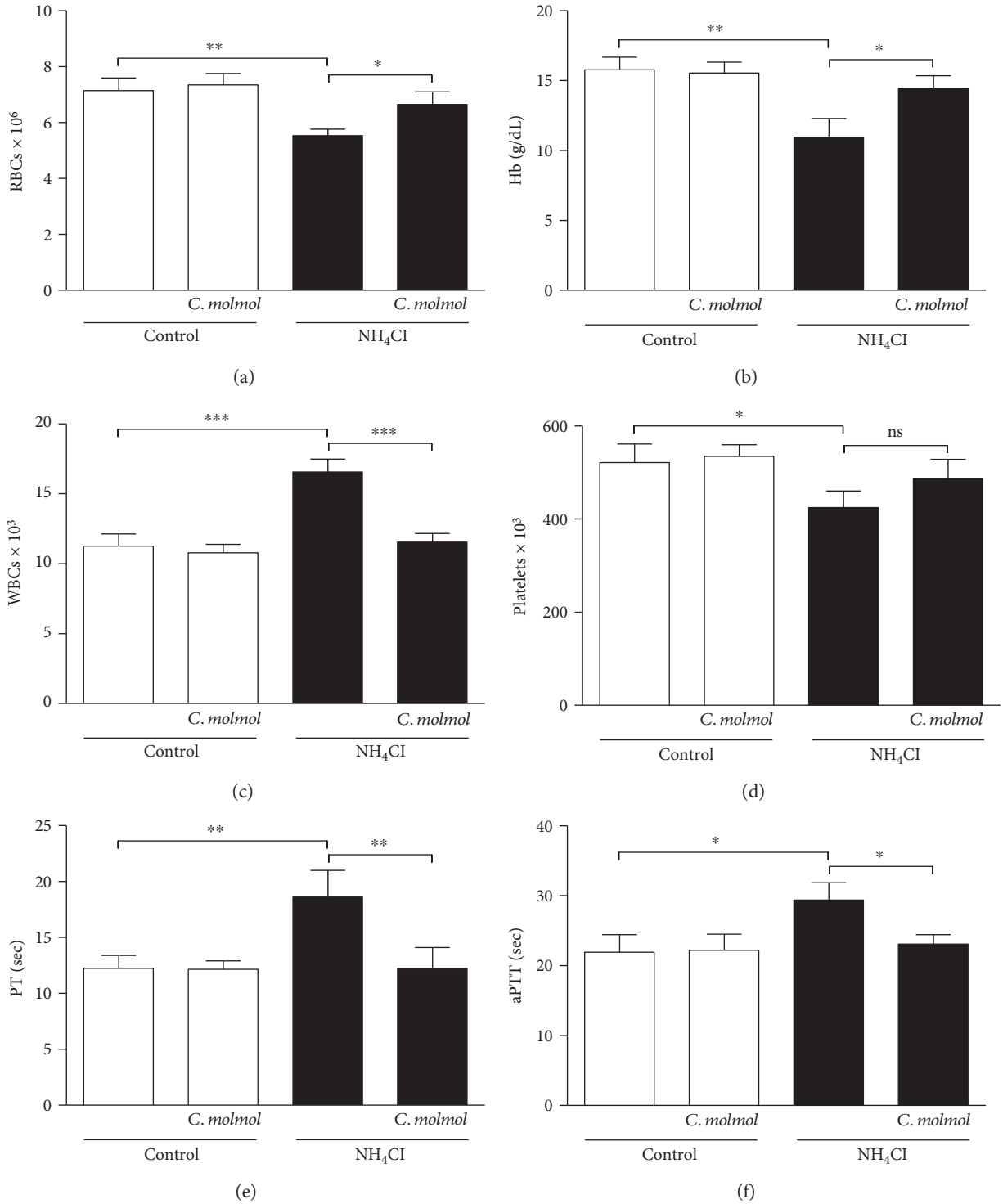

Figure 6: Effect *C. molmol* resin extract on hematologic and coagulation system parameters in NH_4Cl-induced hyperammonemic rats. Data are expressed as mean ± SEM ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. RBCs, erythrocytes; Hb, hemoglobin; WBCs, leukocytes; PT, prothrombin time; aPTT, activated partial thromboplastin time.

be explained by the decreased body fat content. Hyperammonemia has been reported to alter lipid metabolism and significantly decrease body lipid content, leading to declined body weight [55]. Alleviated body weight by *C. molmol* resin extract in this study could be attributed to the ameliorated lipid metabolism. However, further studies are required to better explain the possible role of *C. molmol* in ameliorating body weight in hyperammonemic rats.

Hyperammonemic rats in the present study exhibited a marked increase in the circulating levels of TNF-α as we recently reported [11]. Although the precise interaction between inflammation and hyperammonemia is unclear,

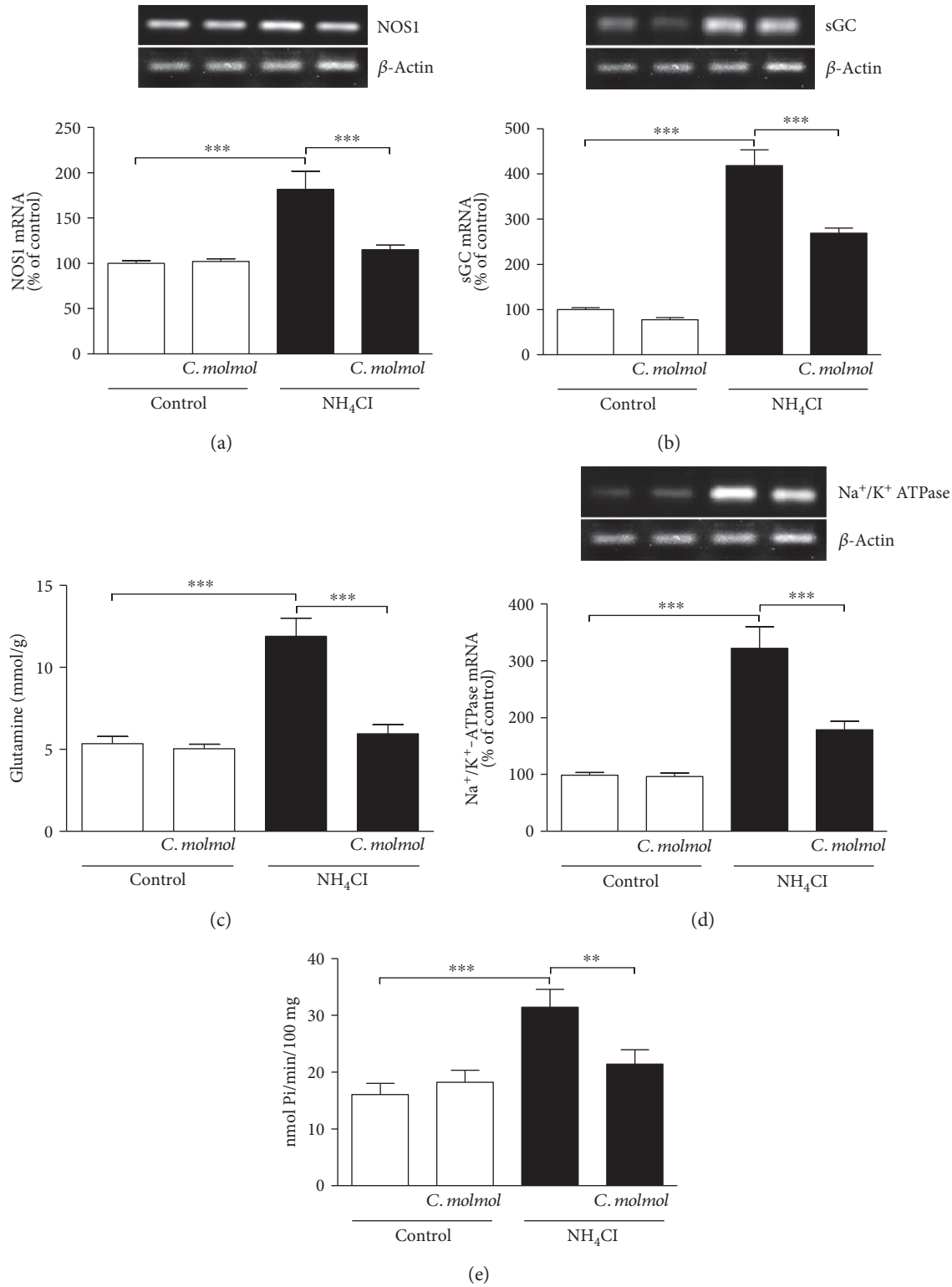

Figure 7: Effect of *C. molmol* resin extract on (a) NOS1 mRNA, (b) sGC mRNA, (c) glutamine levels, (d) Na^+/K^+-ATPase mRNA, and (e) Na^+/K^+-ATPase activity in the cerebrum of NH_4Cl-induced hyperammonemic rats. Data are expressed as mean ± SEM ($N = 6$); $^{**}P < 0.01$ and $^{***}P < 0.001$. NOS1, neuronal nitric oxide synthase; sGC, soluble guanylate cyclase.

inflammation appears to play important role in the pathogenesis of HE [56–58]. In this context, studies have showed a strong positive correlation between inflammation and HE [59, 60]. In patients with liver cirrhosis, elevated circulating levels of the proinflammatory cytokine TNF-α were recorded [59, 60]. Shawcross et al. [61] proposed the crucial role of inflammation in hyperammonemia-associated neuropsychological alterations. Hence, attenuation of inflammation,

particularly mediated by TNFα, may reduce or prevent hyperammonemia. This notion is supported by the findings of Chung et al. [62] who showed the ability of indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), to prevent ammonia-induced brain edema after portacaval anastomosis in rats. In addition, we have demonstrated that the ameliorated blood ammonia levels was associated with decreased circulating levels of TNF-α in NH_4Cl-induced hyperammonemia in rats [11].

Here, *C. molmol* resin extract significantly decreased serum levels of TNF-α, demonstrating that its anti-inflammatory effect plays a role in attenuating hyperammonemia. The anti-inflammatory efficacy of *C. molmol* has been reported in different studies. In a rat model of formalin-induced hind paw edema, Shalaby and Hammouda [35] showed that *C. molmol* exerted a potent anti-inflammatory effect. This effect has been attributed to the reduced production of prostaglandins as reported by Su et al. [63]. Also, Ahmad et al. [53] showed that myrrh attenuated inflammation in a rat model of D-GalN/LPS-induced hepatic injury. Recently, Fatani et al. [64] showed the anti-inflammatory efficacy of *C. molmol* in acetic acid-induced ulcerative colitis in rats. We have recently demonstrated the potent anti-inflammatory effect of *C. molmol* resin extract in a rat model of chemically induced hepatocarcinogenesis [54]. These studies support the idea that attenuation of inflammation has a role in the protective mechanism of *C. molmol* against hyperammonemia.

Attenuation of oxidative/nitrosative stress is another mechanism we hypothesized to mediate the protective effect of *C. molmol* resin extract against NH_4Cl-induced hyperammonemia in rats. In animal models, excess ammonia provokes excessive production of ROS [15] which initiate lipid peroxidation. Here, hyperammonemic rats exhibited marked increase in lipid peroxidation levels in the liver, kidney, and cerebrum. Previous research from our lab showed significant increase in lipid peroxidation in the liver, brain, and kidney of NH_4Cl-induced hyperammonemic rats [10, 11]. Other studies have reported increased lipid peroxidation in animal models of hyperammonemia [16, 65, 66]. Moreover, NH_4Cl-induced hyperammonemic rats showed a marked increase in NO levels in the liver, kidney, and cerebrum. The increased NO production in the brain is a direct result of the upregulated nNOS in hyperammonemic rats. Accordingly, Ramakrishnan et al. [16] have reported increased expression of nNOS in the brain of NH_4Cl-induced rats. Hyperammonemia is known to activate nNOS and increase NO production in the brain. The excessive production of NO can induce neuronal damage [12].

Interestingly, *C. molmol* resin significantly alleviated lipid peroxidation in the liver, kidney, and cerebrum of hyperammonemic rats, demonstrating a radical scavenging efficacy. In addition, *C. molmol* significantly decreased NO production which is associated with the decreased nNOS expression. In accordance, supplementation of *C. molmol* to rats with ulcerative colitis [64], ethanol- [51] and lead-induced liver injury [32], and hepatocarcinogenesis [54] significantly decreased lipid peroxidation and NO levels. Moreover, *C. molmol* significantly enhances both enzymatic and nonenzymatic antioxidant defenses in the liver, kidney, and cerebrum of hyperammonemic rats. These antioxidants play key roles in protecting the body against free radicals. Previous studies have reported declined GSH, SOD, CAT, and GPx in the liver, brain, and kidney of NH_4Cl-induced hyperammonemic rats [10, 11, 16]. Declined antioxidant defense mechanisms in hyperammonemia can aggravate ROS-induced tissue damage. Along with reducing lipid peroxidation and NO, *C. molmol* has been reported to enhance the antioxidant defenses in different tissues of rats [32, 51, 54, 64].

The in vivo antioxidant activity of *C. molmol* resin extract is in positive correlation with the in vitro data. Our findings showed a significant antioxidant and radical scavenging efficacies of *C. molmol* resin extract evidenced by the DPPH assay (Supplementary Figure I available online at https://doi.org/10.1155/2017/7369671). The antioxidant activity of *C. molmol* is due to its rich content of bioactive molecules. Phytochemical analysis of *C. molmol* resin showed the presence of active constituents with antioxidant activity. These bioactive constituents include limonene, m-cresol, eugenol, commiphoric acids, furanosesquiterpenes, pinene, terpenoids, and cuminic aldehyde [67, 68]. In addition, the study of Mahboubi and Kazempour [69] showed the presence of phenolic and flavonoid compounds in *C. molmol* resin ethanolic extract. Phenolic compounds are well known for their potent antioxidant and radical scavenging properties [10, 34, 70, 71].

In addition to its radical scavenging property, we hypothesized that *C. molmol* resin extract can enhance antioxidant defenses and abrogate oxidative stress through activation of Nrf2/HO-1 signaling. The possible involvement of Nrf2 activation in mediating the protective activity of *C. molmol* resin against hyperammonemia has not been previously investigated. The current findings showed a significant decline in Nrf2 expression in the cerebrum of hyperammonemic rats. This downregulation has been markedly reversed following treatment with *C. molmol* resin extract. The results also showed that HO-1 gene expression was upregulated in the cerebrum of hyperammonemic rats treated with *C. molmol* resin extract. These findings highlight that activation of the Nrf2/ARE/HO-1 signaling pathway participates in the neuroprotective effect of *C. molmol* resin extract against hyperammonemia-induced injury. Nrf2 is known to be activated by ROS and then dissociates from Keap1 and translocates to the nucleus where it binds to ARE and activates the transcription of antioxidant and cytoprotective proteins including HO-1, CAT, SOD, and GPx [21]. Although activated by ROS, Nrf2 showed a significant decline in the cerebrum of hyperammonemic rats exhibiting oxidative stress. This was further confirmed by the declined HO-1 expression in the cerebrum of hyperammonemic rats. Moreover, the activity of antioxidant defenses in the cerebrum was strongly correlated with these findings. An explanation for this declined expression of Nrf2 could be the chronic and surplus production of ROS. This notion is supported by our recent findings where we demonstrated downregulation of the Nrf2/ARE/HO-1 pathway in different conditions with excessive production of ROS [34, 48, 72–74]. Furthermore, the anti-inflammatory effect of *C. molmol* resin extract could be attributed, at least in part, to the activation of Nrf2.

Multiple studies have demonstrated the anti-inflammatory role of Nrf2. Knockout of Nrf2 has been associated with reduced anti-inflammatory efficacy of the antioxidant curcumin [75]. In addition, activation of Nrf2 blocked the transcription of IL-6 and IL-1β in macrophages [76].

The effect of *C. molmol* resin on hematological alterations in hyperammonemia was one of our targets in this study; however, reports about hematological alterations in hyperammonemia are very few. Assessment of hematological parameters represents a powerful tool and an earlier indicator to evaluate the deleterious effects of drugs [77]. In the present study, administration of *C. molmol* to normal rats did not affect the hematological and coagulation system parameters, whereas hyperammonemic rats exhibited anemia, leukocytosis, thrombocytopenia, and prolonged PT and aPTT. Hematological abnormalities occur frequently in liver disease conditions [78]. In support of our findings, Kalaitzakis et al. [79] have demonstrated that HE is related to anemia in liver transplant candidates with cirrhosis. The recorded anemia in hyperammonemic rats could also be a consequence of the increased ROS production. ROS can decrease cellular deformability and damage erythrocytes via induction of membrane lipid peroxidation and rigidity [80]. In addition, liver disease is known to be associated with defects of blood coagulation as a consequence of thrombocytopenia, endothelial dysfunction, and deficiencies of coagulation factors. Low circulating levels of the coagulation factors are associated with prolongation of PT and aPTT [81]. Here, hyperammonemic rats exhibited thrombocytopenia and prolonged PT and aPTT. Furthermore, hyperammonemic rats showed leukocytosis. Accordingly, Choi et al. [82] reported mild leukocytosis in hyperammonemic patients with ornithine carbamoyltransferase deficiency and Aggarwal et al. [78] demonstrated a significant association of leukocytosis with HE. Interestingly, *C. molmol* prevented all hematological alterations in hyperammonemic rats. These findings could be a direct result of the prevention of hyperammonemia and attenuation of liver injury, oxidative stress, and inflammation.

Glutamine is a neutral amino acid and functions normally as ammonia carrier in the CNS [83]. Because the brain does not convert ammonia into urea, ammonia is exclusively removed by GS located in astrocytes. Thus, glutamine synthesis is an essential process for the brain to detoxify excess ammonia in liver failure [84]. In hyperammonemic conditions, the metabolism of ammonia to glutamine is followed by an osmotic disturbance, altered cerebral blood flow, oxidative stress, and edema. Other factors including systemic inflammation may contribute to the excess ammonia-induced cerebral alterations [85]. NH_4Cl-induced hyperammonemic rats in the present investigation showed a marked increase in cerebral glutamine levels and the expression of sGC. Excess ammonia has been reported to activate NMDA receptors in the brain cortex [23], followed by increased intracellular Ca^{2+}, increased NO production, and activation of sGC [24]. Interestingly, *C. molmol* supplemented hyperammonemic rats showed a marked decrease in cerebral glutamine levels and downregulated sGC. These findings could be attributed to the ability of *C. molmol* to decrease ammonia levels, oxidative stress, and nNOS expression. Therefore, the protective effect of *C. molmol* resin extract against hyperammonemia is illustrated, at least in part, through inhibiting NMDA receptors and modulation of the glutamate-NO-cGMP pathway.

Excess ammonia can also alter the ionic shifts and affect the membrane potential of nerve cells. Here, NH_4Cl-induced hyperammonemic rats exhibited significantly increased expression and activity of the cerebral Na^+/K^+-ATPase. Previous studies have showed similar findings in hyperammonemic brain conditions [16, 25, 26]. The exact underlying mechanism of the activated Na^+/K^+-ATPase in hyperammonemia is not fully understood. Kosenko et al. [86] proposed that the activation of Na^+/K^+-ATPase is a result of decreased phosphorylation by protein kinase C (PKC). In addition, the activity of Na^+/K^+-ATPase was increased in cerebral cortex following exposure to millimolar concentrations of NH_4Cl [87]. *C. molmol* supplementation markedly ameliorated both the expression and the activity of Na^+/K^+-ATPase in the cerebrum of hyperammonemic rats. These findings added support to the protective role of *C. molmol* resin against the deleterious effects of hyperammonemia.

In conclusion, our study shows for the first time that *C. molmol* resin extract protects against excess ammonia through attenuation of oxidative stress and inflammation and modulation of the glutamate-NO-cGMP pathway. This investigation also confers information that *C. molmol* may be an effective neuroprotective therapeutic agent with a potential mechanism of upregulation Nrf2/ARE/HO-1 pathway and consequently enhances the antioxidant defenses. *C. molmol* ameliorated both the activity and the expression of Na^+/K^+-ATPase and prevented hematological alterations in cases of hyperammonemia and hepatic failure. Therefore, *C. molmol* resin may represent a promising protective agent against hyperammonemia, pending further detailed mechanistic studies. This study may serve as a base for future investigations exploring Nrf2-activating agents as therapeutics for hyperammonemia.

Conflicts of Interest

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

NRF2 Plays a Critical Role in Both Self and EGCG Protection against Diabetic Testicular Damage

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Activation of nuclear factor erythroid 2-related factor 2 (NRF2) has been found to ameliorate diabetic testicular damage (DTD) in rodents. However, it was unclear whether NRF2 is required for these approaches in DTD. Epigallocatechin gallate (EGCG) is a potent activator of NRF2 and has shown beneficial effects on multiple diabetic complications. However, the effect of EGCG has not been studied in DTD. The present study aims to explore the role of NRF2 in both self and EGCG protection against DTD. Therefore, streptozotocin-induced diabetic C57BL/6 wild type (WT) and *Nrf2* knockout (KO) mice were treated in the presence or absence of EGCG, for 24 weeks. The *Nrf2* KO mice exhibited more significant diabetes-induced loss in testicular weight and spermatozoa count, and increase in testicular apoptotic cell death, as compared with the WT mice. EGCG activated NRF2 expression and function, preserved testicular weight and spermatozoa count, and attenuated testicular apoptotic cell death, endoplasmic reticulum stress, inflammation, and oxidative damage in the WT diabetic mice, but not the *Nrf2* KO diabetic mice. The present study demonstrated for the first time that NRF2 plays a critical role in both self and EGCG protection against DTD.

1. Introduction

Diabetes causes damage to multiple organs, including testis [1]. Decreased sperm cell count and velocity were found in patients with diabetes [2]. Moreover, diabetics had increased sperm nuclear and mitochondrial DNA damage [3], along with increased level of advanced glycation end products (AGEs) in the testis, epididymis, and sperm [4]. Diabetes-induced excessive AGEs can cause oxidative stress, leading to activation of mitochondria or endoplasmic reticulum (ER) stress-related cell death pathways, the effect of which may result in sperm loss [5–8]. Therefore, targeting diabetes-induced oxidative stress is a promising strategy to prevent male infertility in diabetic patients.

Nuclear factor erythroid 2-related factor 2 (NRF2) is a master factor in the cellular antioxidant system [9, 10]. NRF2 activates the transcription of a cohort of antioxidant genes, such as heme oxygenase-1 (*Ho1*) and NAD(P)H dehydrogenase quinone 1 (*Nqo1*) [11], the proteins of which work as scavengers of diabetes-induced free radicals. Notably, the

Nrf2 gene knockout (KO) male mice developed infertility in an age-dependent manner [12]. NRF2 and its downstream antioxidants were found to be expressed in the male reproductive tract and played a critical role in defence against oxidative stress [12, 13]. Furthermore, low *Nrf2* mRNA was found to be associated with impaired sperm function parameters in human individuals, including concentration, motility, vitality, and morphology [14]. All these findings suggested that NRF2 plays a pivotal protective role in male infertility. Approaches in activating NRF2, such as administration of the NRF2 activator sulforaphane (SFN) [5, 6], supplementation of zinc [15], and exposure to low-dose X-irradiation [8], effectively ameliorated diabetes-induced testicular inflammation, ER stress, and apoptotic cell death, in rodent models of both type 1 and type 2 diabetes. However, it was unclear whether NRF2 was required for the protective effect of these approaches.

Epigallocatechin gallate (EGCG) is the most abundant and effective green tea catechin and is known to be a potent NRF2 activator [16–20]. Despite showing beneficial effects in a variety of diseases [21–24], EGCG has not been studied for its effect in diabetic testicular damage (DTD). It was also not previously known whether NRF2 is required for this possible action of EGCG. Therefore, the present study aims to answer the following questions: (1) whether or not EGCG has a protective role in ameliorating DTD and (2) whether or not NRF2 is required for self and EGCG protection against DTD. To these ends, diabetes was induced in 8-week-old male C57BL/6 wild-type (WT) and *Nrf2* KO mice by streptozotocin (STZ). Both the diabetic and nondiabetic mice were treated in the presence or absence of EGCG, for a total period of 24 weeks.

2. Methods

2.1. Animal Treatment. C57BL/6 WT (*Nrf2*+/+) and *Nrf2* KO (*Nrf2*−/−) mice were obtained through breeding of heterozygotes (*Nrf2*+/−) [10, 25]. All mice were housed in the Animal Center of Changchun University of Chinese Medicine at 22°C, on a 12 : 12-hour light-dark cycle with free access to rodent feed and tap water. The Institutional Animal Care and Use Committee at Changchun University of Chinese Medicine approved all the experimental procedures, which were consequently in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences. Eight-week-old male mice received either sodium citrate or STZ (Sigma-Aldrich, St. Louis, MO; 50 mg/kg daily, dissolved in 0.1 M sodium citrate, pH 4.5) through intraperitoneal injection for 5 consecutive days. Fasting glucose levels (4-hour fast) were measured a week after the last injection of STZ. Mice with a fasting glucose level higher than 250 mg/dl were considered diabetic. In order to observe the chronic effect of diabetes, the mice were kept for 24 weeks post diabetes onset. Thus, the diabetic mice and age-matched controls were treated daily by subcutaneously injected EGCG (100 mg/kg [26], ≥98%, dissolved in normal saline, PureOne Biotechnology, Shanghai, PRC) or normal saline daily, for a total period of 24 weeks. Blood glucose was recorded on days 0, 28, 56, 84, 112, 140, and 168, after diabetes onset. The mice were then euthanized under anaesthesia by intraperitoneal injection of chloral hydrate at 0.3 mg/kg [27]. The testes and cauda epididymides were harvested for analysis. Six mice in each group were studied.

2.2. Sperm Density Assessment. The cauda epididymis from each mouse was placed in 2 ml Earle's balanced salt solution (Sigma-Aldrich) supplemented with 0.1% bovine serum albumin (Sigma-Aldrich). The epididymis was gently teased with a bent needle to release spermatozoa under observation through a stereomicroscope (Olympus). Sperm density was assessed with a haemocytometer and was presented by spermatozoa count per epididymis [28–30].

2.3. Hematoxylin and Eosin (H&E) Staining, Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Assay, and Immunohistochemical (IHC) Staining. Testes were fixed immediately in 10% buffered formalin solution after harvesting and were embedded in paraffin and sectioned into 5 μm thick sections onto glass slides. The sections were processed for H&E staining and TUNEL staining, as previously described [6]. For TUNEL staining, apoptotic cells exhibited a brown nuclear stain as TUNEL positive cells and were counted manually under a microscope. Four sections from each testis were counted. On each section, 30 seminiferous tubule's cross sections from each testis were selected in the same pattern, moving each slide without repetitive counting in a blinded fashion [31]. Results were presented as TUNEL positive cells per 10^3 cells. For IHC staining, the sections were incubated with anti-3-nitrotyrosine (3-NT, Millipore, Temecula, CA, USA, 1 : 100) overnight at 4°C. After washing with PBS, the sections were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA, 1 : 300 dilutions in PBS) for 2 hours at room temperature. The sections were then treated with peroxidase substrate 3,3′-diaminobenzidine in the developing system provided by Vector Laboratories (Burlingame, CA, USA) and counterstained with hematoxylin.

2.4. Reactive Oxygen Species (ROS) Assay. Testicular ROS generation was measured using a ROS assay kit provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, PRC), following the manufacturer's instructions.

2.5. Isolation of Nuclei. Nuclei were isolated from testicular tissue of each mouse using a nuclei isolation kit provided by Sigma-Aldrich, following the manufacturer's instructions, as previously described [25, 30, 32].

2.6. Western Blot Analysis. Western blot was performed using testicular tissue as described in our previous study [33]. The primary antibodies included anti-activating transcription factor 4 (ATF4, Cell Signaling Technology, Danvers, MA, USA, 1 : 1000), anti-Bcl-2-associated X protein (Bax, Cell Signaling Technology, 1 : 1000), anti-B-cell lymphoma 2 (Bcl-2, Santa Cruz Biotechnology, 1 : 2000), anti-binding immunoglobulin protein (BIP, Cell Signaling Technology, 1 : 1000),

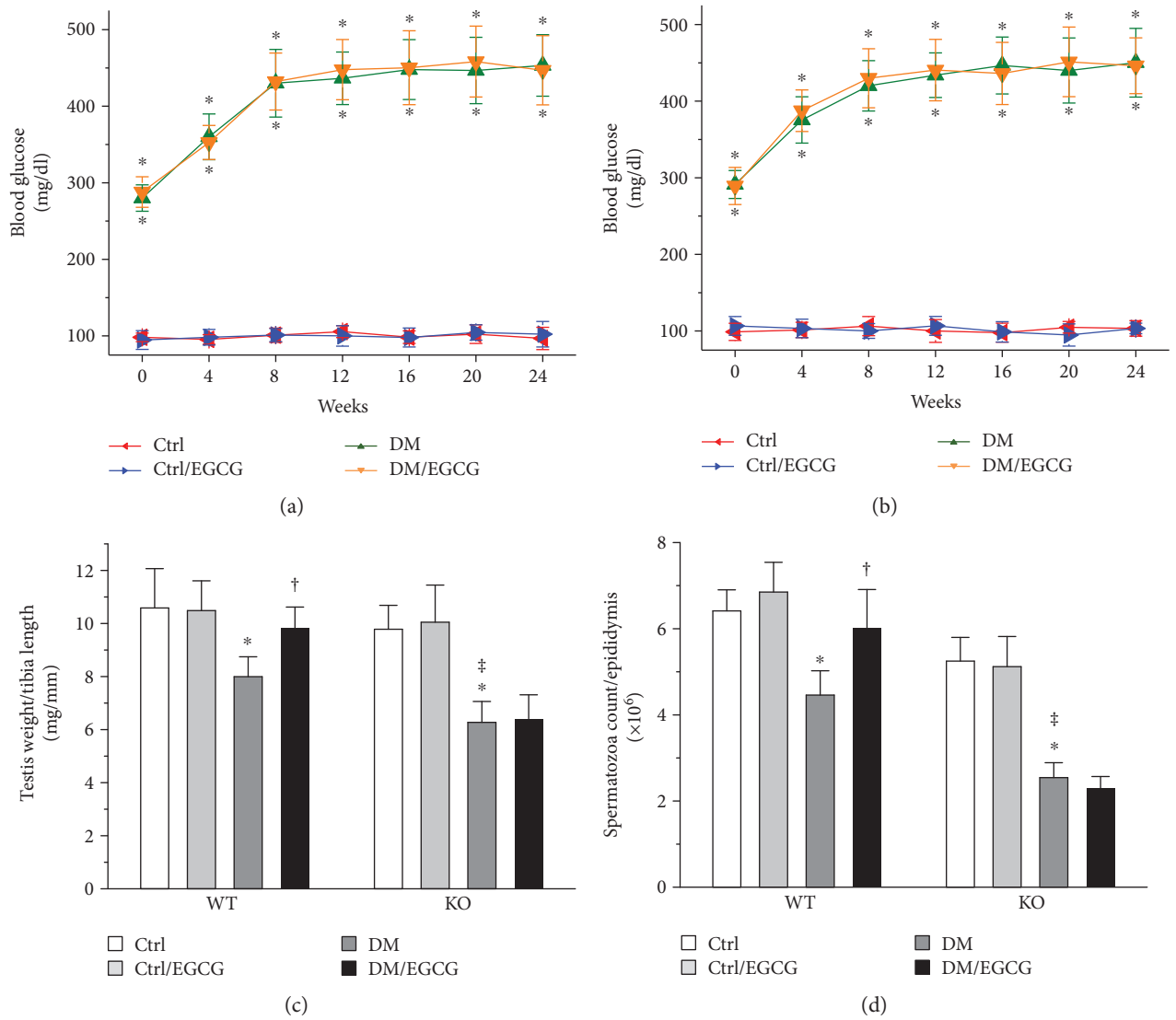


Figure 1: Deletion of the *Nrf2* gene completely abrogated both self and EGCG protection against diabetes-induced testicular weight loss and reduction in spermatozoa count. Diabetes was induced in 8-week-old male C57BL/6 WT and *Nrf2* KO mice by streptozotocin. Blood glucose was monitored in both the (a) WT and (b) *Nrf2* KO mice at the multiple time points 0, 4, 8, 12, 16, 20, and 24 weeks post diabetes onset. (c) Testis weight to tibia length ratio and (d) spermatozoa count were calculated at the time, 24 weeks post diabetes onset, at which the mice were killed. Data were presented as means ± SD ($n = 6$). $^{*}p < 0.05$ versus Ctrl; $^{\dagger}p < 0.05$ versus DM; $^{\ddagger}p < 0.05$ versus WT DM. WT: wild type; KO: knockout; Ctrl: control; DM: diabetes mellitus.

anti-caspase12 (Cell Signaling Technology, 1 : 1000), anti-cleaved caspase3 (c-caspase3, Cell Signaling Technology, 1 : 1000), anti-C/EBP homologous protein (CHOP, Cell Signaling Technology, 1 : 1000), anti-GAPDH (Santa Cruz Biotechnology, 1 : 2000), anti-histone H3 (Santa Cruz Biotechnology; 1 : 1000), anti-intercellular adhesion molecule 1 (ICAM-1, Santa Cruz Biotechnology, 1 : 500), anti-inducible nitric oxide synthase (iNOS, Cell signaling Technology, 1 : 1000), anti-NRF2 (Santa Cruz Biotechnology, 1 : 1000), anti-pro-caspase3 (Cell Signaling Technology, 1 : 1000), and anti-vascular cell adhesion molecule 1 (VCAM-1, Santa Cruz Biotechnology, 1 : 500).

2.7. Quantitative Reverse Transcription PCR (qPCR). qPCR was performed as previously described [34, 35]. Primers for *Ho1* (product number: Mm00516005_m1) and *Nqo1* (product number: Mm01253561_m1) were purchased from Life Technologies (Grand Island, NY, USA). Thermal cycling was carried out as the following: 95°C for 3 minutes (m) as initial denaturing, 45 cycles at 94°C for 30 seconds (s), 60°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 2 m.

2.8. Quantitative Analysis of Lipid Peroxides. Testicular malondialdehyde (MDA) concentration was measured using a lipid peroxidation assay kit (Sigma-Aldrich), following the manufacturer's instructions, as previously described [6].

2.9. Statistical Analysis. Six mice in each group were researched. Indices in each group were measured and

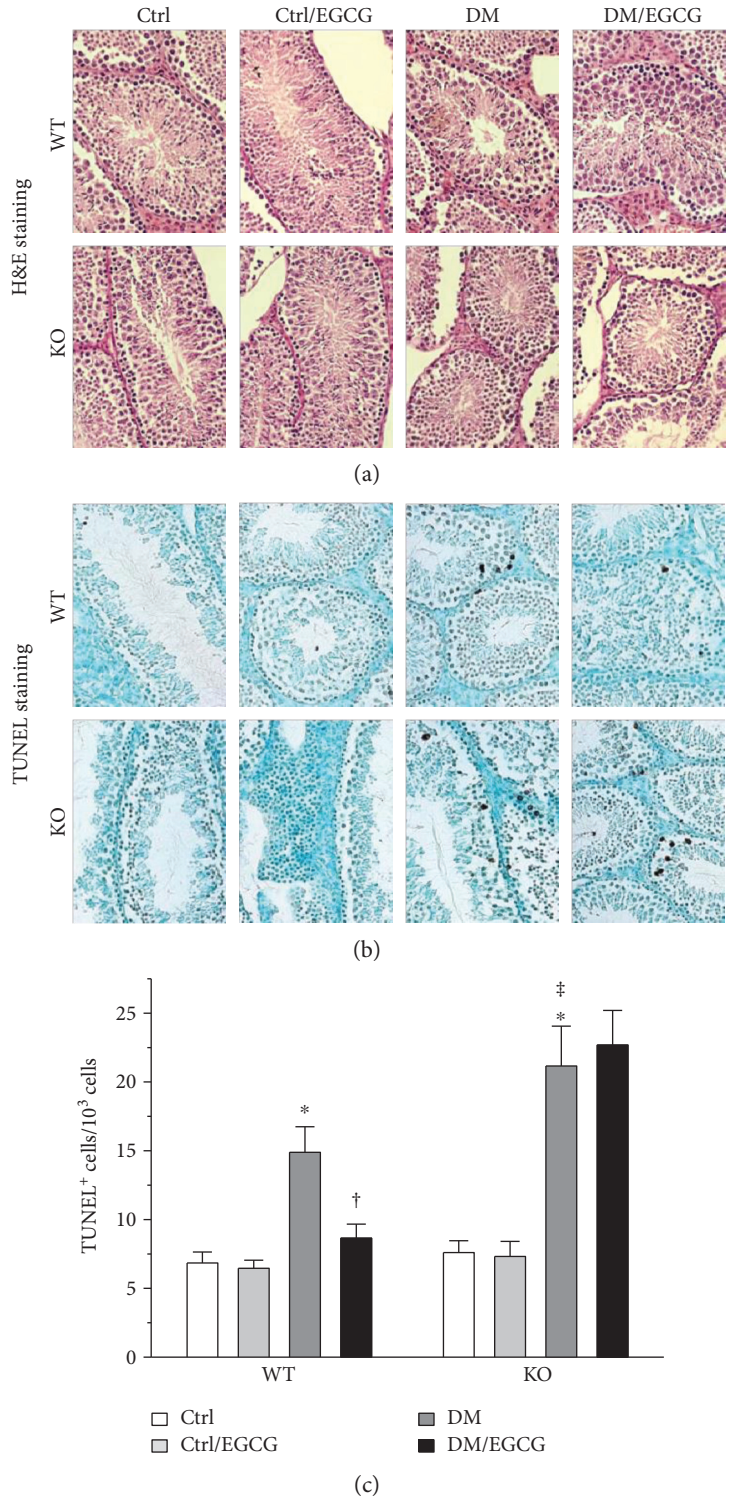


Figure 2: NRF2 was required for both self and EGCG protection against diabetes-induced testicular apoptotic cell death. (a) H&E staining was conducted for observation of morphological change. (b) Testicular apoptotic cell death was detected by TUNEL assay, from which (c) TUNEL$^+$ cells were calculated. Data were presented as means ± SD ($n = 6$). $^*p < 0.05$ versus Ctrl; $^\dagger p < 0.05$ versus DM; $^\ddagger p < 0.05$ versus WT DM. H&E: hematoxylin and eosin; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; $^+$: positive; other abbreviations are the same as those in Figure 1.

summarized as means ± SD. Image Quant 5.2 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) was used to analyse the density of Western blot images. IHC positive area was quantified by the Image-Pro Plus Version 6.0 software (Media Cybernetics, Rockville, MD, USA). One-way ANOVA was performed for comparisons among different

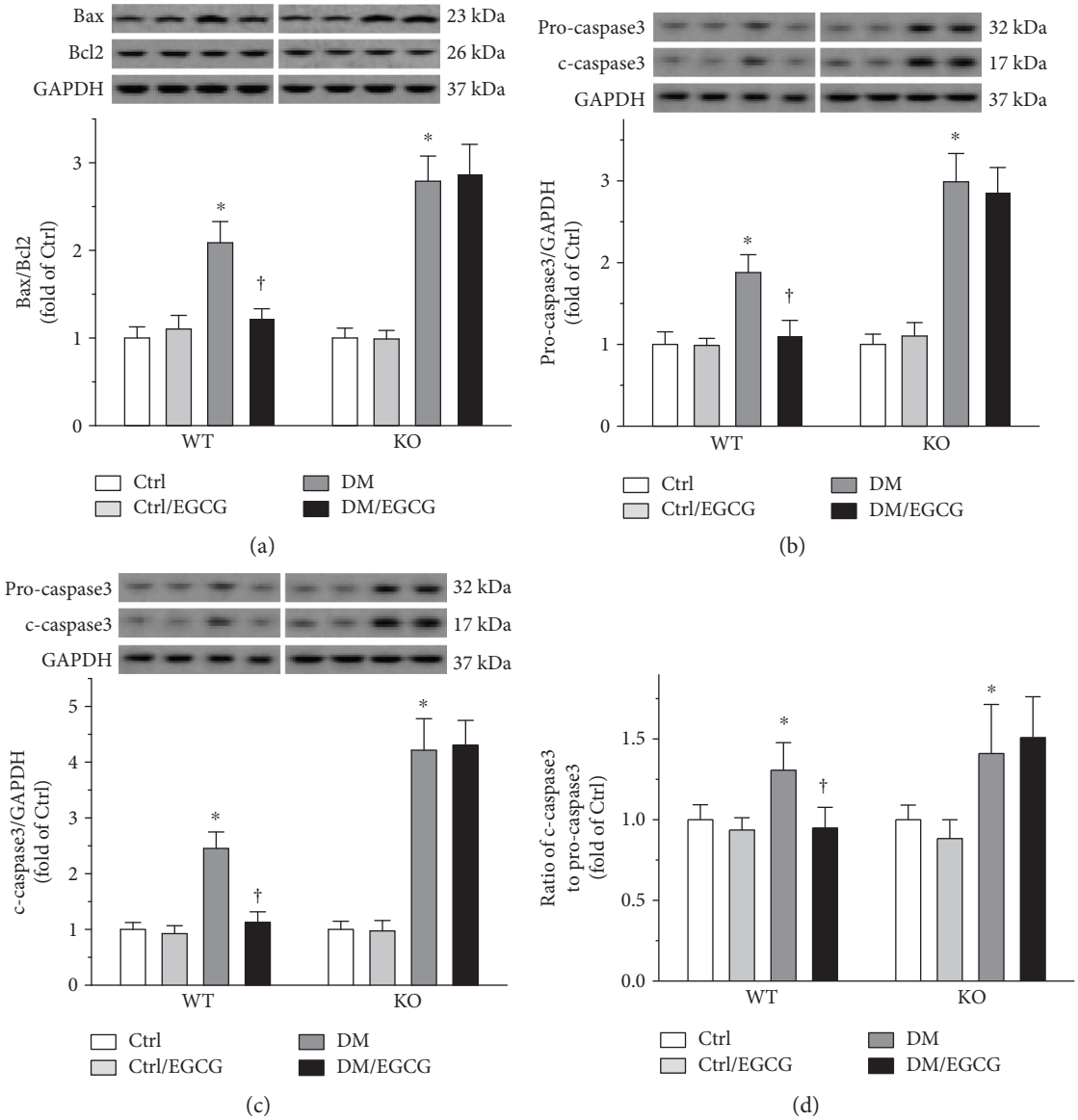


Figure 3: EGCG prevented diabetes-induced activation of testicular apoptotic cell death signaling via NRF2. Testicular apoptotic signaling was further evaluated by determining (a) the ratio of Bax protein level to Bcl2 protein level, along with the protein levels of (b) pro-caspase3 and (c) c-caspase3. To further assess the activity of caspase3, (d) the ratio of c-caspase3 to pro-caspase3 was calculated. For (b) and (c), the protein levels were normalized with GAPDH. Data were normalized as fold variation to Ctrl and were presented as means ± SD ($n = 6$). $^{*}p < 0.05$ versus Ctrl; $^{\dagger}p < 0.05$ versus DM. Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; c-caspase3: cleaved caspase3; other abbreviations are the same as those in Figure 1.

groups, followed by post hoc pairwise comparisons using Tukey's test with Origin 8.6 data analysis and graphing software Lab (OriginLab, Northampton, MA, USA). A test was significant if $p < 0.05$.

3. Results

3.1. Deletion of the Nrf2 Gene Completely Abrogated Both Self and EGCG Protection against Diabetes-Induced Testicular Weight Loss and Reduction in Spermatozoa Count. Both the WT and the *Nrf2* KO diabetic mice developed significantly higher blood glucose levels at 0, 4, 8, 12, 16, 20, and 24 weeks post diabetes onset, as compared with respective controls (Figures 1(a) and 1(b)). EGCG had no impact on blood glucose levels in either type of the mice, under either diabetic or nondiabetic conditions (Figures 1(a) and 1(b)). Diabetes caused a significant decrease in the ratio of testis weight to tibia length and spermatozoa count in either type of the mice (Figures 1(c) and 1(d)). Notably, the *Nrf2* KO diabetic mice suffered from more marked decrease in the two indices, as compared with the WT diabetic mice (Figures 1(c) and 1(d)). The WT diabetic mice, but not the *Nrf2* KO diabetic mice, were protected by EGCG from diabetes-induced reduction in testicular weight and spermatozoa count (Figures 1(c) and 1(d)). These findings suggested that NRF2 plays a critical role in both self-protection and EGCG

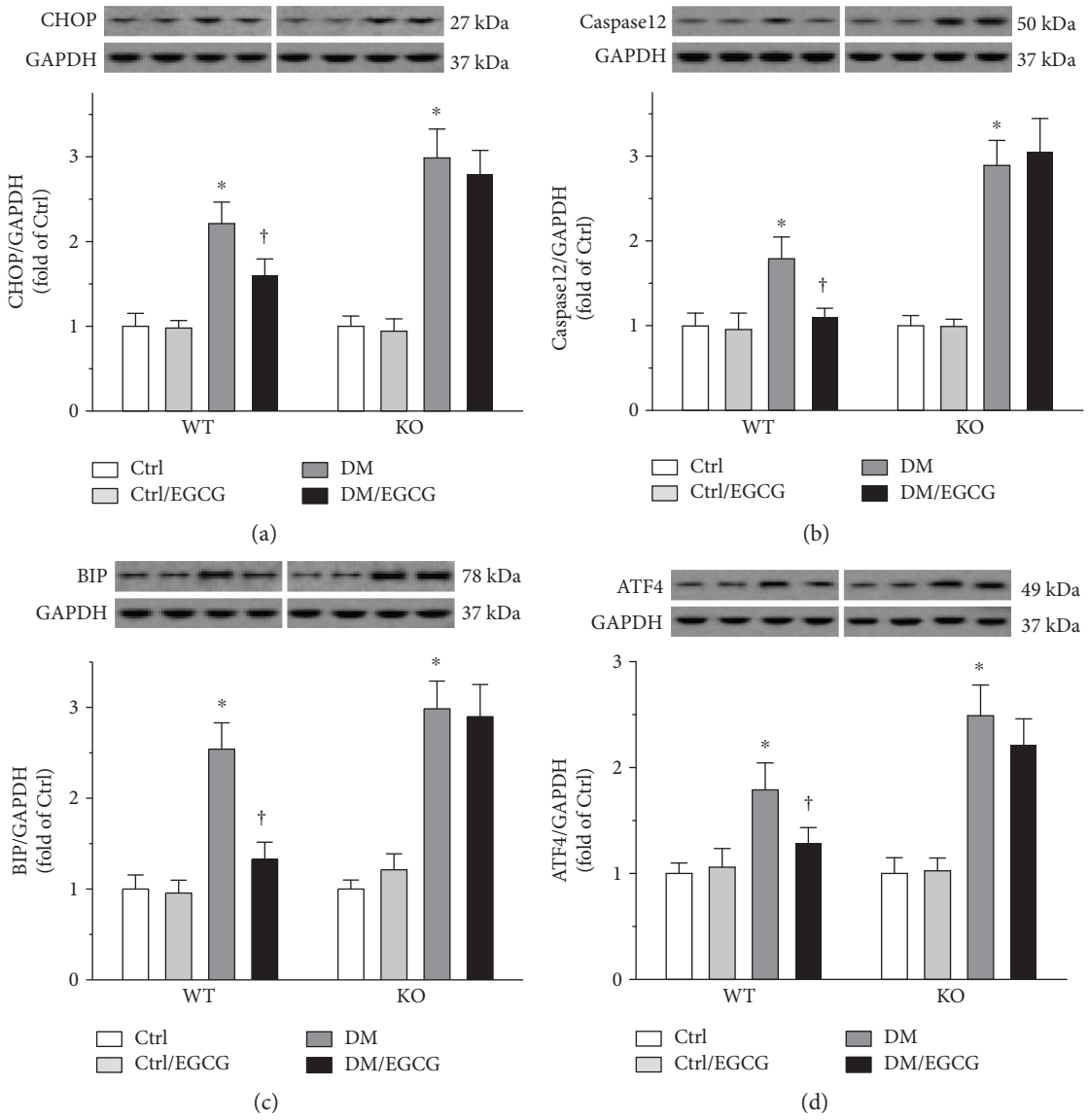
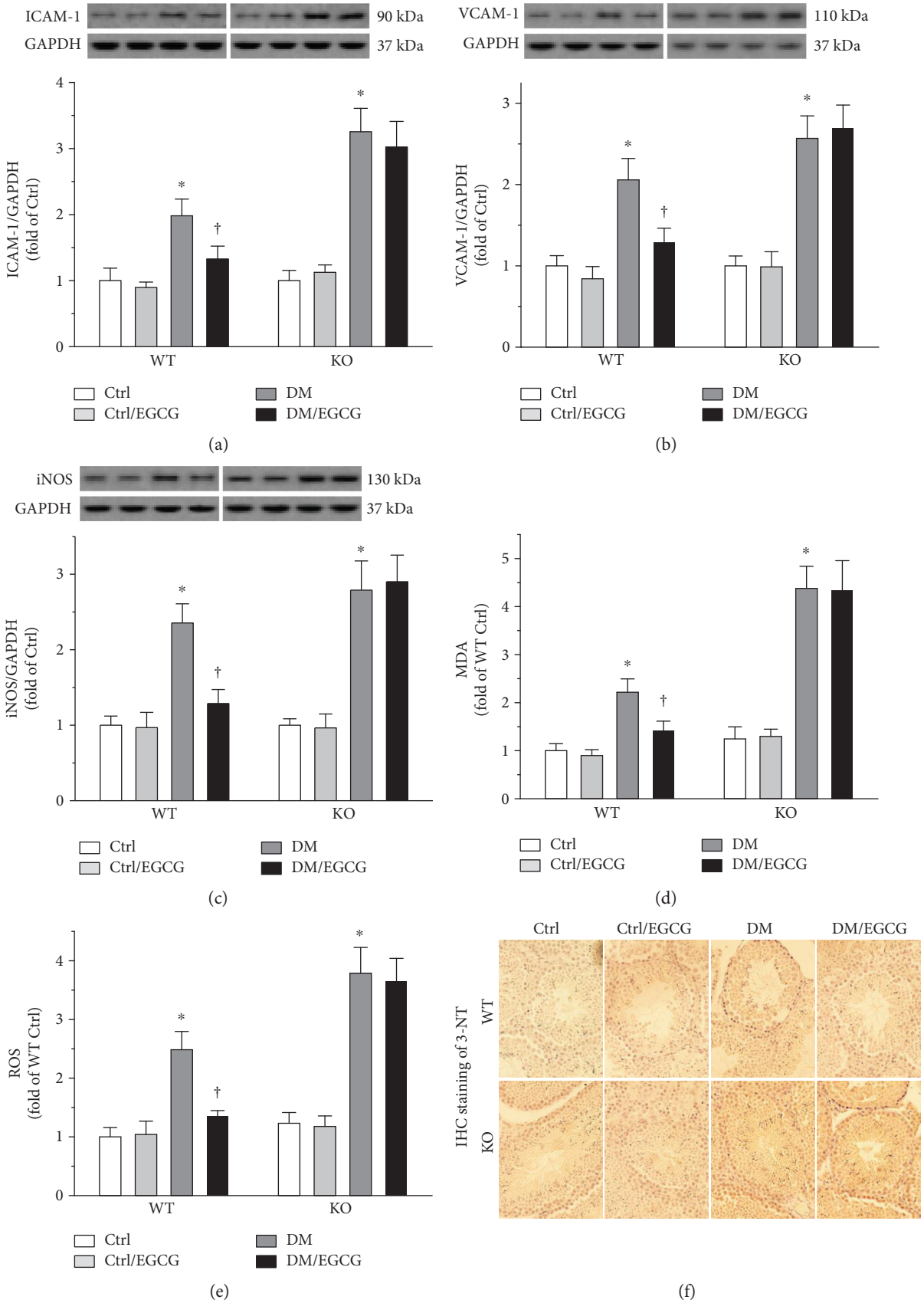


Figure 4: NRF2 mediated EGCG prevention of diabetes-induced testicular ER stress. The status of ER stress was determined by measuring protein levels of (a) CHOP, (b) caspase12, (c) BIP, and (d) ATF4, using Western blot. The protein levels were normalized with GAPDH. Data were normalized as fold variation to Ctrl and were presented as means ± SD ($n = 6$). $^{*}p < 0.05$ versus Ctrl; $^{\dagger}p < 0.05$ versus DM. ER: endoplasmic reticulum; CHOP: C/EBP homologous protein; BIP: binding immunoglobulin protein; ATF4: activating transcription factor 4; other abbreviations are the same as those in Figure 1.

protection against diabetes-induced loss in testis weight and spermatozoa count.

3.2. NRF2 Was Required for Both Self and EGCG Protection against Diabetes-Induced Testicular Apoptotic Cell Death. Diabetes did not lead to obvious testicular pathological changes, as presented by H&E staining (Figure 2(a)). However, apoptotic cell death was prominent in the diabetic testes of either type of the mice, as shown by TUNEL staining (Figure 2(b)). Notably, diabetes-induced testicular apoptotic cell death was more significant in the *Nrf2* KO mice, as compared to the WT mice (Figure 2(c)). EGCG significantly decreased the number of testicular TUNEL positive cells in the WT diabetic mice, but not the *Nrf2* KO diabetic mice (Figure 2(c)).

3.3. EGCG Prevented Diabetes-Induced Activation of Testicular Apoptotic Cell Death Signaling via NRF2. The status of testicular apoptotic cell death was further confirmed by determining the ratio of Bax to Bcl2 (Bax/Bcl2, Figure 3(a)) and the protein levels of pro-caspase3 and c-caspase3 (Figures 3(b) and 3(c)). Bax/Bcl2 and the protein levels of pro-caspase3 and c-caspase3 were all significantly elevated in the testes of the diabetic mice (Figures 3(a), 3(b), and 3(c)), the effects of which were almost completely prevented by EGCG in the WT mice (Figures 3(a), 3(b), and 3(c), left panels). However, deletion of the *Nrf2* gene completely abrogated these efficacies of EGCG (Figures 3(a), 3(b), and 3(c), right panels). To further evaluate caspase3 activity, the ratio of c-caspase3 to pro-caspase3 was calculated in all groups and comparisons were constructed between the groups



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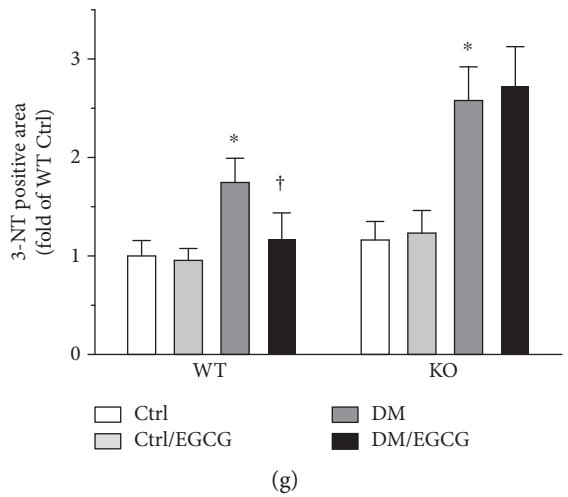


Figure 5: EGCG completely lost the efficacy in ameliorating diabetes-induced testicular inflammation and oxidative damage in the absence of NRF2. Testicular inflammation was assessed by determining protein levels of (a) ICAM-1 and (b) VCAM-1, using Western blot. To determine testicular oxidative stress, (c) iNOS protein was determined by Western blot. The protein levels were normalized with GAPDH. Data were normalized as fold variation to Ctrl and were presented as means ± SD ($n = 6$). To further evaluate testicular oxidative stress, ELISAs were performed to detect (d) MDA and (e) ROS levels, and (f) IHC staining for 3-NT was also performed. Data were normalized as fold variation to WT Ctrl and were presented as means ± SD ($n = 6$). $^{*}p < 0.05$ versus Ctrl; $^{\dagger}p < 0.05$ versus DM. ICAM-1: intercellular adhesion molecule 1; VCAM-1: vascular cell adhesion molecule 1; iNOS: inducible nitric oxide synthase; MDA: malondialdehyde; ROS: reactive oxygen species; IHC: immunohistochemical; 3-NT: 3-nitrotyrosine; other abbreviations are the same as those in Figure 1.

(Figure 3(d)). As shown in Figure 3(d), EGCG prevented the diabetes-elevated ratio of c-caspase3 to pro-caspase3 (Figure 3(d), left panel) in the WT mice, but not in the *Nrf2* KO mice (Figure 3(d), right panel).

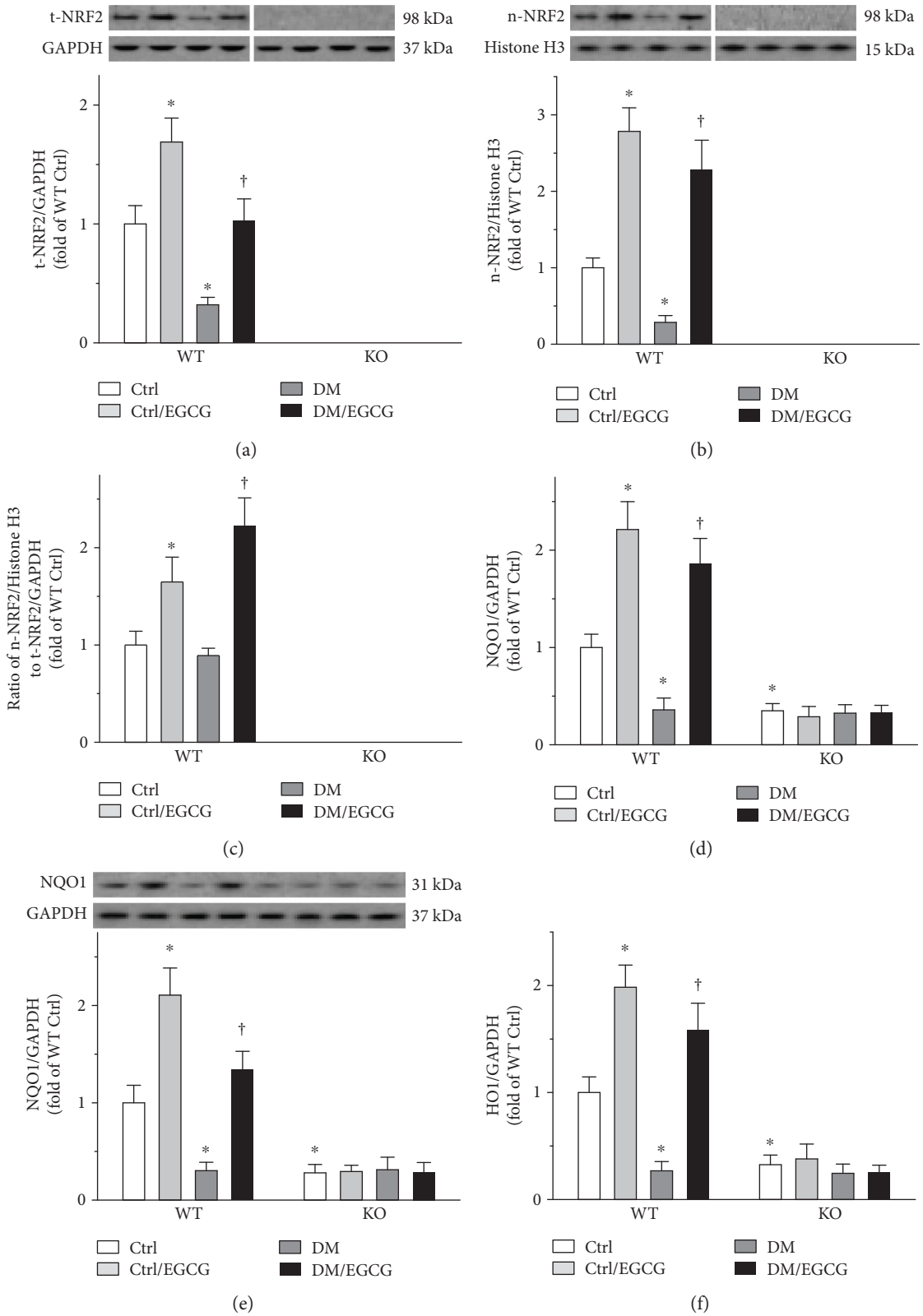
3.4. NRF2 Mediated EGCG Prevention of Diabetes-Induced Testicular ER Stress. ER stress was evaluated by determining the protein levels of CHOP (Figure 4(a)), caspase12 (Figure 4(b)), BIP (Figure 4(c)), and ATF4 (Figure 4(d)), all of which were elevated in the diabetic testes. EGCG decreased these indices in the WT, but not the *Nrf2* KO, diabetic mice (Figures 4(a), 4(b), 4(c), and 4(d)). The results indicated NRF2 to be the key factor through which EGCG prevented diabetes-induced testicular ER stress.

3.5. EGCG Completely Lost the Efficacy in Ameliorating Diabetes-Induced Testicular Inflammation and Oxidative Damage in the Absence of NRF2. Testicular inflammation was evaluated by determining protein levels of ICAM-1 (Figure 5(a)) and VCAM-1 (Figure 5(b)). Testicular oxidative damage was determined by measuring iNOS protein level (Figure 5(c)), MDA level (Figure 5(d)), and ROS generation (Figure 5(e)). In the WT mice, EGCG markedly decreased these diabetes-elevated indices (Figures 5(a), 5(b), 5(c), 5(d), and 5(e), left panels). These effects of EGCG were completely lost in the absence of NRF2 (Figures 5(a), 5(b), 5(c), 5(d), and 5(e), right panels). The status of testicular oxidative stress was further evaluated by immunohistochemical staining of 3-NT (Figure 5(f)), an indicator of oxidative/nitrosative damage. As shown in Figure 5(g), EGCG completely lost the protective role in attenuating the diabetes induction of 3-NT in the absence of NRF2.

3.6. EGCG Enhanced Testicular NRF2 Expression and Function. Testicular whole cell NRF2 (total NRF2, t-NRF2) and nuclear NRF2 (n-NRF2) were both increased by EGCG in the WT mice, under either diabetic or nondiabetic conditions (Figures 6(a) and 6(b), left panels). NRF2 protein was not detectable in the testes of the *Nrf2* KO mice (Figures 6(a) and 6(b), right panels), the result of which confirmed the deletion of the *Nrf2* gene. The ratio of n-NRF2/Histone H3 to t-NRF2/GAPDH (Figure 6(c)) was calculated to reflect the proportion of NRF2 that translocated to the nucleus. This ratio was found to be increased by EGCG in either diabetic or nondiabetic WT mice (Figure 6(c), left panel). In order to evaluate NRF2 function, the expression of *Nqo1* (Figures 6(d) and 6(e)) and *Ho1* (Figures 6(f) and 6(g)) was determined. As shown in Figures 6(d), 6(e), 6(f), and 6(g), the mRNA and protein levels of *Nqo1* and *Ho1* were all elevated by EGCG in the WT mice (Figures 6(d), 6(e), 6(f), and 6(g), left panels), but not in the *Nrf2* KO mice (Figures 6(d), 6(e), 6(f), and 6(g), right panels). Moreover, the *Nrf2* KO mice had lower basal expression of *Nqo1* and *Ho1*, as compared with the WT mice (Figures 6(d), 6(e), 6(f), and 6(g)).

4. Discussion

The present study determined the effect of EGCG on the prevention of DTD. The results showed that diabetes caused significant testicular weight loss, decreased spermatozoa count, and increased testicular apoptotic cell death, ER stress, and oxidative damage, as compared with control. Notably, these detrimental outcomes were more prominent in the *Nrf2* KO mice, as compared with the WT mice. EGCG activated NRF2 signaling and produced a significant attenuation of



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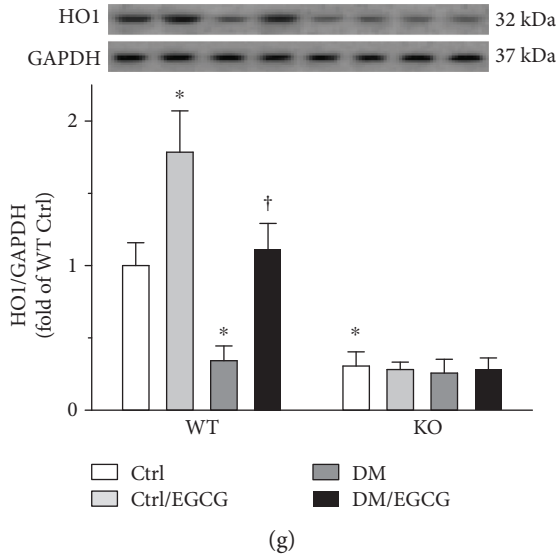

Figure 6: EGCG enhanced testicular NRF2 expression and function. Testicular (a) t-NRF2 and (b) n-NRF2 protein were determined by Western blot in all the mice. (c) The ratio of n-NRF2 to t-NRF2 was calculated to indicate NRF2 nuclear translocation. To evaluate NRF2 function, the expression of *Nqo1* and *Ho1* were further determined, by measuring *Nqo1* (d) mRNA and (e) protein levels, as well as *Ho1* (f) mRNA and (g) protein levels. t-NRF2 protein and the expression of *Nqo1* and *Ho1* were normalized to GAPDH. n-NRF2 was normalized to Histone H3. Data were normalized as fold variation to WT Ctrl and were presented as means ± SD ($n = 6$). $^{*}p < 0.05$ versus WT Ctrl; $^{\dagger}p < 0.05$ versus WT DM. t-NRF2: total NRF2; n-NRF2: nuclear NRF2; *Nqo1*: NAD(P)H dehydrogenase quinone 1; *Ho1*: heme oxygenase-1; other abbreviations are the same as those in Figure 1.

the testicular damage caused by diabetes in the WT mice. However, deletion of the *Nrf2* gene completely abolished the protective effect of EGCG.

Oxidative stress is considered to be one of the main mechanisms through which diabetes causes long-term complications [36–38]. Significant oxidative damage was observed in the testes of diabetic mice [5–8, 15]. Given that NRF2 plays a critical role in cellular defence against diabetes-induced oxidative stress, approaches to activate testicular NRF2 have been tested in diabetic mice, including administration of the NRF2 activator SFN [5, 6], supplementation of zinc [15], and exposure to low-dose X-irradiation [8]. Although the effects of the approaches were promising, it was still unclear whether NRF2 activation is required for the protective effect of the approaches. In the present study, by using *Nrf2* KO mice, NRF2 was found to be the key factor through which EGCG ameliorated DTD. In addition, enhanced oxidative stress status was observed in a rat model of prediabetes [39, 40], and white tea consumption restored sperm quality in the prediabetic rats by ameliorating testicular oxidative damage [40]. The present study supports the previous report by Oliveira et al. [40], with an emphasis on the long-term DTD.

One novel finding of the present study was the protective role of NRF2 in self-prevention of the pathogenesis of DTD (Figures 1(c), 1(d), 2(b), and 2(c)), in addition to the finding that NRF2 was required for the protective effect of EGCG on DTD. The self-protective role of NRF2 observed in the present study is in accordance with the previous findings which showed that NRF2 played a key preventive role in diabetic cardiomyopathy [41] and nephropathy [32, 42, 43]. The beneficial role of NRF2 in multiple organs or systems under diabetic condition [38] may support the use of NRF2 activators, even though the activators may not be specific to an organ, tissue, or cell type.

NRF2 activators have been developed based on different regulatory mechanisms. Zinc was reported to upregulate NRF2 protein in the testes of diabetic rats [15], although the mechanism by which zinc increased NRF2 was not investigated. The finding that zinc enhanced NRF2 expression and function via activating protein kinase B- (PKB- or AKT-) mediated inhibition of Fyn function [44] might provide a clue for the possible mechanism by which zinc activated NRF2 in the testes of diabetic rats. Low-dose radiation was also recently reported to attenuate testicular apoptosis in diabetic rats [8]. The study indicated that low-dose radiation inhibited protein tyrosine phosphatase-1B and tribbles homologue 3, the effect of which resulted in AKT-mediated activation of testicular NRF2 signaling [8]. Therefore, zinc and low-dose radiation shared the same AKT signaling pathway to activate testicular NRF2. SFN is a potent NRF2 activator. Kelch-like ECH-associated protein 1 (KEAP1) is the key negative cytoplasmic regulator of NRF2 [11, 45]. SFN activates NRF2 signaling by modulating the structure of KEAP1 protein, resulting in the release of NRF2 from the KEAP1-NRF2 complex [11, 45]. Although previous studies showed that SFN activated NRF2 and ameliorated diabetes-induced testicular apoptotic cell death without knowing the expression of *Keap1* [5, 6], we speculate that inhibition of KEAP1 function by SFN could be the mechanism through which SFN activated NRF2 in these studies. Similar to SFN, EGCG is also known to activate NRF2 by inactivating KEAP1 [46, 47]. EGCG is speculated to directly interact with the cysteine residues present in KEAP1, thereby stimulating NRF2

dissociation from KEAP1 [48]. However, another study indicated that EGCG might induce NRF2 via activation of AKT and ERK in human mammary epithelial cells [18]. Future studies are needed to elucidate the exact mechanisms of EGCG and other NRF2-activating approaches in the regulation of NRF2 in DTD.

The NRF2 activator SFN has already been tested in several clinical trials [49]. Furthermore, the approval of dimethyl fumarate (BG-12), another NRF2 activator, for use in the treatment of multiple sclerosis [50] is the confirmation of NRF2 being a viable drug target in disease. However, to date, no NRF2 activator has been applied in clinical trials for DTD or diabetes-induced male infertility. Hence, attention should be paid to the critical role of NRF2 in this diabetic complication.

Taken together, the present study demonstrates, for the first time, that NRF2 plays a key role in self and EGCG protection against diabetic testicular damage. Therefore, this study may provide a basis for potential application of EGCG or other NRF2 activators in future clinical trials.

Conflicts of Interest

The authors declare that there is no conflict of interest in this work.

Authors' Contributions

Hao Wu conceived the idea and designed the project. Chenyu Pan, Shengzhu Zhou, Junduo Wu, Lingyun Liu, Yanyan Song, Tie Li, Lijuan Ha, Xiaona Liu, Fuchun Wang, and Jingyan Tian researched and interpreted the data. Hao Wu and Jingyan Tian wrote the manuscript. Chenyu Pan, Shengzhu Zhou, Junduo Wu, Lingyun Liu, Yanyan Song, Tie Li, Lijuan Ha, Xiaona Liu, Fuchun Wang, Jingyan Tian, and Hao Wu reviewed and revised the manuscript. Hao Wu and Fuchun Wang provided the funding for this work. Hao Wu and Jingyan Tian are guarantors of this work, had full access to the data, and take responsibility for the integrity of the data and the accuracy of the data analysis. All the authors approve the version to be published.

Acknowledgments

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Research Article

Genetic Nrf2 Overactivation Inhibits the Deleterious Effects Induced by Hepatocyte-Specific c-met Deletion during the Progression of NASH

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We have recently shown that hepatocyte-specific c-met deficiency accelerates the progression of nonalcoholic steatohepatitis in experimental murine models resulting in augmented production of reactive oxygen species and accelerated development of fibrosis. The aim of this study focuses on the elucidation of the underlying cellular mechanisms driven by Nrf2 overactivation in hepatocytes lacking c-met receptor characterized by a severe unbalance between pro-oxidant and antioxidant functions. Control mice (c-met$^{fx/fx}$), single c-met knockouts (c-met$^{\Delta hepa}$), and double c-met/Keap1 knockouts (met/Keap1$^{\Delta hepa}$) were then fed a chow or a methionine-choline-deficient (MCD) diet, respectively, for 4 weeks to reproduce the features of nonalcoholic steatohepatitis. Upon MCD feeding, met/Keap1$^{\Delta hepa}$ mice displayed increased liver mass albeit decreased triglyceride accumulation. The marked increase of oxidative stress observed in c-met$^{\Delta hepa}$ was restored in the double mutants as assessed by 4-HNE immunostaining and by the expression of genes responsible for the generation of free radicals. Moreover, double knockout mice presented a reduced amount of liver-infiltrating cells and the exacerbation of fibrosis progression observed in c-met$^{\Delta hepa}$ livers was significantly inhibited in met/Keap1$^{\Delta hepa}$. Therefore, genetic activation of the antioxidant transcription factor Nrf2 improves liver damage and repair in hepatocyte-specific c-met-deficient mice mainly through restoring a balance in the cellular redox homeostasis.

1. Introduction

Formation of reactive oxygen species (ROS) has been considered classically a pathophysiological phenomenon critically involved in the progression from simple hepatic steatosis to steatohepatitis. Upon triglyceride accumulation, cellular compartments responsible for lipid catabolism such as mitochondria and lysosomes increase their activity with a consequent generation of free radicals that trigger molecular signals leading to cell death and release of proinflammatory mediators. In this context, the use of antioxidant buffering against the generation of ROS has been shown to partially reduce the progression of nonalcoholic steatohepatitis. A growing body of evidence indicates the HGF (hepatocyte growth factor)/c-met axis as a molecular pathway linked to the control of the cellular redox homeostasis. However, data concerning the consequences of HGF stimulation on the cellular generation of free radicals are still quite controversial. Whereas in primary cell lines and tissues such as cardiomyocytes [1] and neurons [2], stimulation with HGF was shown

to dampen ROS production and to reduce oxidative stress-dependent apoptosis; in cancer cell lines [3] and other in vitro conditions [4], HGF treatment resulted in augmented cell motility accompanied by increased ROS production. Our group and others recently showed that disruption of c-met functionality aggravates the onset of NASH through the impairment of mechanisms regulating cell sensitivity to lipotoxicity, ROS production, and cell proliferation [5, 6]. In particular, data emerging from genomic array analysis clearly indicated an aberrant regulation of a pattern of genes responsible for increased pro-oxidant environment, amongst them the transcription factor Nrf2 (nuclear factor erythroid-derived 2-like 2) [5].

Under conditions of oxidative or electrophilic stress, Nrf2 degradation is inhibited through oxidant-dependent modifications of specific cysteine residues within Keap1, a protein that under quiescent conditions facilitates the marking of Nrf2 for degradation via the proteasome [7]. It is now well established that stabilization and activation of Nrf2 through pharmacological or genetic targeting improves cellular redox homeostasis and survival through transcriptional upregulation of antioxidant and detoxifying genes [8]. In line with these studies, we recently showed that activation of Nrf2 in hepatocytes afforded by genetic deletion of Keap1 was able to reduce triglycerides accumulation and ROS generation in mice subjected to experimental models of NASH [9]. This former observation leads to the generation of a double knockout mouse lacking simultaneously the receptor c-met and Keap1 specifically in hepatocytes to investigate the effects of Nrf2 overexpression in cells displaying an impaired control of the redox functions. In fact, the purpose of this study pointed towards uncovering a bridge between the HGF/c-met axis and the Keap1/Nrf2 system in the context of metabolic liver disturbances (schematic overview in Suppl. Fig. 4 available online at https://doi.org/10.1155/2017/3420286). The results emerging indicated that overexpression of Nrf2 was able to suppress the levels of liver steatosis and fibrosis in c-met-deficient hepatocytes to the level of the control group, with a drastic reduction of triglyceride (TG) accumulation and ROS production. Considering that preliminary clinical data indicate a reduction of c-met expression in patients diagnosed with NASH, this study provides further evidence for strategies for therapeutic interventions in this field.

2. Materials and Methods

2.1. Animals and Experimental Model. Hepatocyte-specific Keap1 knockout mice were generated by breeding Keap1-floxed mice with albumin-Cre (Alb-Cre) C57BL/6 transgenic mice as previously described [10]. Similarly, floxed wild type (c-met$^{fx/fx}$) and hepatocyte-specific conditional c-met knockout (c-met$^{\Delta hepa}$) mice were generated under control of a postnatal activated albumin promoter (C57BL/6), as indicated elsewhere [11]. These two strains were then crossed to generate double mutant c-met/Keap1 (met/Keap1$^{\Delta hepa}$) harboring the same genetic background. Male age-matched wild type (c-met$^{fx/fx}$) and littermate hepatocyte-specific conditional c-met-knockout (c-met$^{\Delta hepa}$) were then cohoused with genetically derived met/Keap1$^{\Delta hepa}$ in 12-hour light/dark cycle and allowed to free food and water. At the age of 8–10 weeks, the animals were split in three groups ($N = 5$) and fed a chow and MCD diet (E15652-94, ssniff Spezialdiäten GmbH), respectively, for a period of 4 weeks. Food intake and body weights were measured weekly, and all animals consumed similar amounts of diets. At the end of the indicated time point, blood and liver samples were collected, fixed in formalin, and cryopreserved for biochemical and histological analyses. All animal experiments were carried out in accordance with the regulations of the German legal requirements on laboratory animal care (LANUV).

2.2. Histological and Morphological Analyses. After explant, liver tissues were fixed in formaldehyde-buffered solution for 24 h and then embedded in paraffin. Blocks containing preserved hepatic tissues were then cut into 5 μm sections and stained with hematoxylin and eosin (H&E) for microscopic examination and morphological analyses. Similarly, 8 μm thin liver sections were stained, upon deparaffinization, with a Sirius red/picric acid (Sigma-Aldrich) solution for 1 h as previously described [9]. Upon dehydration and mounting, photomicrographs of stained sections were randomly taken in a 200x magnification and positive areas were quantified using the open source software ImageJ.

2.3. Serum and Liver Biochemical Measurements. Blood samples were collected and centrifuged in heparin-embedded tubes, and serum transaminase levels (ALT/AST) were measured according to the standard procedures of the Central Laboratory Facility of the University Hospital RWTH of Aachen as index of liver damage. For the evaluation of intrahepatic triglyceride content, liver samples were homogenized in a specific Tris buffer (10 mM Tris, 2 mM EDTA, 0.25 M sucrose, and pH 7.5) and successively processed according to the manufacturer's instructions of a commercial colorimetric kit (10724600, Human Diagnostics). For biochemical quantification of hepatic collagen deposition, 50 mg of liver samples were homogenized in 1 ml of 6 N HCl solution and incubated o.n. at 110°C. Homogenates were then treated with a chloramine-T solution and incubated with Ehrlich's reagent to measure hydroxyproline content by biochemical photometric assay as indicated in previous publication [12].

2.4. RNA Isolation and qPCR Analysis. Total RNA from hepatic tissue was isolated with peqGold RNAPure solution (30–1020, PeqLab, VWR, Germany) according to the manufacturer instructions. An amount of 500 ng of isolated RNA was transcribed into cDNA using the Omniscript Reverse Transcription kit (205111, Qiagen). Quantitative qPCR was performed by using a Real-time PCR System 7300 (Applied Biosystems) and Fast SYBR Green Master Mix qPCR (Thermo Fisher Scientific). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ quantification formula normalizing each gene with the expression of the housekeeping gene 18S (ribosomal subunit). The primers used in this study are reported in Suppl. Table 1.

2.5. Western Blot Analyses. Total hepatic homogenates were performed by lysing liver samples with an Ultra-Turrax

homogenizer in Tris/HCl-lysis buffer containing inhibitors of proteases and phosphatases as described before [9]. Fifty to eighty micrograms of total lysates were denatured in Laemmli sample buffer and separated in 10% and 12% SDS-PAGE gel. Upon electrophoresis and transfer blotting, Ponceau-Red staining was used to verify transfer efficiency and equal protein loading. Membranes were then blocked in blocking buffer and incubated overnight at 4°C with primary antibodies (Suppl. Table 2). Therefore, secondary antibodies were incubated on the membranes for one hour at room temperature. The following secondary antibodies have been used in this study: HRP-linked anti-rabbit immunoglobulin G (7074, Cell Signaling) and HRP-linked anti-mouse immunoglobulin G (sc-2005, Santa Cruz). Enhanced chemiluminescence (ECL) method was used to detect protein bands and the software ImageJ was employed for densitometric analysis of band intensity.

2.6. FACS Analysis of Myeloid and Lymphoid Hepatic Infiltrates. A nonparenchymal cell fraction from whole liver extracts was isolated upon collagenase and mechanical digestion followed by Percoll (GE Healthcare Life Sciences) gradient centrifugation as previously described [5]. In parallel, blood samples were collected in EDTA-containing tubes and treated with red blood cell lysis buffer (Pharm-Lyse, BD Biosciences, Germany). Upon removal of red bodies and centrifugation, immune cells were incubated with fluorochrome-conjugated antibodies and characterized according to two different panels, a myeloid panel: CD45-BV510 (103138, BioLegend), 7AAD-PE-Cy5-YG (420404, BioLegend), CD11b-BV711 (101242, BioLegend), F4/80-APC (17-4801-82, eBiosciences), MHC2-Alexa700 (107622, BioLegend), CD11c-PE-Cy7 (25-0114-81, eBiosciences), and Ly6G-FITC (551460, BD Pharmingen) and a lymphoid panel: CD45-BV510 (103138, BioLegend), 7AAD-PE-Cy5-YG (420404, BioLegend), CD3-PE-Cy7 (25-0031-82, eBiosciences), CD4-FITC (11-0041-85, eBiosciences), CD8-PerCpCy5.5 (126610, BioLegend), and NK1.1-BV711 (108745, BioLegend). Labeled cells were then subjected to flow cytometry using a BD Canto II (BD Biosciences) and relative cell numbers were analyzed using FlowJo software (Tree Star).

2.7. Immunohistochemistry and TUNEL Assay. For 4-HNE immunostaining, paraffin-embedded sections were used. Upon antigen retrieval in sodium citrate buffer, endogenous peroxidases were inhibited through incubation with 3% H_2O_2 in PBS buffer for 10 minutes. Blocking was performed by incubating the sections with 5% goat serum in PBS buffer for 1 hour. Sections were then incubated with primary antibody in blocking solution overnight at 4°C and successively 1 hour at room temperature with secondary antibody (anti-mouse biotinylated). Antigen was visualized using a peroxidase substrate DAB kit (di-amino benzidine) (DAKO). For immunofluorescence staining, hepatic 5 μm cryosections were fixed in 4% paraformaldehyde-buffered solution. Blocking was performed by incubating the sections with 0.2% BSA in PBS buffer for 5 minutes. Then sections were incubated with primary antibody for 1 h at RT in PBS buffer containing 1% mouse serum. After washing and further blocking, slides were incubated with secondary antibody for one hour at RT. DAPI (4′,6-diamidino-2-phenylindole) was used to visualize cell nuclei. The secondary antibody used in this section is anti-rat Alexa Fluor 594-conjugated antibody (Molecular Probes/Invitrogen). Primary antibodies are indicated in Suppl. Table 2.

For the detection of apoptotic cells, a TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay was performed by using the in situ cell death detection kit (Fluorescein, 11684795910, Roche). Analysis of quantification of positive cells was performed by using the open source software ImageJ.

2.8. Statistical Analysis. All results are expressed as mean ± SE and represent data from 5 animals per group. All significant p values were measured by one-way ANOVA test, followed by Bonferrroni's posttest for the comparison between groups. A value of $p < 0.05$ was considered significant ($^{*}p < 0.05$, $^{**}p < 0.01$).

3. Results

3.1. Nrf2 Overexpression in Hepatocytes Lacking c-met Receptor Results in Reduced Triglycerides Accumulation upon MCD Feeding. As previously described, deletion of the exon 15 in c-met$^{\Delta hepa}$ mice operated by the cre-recombinase under control of the albumin promoter resulted in a defective intracellular activation of the receptor as indicated by impaired phosphorylation of specific tyrosine residues (data not shown). The effective deletion of c-met exon 15 was confirmed by reverse transcriptase-PCR analysis (Figure 1(a), upper graph). As expected, selective hepatic deletion of Keap1 resulted in increased protein levels of the transcription factor Nrf2 (Figure 1(a), lower graph). This stabilization correlated with augmented transcriptional activity as assessed by expression of a well-known target gene Nqo1 (Suppl. Fig. 1a). Displaying no differences of total body weight, hepatocyte c-met deletion resulted in a slight reduction of the hepatic mass. Further, deletion of Keap1 induced a moderate but significant increase of liver weight as compared to control mice, under normal chow feeding, only subtly depending on changes of the cell proliferation rate (Suppl. Fig. 1b). However, microscopically, we could not detect any obvious alteration as emerged from histological analysis of hematoxylin and eosin (H&E) staining (Figure 1(b), left panels). After 4 weeks of MCD feeding, met/Keap1$^{\Delta hepa}$ still displayed a significant increase of the liver mass without significant alterations of body weight that progressively declined in all groups (Figures 1(c) and 1(d)). Interestingly, double knockouts showed decreased accumulation of lipid droplets and a lower grade of steatosis as confirmed by histological analyses of H&E staining (Figure 1(b), right panels). Biochemical analyses revealed that met/Keap1$^{\Delta hepa}$ accumulated about 50% less TG in the liver as compared to other experimental groups (Figure 1(e)). Accordingly, gene expression of the lipid droplet associated protein, Plin2, showed an absolute increase of steatohepatitis progression in c-met$^{\Delta hepa}$ mice. In contrast, the same gene was significantly downregulated

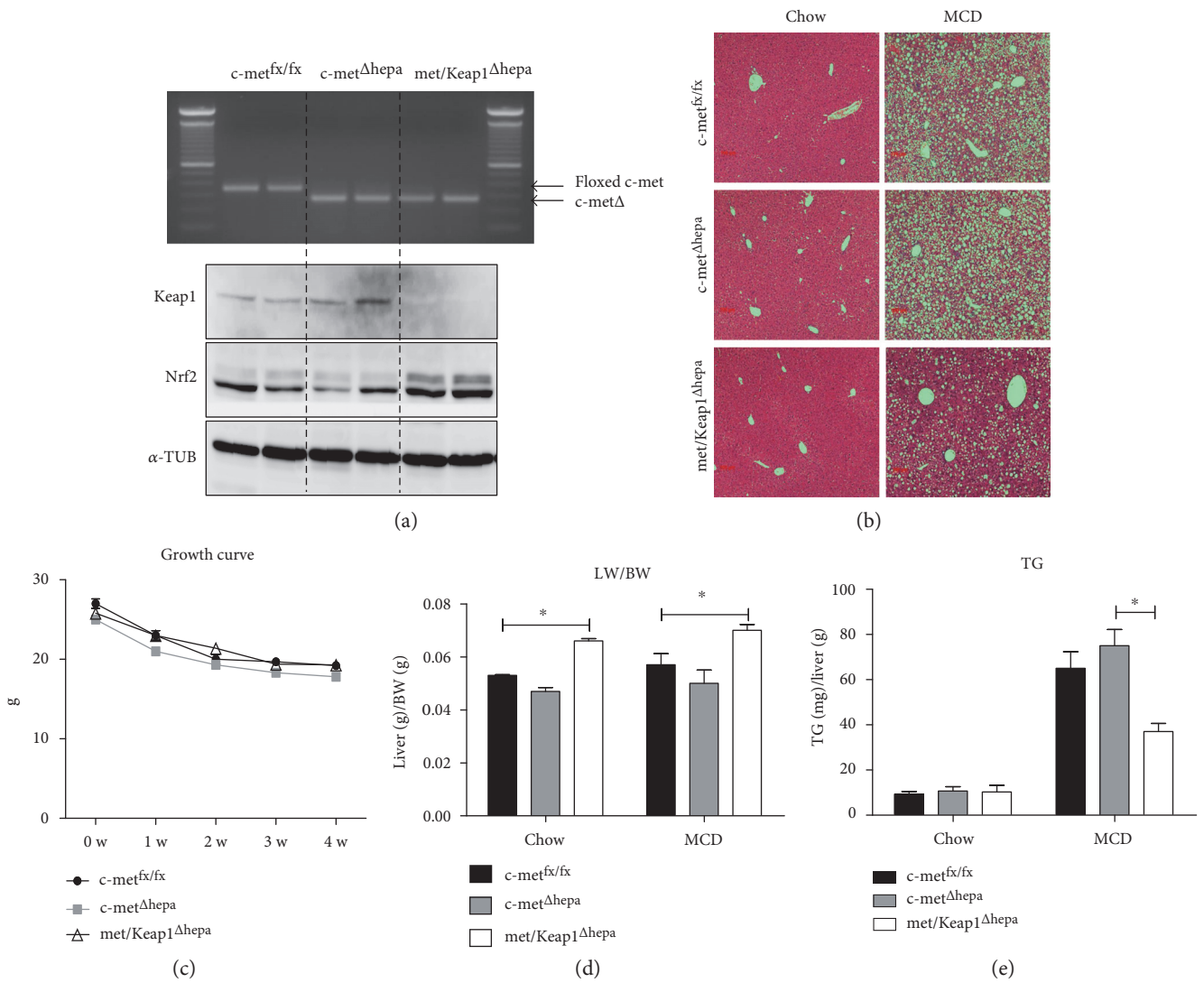


FIGURE 1: Genotyping of c-met gene performed on cDNA transcribed from hepatic RNA showing amplification of the floxed region of the gene with and without cre-recombinase activation ((a), upper panel). Western blot panel for Keap1 and Nrf2 protein expression analysis ((a), lower panels). Representative pictures of hematoxylin and eosin (H&E) staining on liver section of c-met$^{fx/fx}$, c-met$^{\Delta hepa}$, and met/Keap1$^{\Delta hepa}$ mice after 4 weeks chow (left panels) and MCD (right panels) diet (b). Weekly growth curve of c-met$^{fx/fx}$, c-met$^{\Delta hepa}$, and met/Keap1$^{\Delta hepa}$ mice during a 4-week course of MCD treatment. *Data are expressed as mean ± SE, Student's *t*-test with $p < 0.05$ ($N = 5$) (c). Liver body weight ratio (d) and intrahepatic triglyceride content measurement (e) after 4-week chow and MCD diet feeding. *Data are expressed as mean ± SE, ANOVA test with $p < 0.05$ ($N = 5$).

in met/Keap1$^{\Delta hepa}$ hepatocytes as compared with the single mutants (Figure 2(a)). Furthermore, double knockout mice displayed increased hepatic expression and phosphorylation of AMPK and augmented expression of the transcription factor PGC-1α compared to the other experimental groups (Figures 2(b), 2(c), and 2(d)), indicating enhanced fatty acid oxidation and mitochondrial biogenesis. These results confirmed that Nrf2 overactivation is sufficient to enhance hepatic lipid catabolism and mitochondrial functionality as we recently illustrated in detail elsewhere [9].

3.2. Nrf2 Overexpression Dampens the Exacerbation of Oxidative Stress Production in Hepatocytes Lacking c-met Receptor upon MCD Feeding. Whereas under chow diet, oxidative stress was barely detectable, and after 4 weeks of MCD administration, hepatocyte-specific c-met deletion resulted in increased production of oxidative stress compared to wild type, as evidenced by immunostaining analysis of 4-HNE, a bioproduct of lipid peroxidation (Figures 3(a), 3(b), 3(c), 3(d), and 3(e)). In contrast, overactivation of Nrf2 induced by Keap1 deletion resulted in a strong decrease of 4-HNE-positive cells at levels even lower than the control group (c-met$^{fx/fx}$) (Figure 3(f)). Of note, histological analyses indicated that met/Keap1$^{\Delta hepa}$ livers not only showed a decreased number of 4-HNE positive cells but the intensity of the signal was also lower compared to c-met$^{fx/fx}$ and single c-met$^{\Delta hepa}$ (Figures 3(d), 3(e), and 3(f)). More importantly, as also observed in our previous microarray analyses [5], c-met deletion resulted in derepression of pro-oxidant enzymes directly responsible for the generation of cellular

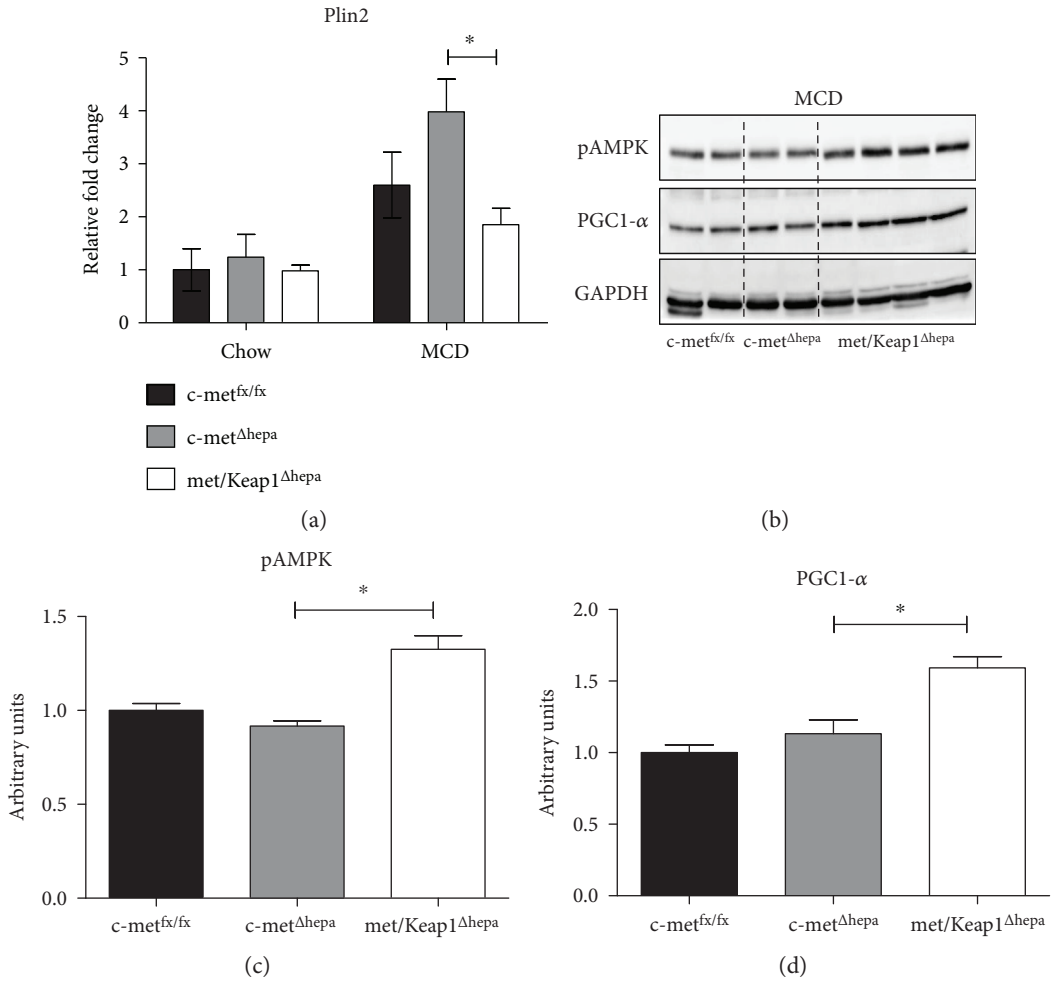


Figure 2: Analysis of hepatic gene expression of proteins involved in lipid droplet formation (a). Hepatic protein expression levels from Western blot (b) and densitometric analysis of band intensities (c and d) performed on total liver lysates of mice after 4 w MCD administration. *Data are expressed as mean ± SE, ANOVA test with $p < 0.05$ ($N = 5$).

ROS, such as Cyp2e1, Cyp4a10, and the NADPH oxidase NOX2 (Figures 4(a), 4(b), and 4(c)). Thus, hepatic induction of these genes as well as the protein expression (Figures 4(g) and 4(h)) was dramatically blunted by overexpression of Nrf2 in met/Keap1$^{\Delta hepa}$ mice. These results seem to offer further evidence for a role of HGF/c-met signaling in the preservation of the cellular redox balance. Furthermore, Nrf2 activation resulted in upregulation of gene expression of enzymes involved in H_2O_2 and free radical scavenging, such as catalase, thioredoxin-1, and the pentose phosphate pathway intermediate enzyme 6-phosphogluconate dehydrogenase (PGD) (Figures 4(d), 4(e), and 4(f)).

3.3. Nrf2 Overexpression Drastically Reduces the Number of Apoptotic Cells in Hepatocytes Lacking c-met Receptor upon MCD Feeding. In association with increased ROS production, TUNEL assay revealed that the number of apoptotic hepatocytes in c-met$^{\Delta hepa}$ livers was augmented as compared to the other experimental groups (Figures 5(a), 5(b), 5(c), and 5(d)), as already observed in other experimental situations. Concomitant Keap1 deletion in c-met$^{\Delta hepa}$ hepatocytes significantly turned down programmed cell death to levels comparable with the control group (Figure 5(d)) although no significant differences in the serum transaminase levels were detected (data not shown). As previously observed, Nrf2 activation correlated with a dramatic increase of the antiapoptotic protein Bcl-2 (Figure 5(e)). Surprisingly, phosphorylation levels of Akt were reduced in c-met$^{\Delta hepa}$ as well as met/Keap1$^{\Delta hepa}$ hepatocytes, ruling out an involvement of this pathway in Nrf2-mediated cell survival (Figure 5(e)). Interestingly, met/Keap1$^{\Delta hepa}$ livers showed a significant increase of phosphorylation of the JNKs—specifically JNK1—kinases (Figures 5(e) and 5(f)) with no evident differences for other MAPK kinases such as ERK1/2 (data not shown).

3.4. Hepatic Nrf2 Overexpression in Livers Lacking c-met Receptor Decreases the Influx of Infiltrating Inflammatory Cells upon MCD Feeding. FACS analysis performed on liver lysates after 4 weeks of MCD treatment revealed that met/Keap1$^{\Delta hepa}$ accumulate less proinflammatory neutrophils compared to the other experimental groups, as indicated by the number of Ly6G$^+$ cells (Figures 6(a), 6(b), 6(c), and 6(d)). Interestingly, the number of circulating neutrophils was also significantly reduced in these animals (Suppl. Fig.

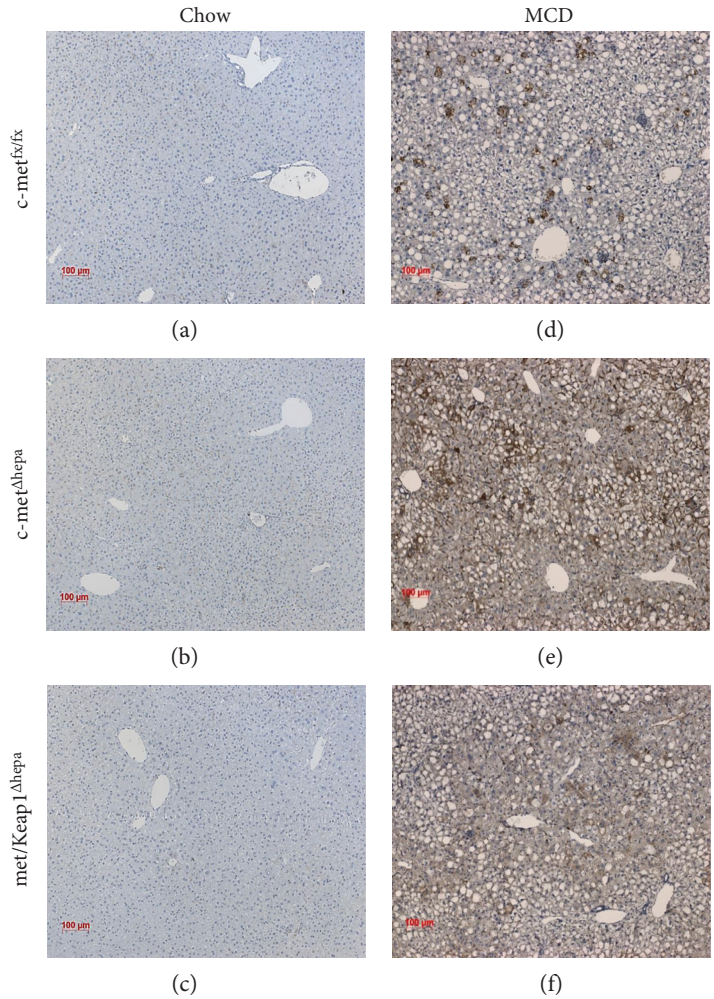


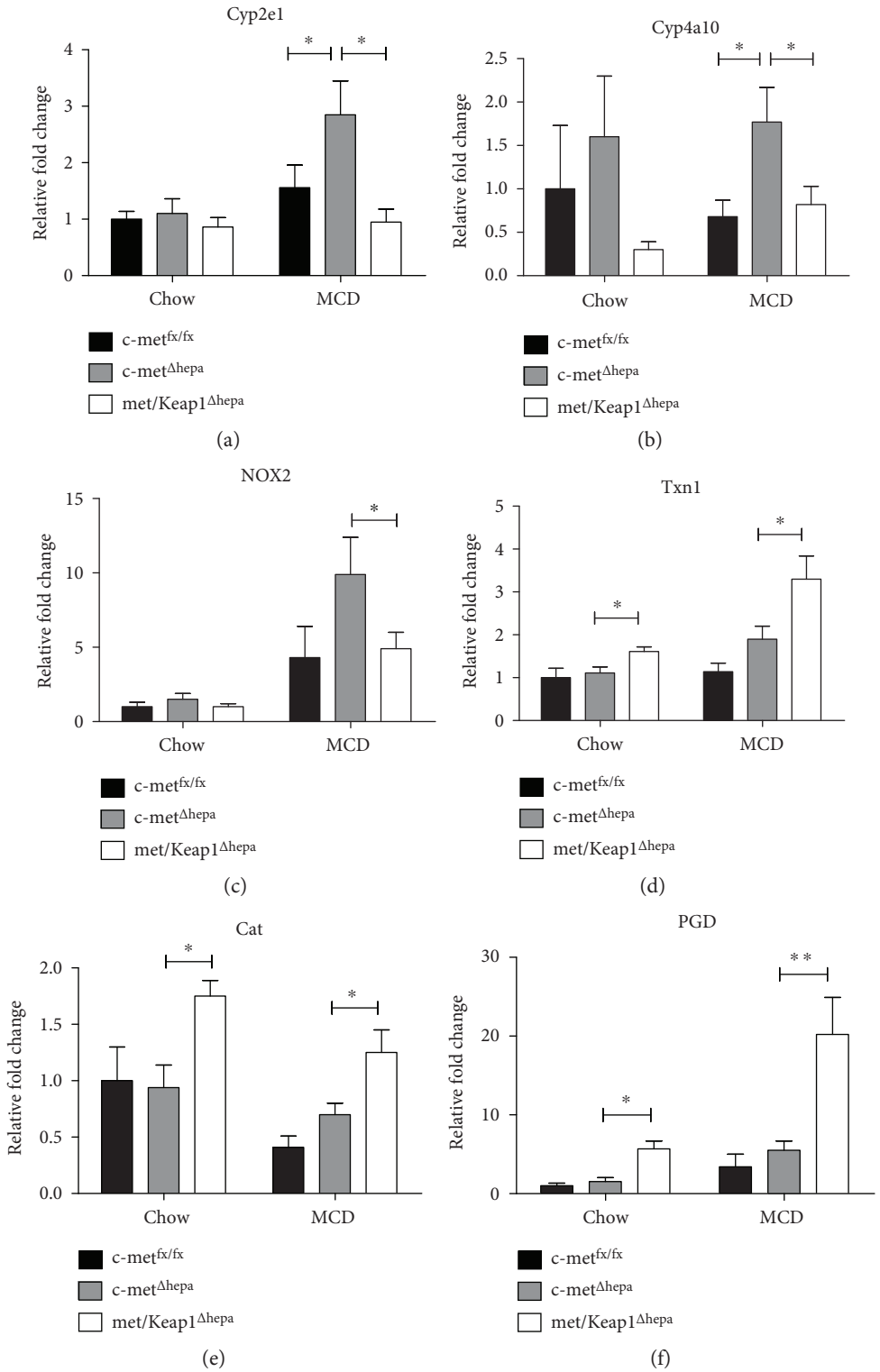
Figure 3: Immunohistochemistry staining for oxidative stress marker 4-HNE on liver sections of c-met$^{fx/fx}$, c-met$^{\Delta hepa}$, and met/Keap1$^{\Delta hepa}$ mice after 4-week chow (a, b, c) and MCD (d, e, f) diet administration, respectively.

3b). Similarly, the number of activated macrophages, measured as F4/80$^+$/CD11b$^+$ cells, was dramatically reduced in the livers of met/Keap1$^{\Delta hepa}$ compared to the other groups. In line with these findings, hepatic gene expression of proinflammatory cytokines, Ccl-2 and TNF-α, was dramatically downregulated in met/Keap1$^{\Delta hepa}$ mice (Suppl. Fig. 1c and data not shown). These results were confirmed by immunofluorescence analyses of CD11b-positive cells (Figures 7(a), 7(b), and 7(c)) and F4/80-positive macrophages (Figures 7(d), 7(e), and 7(f)) supported by morphometric quantifications (Figures 7(g) and 7(h)). Importantly, these histological pictures pointed out the dramatic reduction of the number of inflammatory cell clusters in met/Keap1$^{\Delta hepa}$ livers after MCD administration. Surprisingly, met/Keap1$^{\Delta hepa}$ mice showed a significant increase of CD4$^+$ lymphocytes infiltrating into the liver whereas no changes in the CD8$^+$ lymphocytes number were observed (Suppl. Fig. 2 and Suppl. Fig. 3a).

3.5. Nrf2 Overexpression in Hepatocytes Attenuates the Enhanced Development of Fibrosis Resulting from Hepatocyte-Specific c-met Deletion upon MCD Feeding. As also described in our previous report [5], c-met deletion in hepatocytes accelerates appearance and progression of liver fibrosis in several models of chronic liver injury as well as by feeding a MCD diet. Single c-met knockout livers displayed an increased deposition of collagen fibers as observed in the Sirius red staining (Figures 8(a), 8(b), and 8(c)) and confirmed by related morphometric analysis (Figure 8(d)). This observation was further confirmed by biochemical measurement of intrahepatic hydroxyproline content indicating the highest collagen accumulation in c-met$^{\Delta hepa}$ livers (Figure 8(e)). Interestingly, fibrosis was strongly inhibited in the double mutants where Nrf2 was overactivated. Thus, Sirius red staining and hydroxyproline content were suppressed to the levels of the control group (Figures 8(a), 8(b), 8(c), 8(d), and 8(e)). Concordantly, deletion of Keap1 in hepatocytes lacking c-met also restored the hepatic expression of profibrotic mediators as Col1A1 and TGF-β1 to control levels (Figures 8(f) and 8(g)).

4. Discussion

The generation of reactive oxidative species associated with mitochondrial alterations and the activation of pro-oxidant enzymes still represents an unsolved issue in the context of



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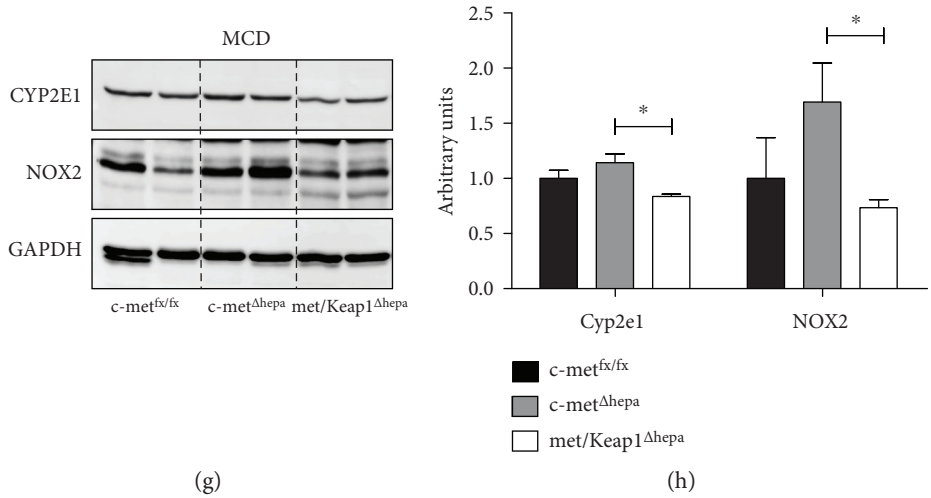


Figure 4: Analysis of hepatic gene expression of proteins involved ROS production (a, b, c) and of proteins responsible for the antioxidant cell defense (d, e). (f) $^{**}p$ values < 0.01. *Data are expressed as mean ± SE, ANOVA test with $p < 0.05$ ($N = 5$). Hepatic protein expression levels of Cyp2e1 and NOX2 from Western blot performed on total liver lysates of mice after 4 w MCD administration (g) with relative densitometric analysis (h).

nonalcoholic steatohepatitis in which it seems to play a pivotal role in the exacerbation of liver injury, inflammation, and repair. Although the mitochondria represent the major source of free radicals already under physiological conditions, other cell compartments such as microsomes and lysosomes participate in ROS production via oxidative reactions under stressful conditions. Increased activity of the cytochrome p450, particularly of the isoform Cyp2e1, has emerged as an important free radical generator during NASH [13]. Indeed, a direct association between Cyp2e1 and Cyp4a10 enzyme expression and the initiation of lipid peroxidation during the progression of NASH has been clearly identified [14]. Moreover, a critical role for NADPH oxidases (such as NOX2) in the development of features related to aggravation of NASH and, in general, of the metabolic syndrome has been convincingly demonstrated by Garcia-Ruiz et al. [15]. Following these considerations, many experimental and clinical studies based on scavenging or buffering of free radicals have begun to show promising results in a more relevant therapeutic context [16, 17]. It is worth to mention that in the context of NASH several therapeutic options targeting oxidative stress via Nrf2 activation already achieved preclinical and clinical phase trials [18]. In particular, beyond classical natural antioxidant such as sulforaphane or resveratrol, the synthetic electrophilic compound oltipraz (dithiolethione) revealed promising effects in the treatment of liver metabolic diseases and is currently being analyzed in a phase II clinical trial.

The role of HGF/c-met axis in liver pathophysiology has been extensively investigated with a particular light on aspects regarding liver regeneration, hepatocyte proliferation, and apoptosis [19]. Moreover, activation of this signaling pathway has been repeatedly reported to exert hepatoprotective effects against experimental conditions characterized by oxidative stress [20, 21]. In isolated hepatocytes, Clavijo-Cornejo et al. showed that HGF exerts a biphasic regulation of the NADPH oxidases with a short-time effect inducing the activation of the enzyme and a long-time effect after which the persistence of a functional HGF/c-met pathway results to be necessary for the suppression of the NADPH oxidases components in an Nrf2-dependent manner [22]. In line with these findings, a recent work from our group [5] demonstrated that c-met-deleted hepatocytes displayed enhanced oxidative stress and increased apoptotic cell death in association with overproduction of superoxide anion in vivo. This led to enhanced progression of hepatic inflammation and fibrosis. Recently, Dominguez-Perez et al. showed that administration of HGF reduces hepatocyte susceptibility to lipotoxicity through an increase of antioxidant defenses such as Υ-GCS and GSH thereby attenuating ROS formation and damage [23]. The generation of double mutant c-met/Keap1$^{\Delta hepa}$ mice further demonstrated that re-establishing a functional antioxidant activity completely reversed the accelerated pathological conditions observed in single c-met$^{\Delta hepa}$ mice. In particular, the reduction of oxidative stress was accompanied by a decrease of the abovementioned pro-oxidant systems, Cyp2e1, Cyp4a10, and NOX2 expression. Conversely, expression of antioxidant cell weapons, such as catalase and thioredoxin-1, in addition to the well-known activation of the pentose phosphate pathway, was strongly upregulated. It is interesting to note that the amelioration of the redox balance occurred concomitantly with a reduced hepatic accumulation of triglycerides related to the inhibition of the LXR-dependent lipogenic program induced by Nrf2 as previously shown [9, 24]. These findings actually consolidate our former data set illustrating reduced TG accumulation and oxidative stress in hepatocytes carrying a genetic activation of Nrf2 in two experimental models of diet-induced steatohepatitis [9]. Moreover, this aspect coherently matches with the activation of the AMPK/PGC-1α pathway observed in met/Keap1$^{\Delta hepa}$ hepatocytes. Although a causal relation with Nrf2 activation remains

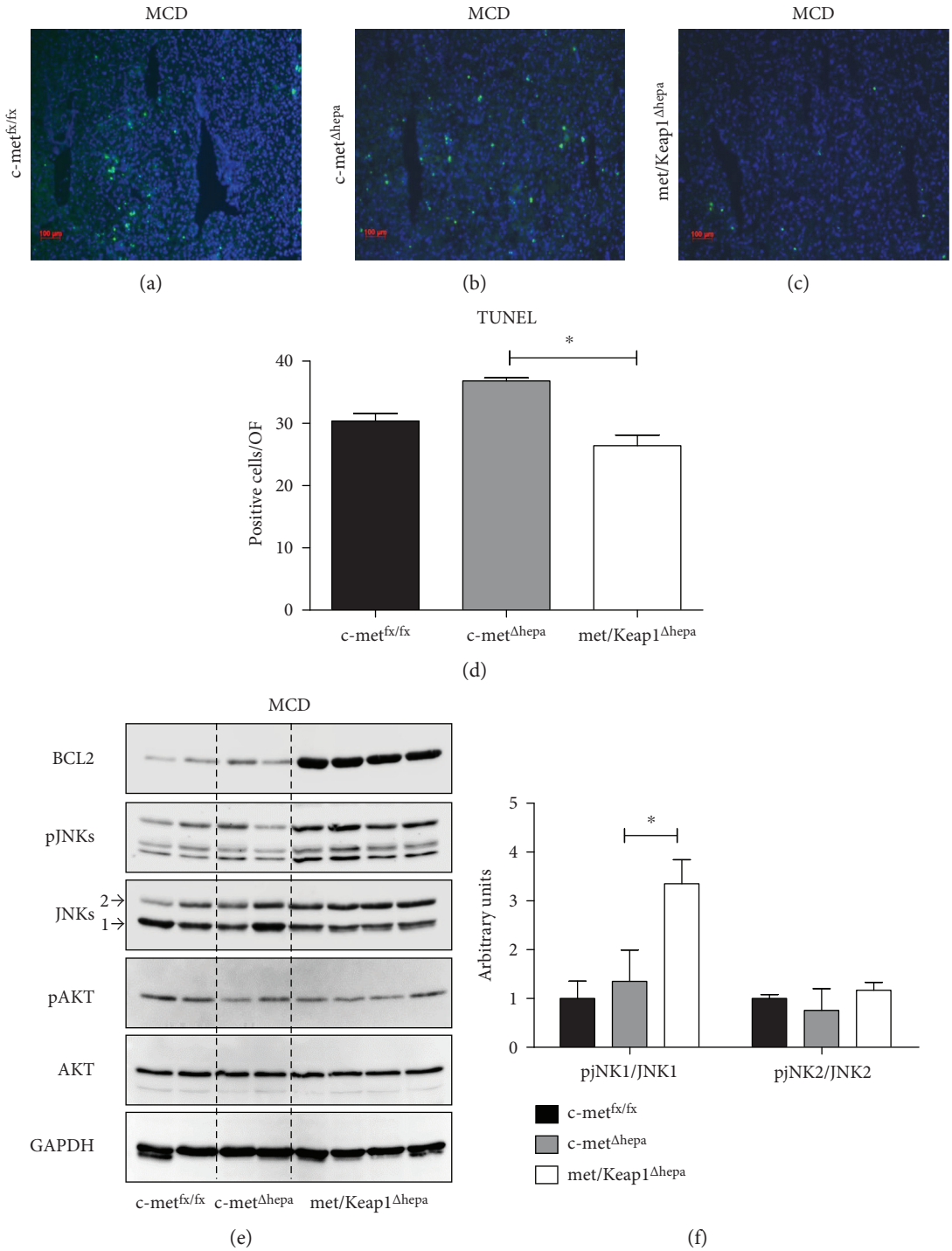
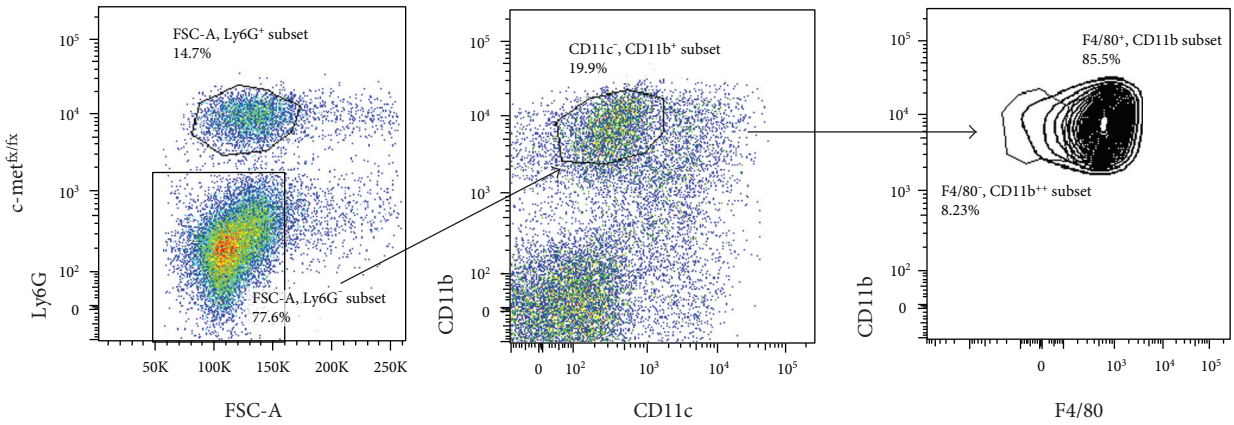


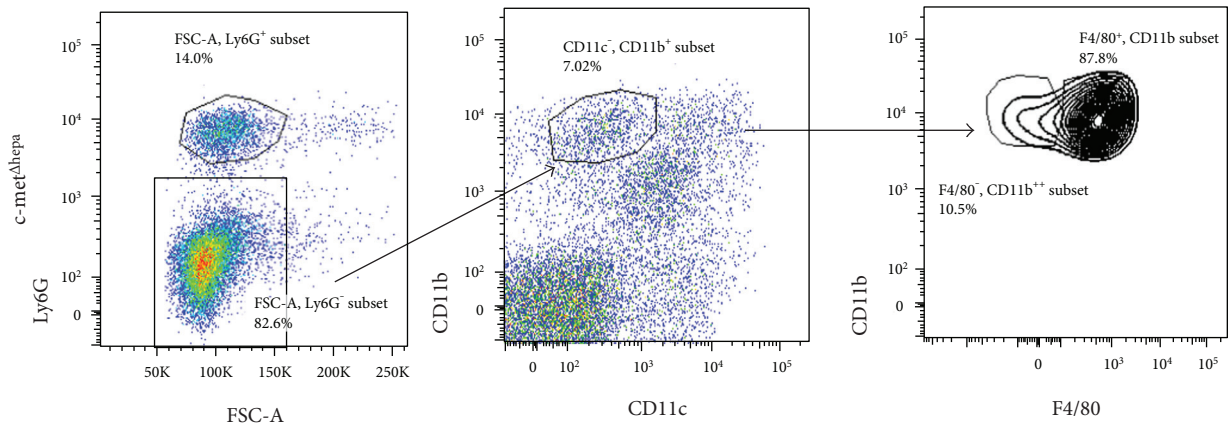
Figure 5: TUNEL immunofluorescence of c-met$^{fx/fx}$ (a), c-met$^{\Delta hepa}$ (b), and met/Keap1$^{\Delta hepa}$ (c) liver sections (magnification 100x) after 4 weeks of MCD feeding, with relative morphometric analysis (d). *Data are expressed as mean ± SE, ANOVA test with $p < 0.05$ ($N = 5$). Hepatic protein expression levels from Western blot (e) and densitometric analysis of band intensities (f) performed on total liver lysates of mice after 4 w MCD administration.

poorly elucidated, Nrf2-dependent negative regulation of the lipogenic gene SCD-1 could contribute to hepatic AMPK activation as shown by Dobrzyn et al. [25]. Lastly, it would be reasonable to connect AMPK activation with maintenance of the NADPH production originating from the pentose phosphate pathway as an antioxidant mechanism in response to metabolic stress, as elegantly proposed elsewhere [26].

In relation to this observation, TUNEL-positive hepatocytes in double knockouts were strongly diminished as compared to those in control and single c-met knockouts. Interestingly, independent of the degree of steatosis, the loss



(a)



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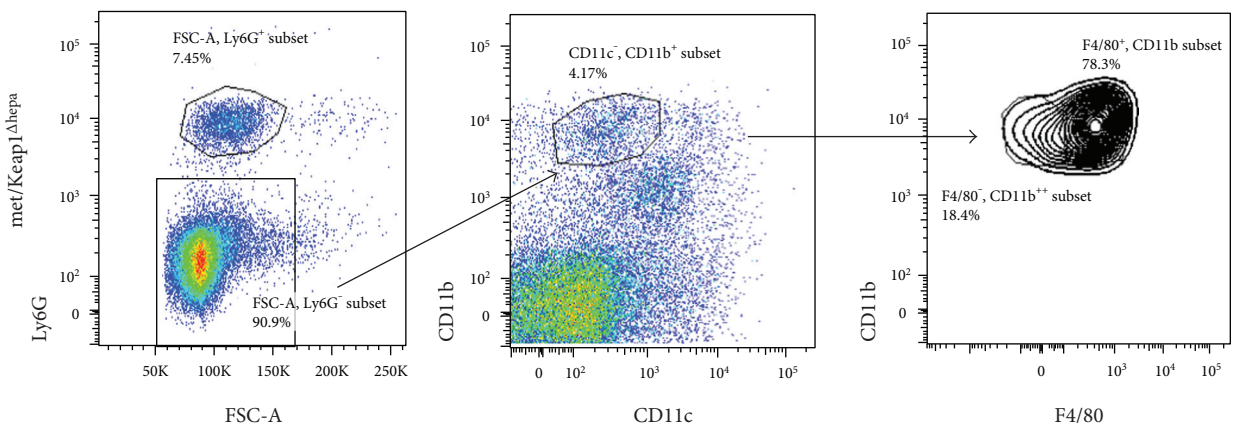

(c)

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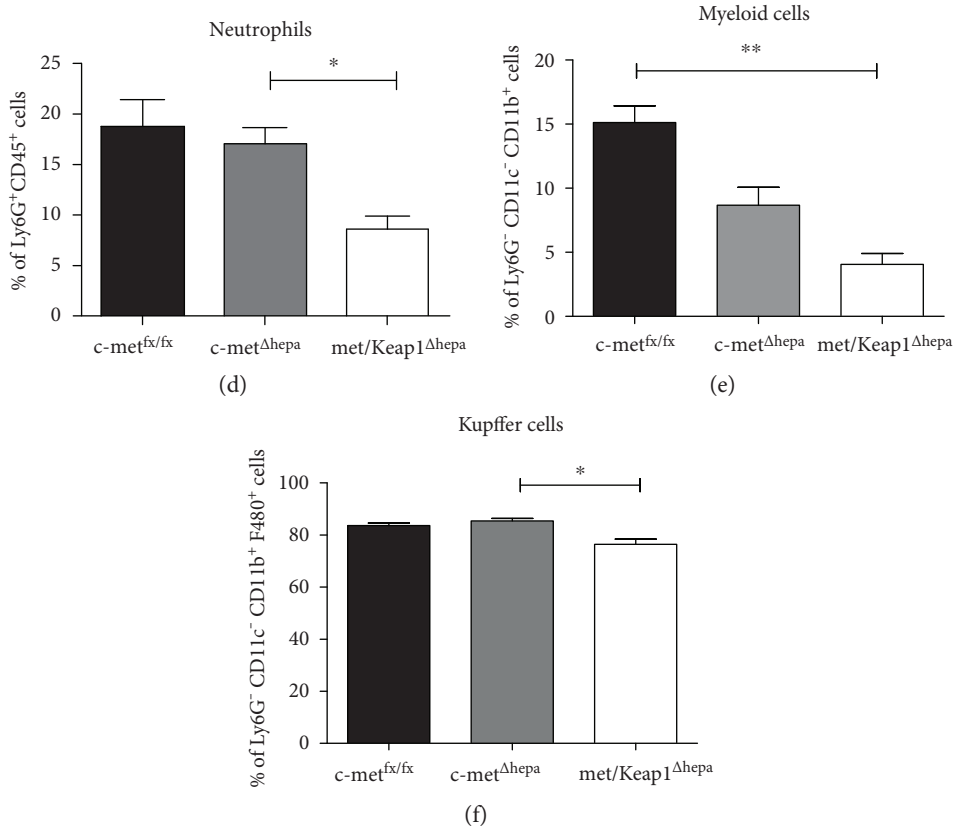

FIGURE 6: Representative gating strategy from flow cytometry analysis of intrahepatic neutrophils and monocyte/macrophage populations performed on total liver lysates of c-met$^{fx/fx}$ (a), c-met$^{\Delta hepa}$ (b), and met/Keap1$^{\Delta hepa}$ (c) after 4 weeks of MCD feeding. Neutrophils were gated by FSC/SSC, CD45$^+$/alive CD11b$^+$, and Ly6G$^+$ cells. Macrophages were gated by FSC/SSC, CD45$^+$/alive, Ly6G$^-$, CD11b$^+$, F4/80$^+$. Quantification of the gated populations reported on histogram (d, f). (e) $^{**}p$ values < 0.01. *Data are expressed as mean ± SE, ANOVA test with $p < 0.05$ ($N = 3-5$).

of c-met impairs hepatocyte capacity to counteract oxidative stress generated by fatty acid oxidation, thereby sensitizing cells to apoptosis. This suggests that reducing oxidative stress via Nrf2 activation might represent a key step in the protection from programmed cell death as we already observed in hepatocyte-specific Keap1 knockout mice [10]. Curiously, in the present work, cell protection was not associated with enhanced activation of the PI3K/AKT survival pathway. Thus, a significant increase of the stress-activated JNK phosphorylation was observed. Although the importance of this point results unclear, JNKs have been recently shown to promote nuclear Nrf2 activation [27]. Moreover, it might be relevant to underline that liver size was not significantly affected by administration of MCD diet in all the experimental groups and the double knockouts already display hepatomegaly under normal conditions. Analyses of immunofluorescence for the proliferation marker Ki67 indicated that hepatocyte proliferation seems to contribute only minimally to this phenotype. Thus, we reasonably believe that under normal chow conditions hepatomegaly might be the result of a synergistic effect of proliferation and cellular hypertrophy. Upon MCD feeding, a compensative proliferation in response to liver injury and hepatocyte cell death is commonly activated. Single hepatocyte-specific c-met deletion constantly results in diminished proliferation rate being partly responsible for the impaired tissue repair as previously reported. Genetic Nrf2 overactivation does not seem to have an impact on the proliferation rate but the extent of cell death is indeed dramatically reduced. Therefore, in conditions of metabolic stress, the increase in liver/body weight ratio observed in double knockouts results to be maintained through reduced cell death and increased cell functionality (hypertrophy).

In line with these findings, simultaneous deletion of c-met and Keap1 resulted in a dramatic decrease of infiltrating neutrophils (Ly6G$^+$) and inflammatory monocytes (CD11b$^+$/F4/80$^+$). This indicates a modulation of the immune response possibly driven by reduced liver injury and through the negative modulation of specific inflammatory mediators. Although the reduction of neutrophils might be beneficial in the context of NASH in terms of a decreased MPO release [28], on the other hand, further studies are required in order to identify the phenotype of monocyte subpopulations since the cell polarization might differently influence the progression of the disease. In contrast, livers of c-met/Keap1$^{\Delta hepa}$ mice display a significant increase of infiltrating CD3$^+$ lymphocytes, characterized by a predominant CD4$^+$ subtype. These findings seem to be consistent with a recent study showing that, in the context of NASH, CD4$^+$ lymphocytes

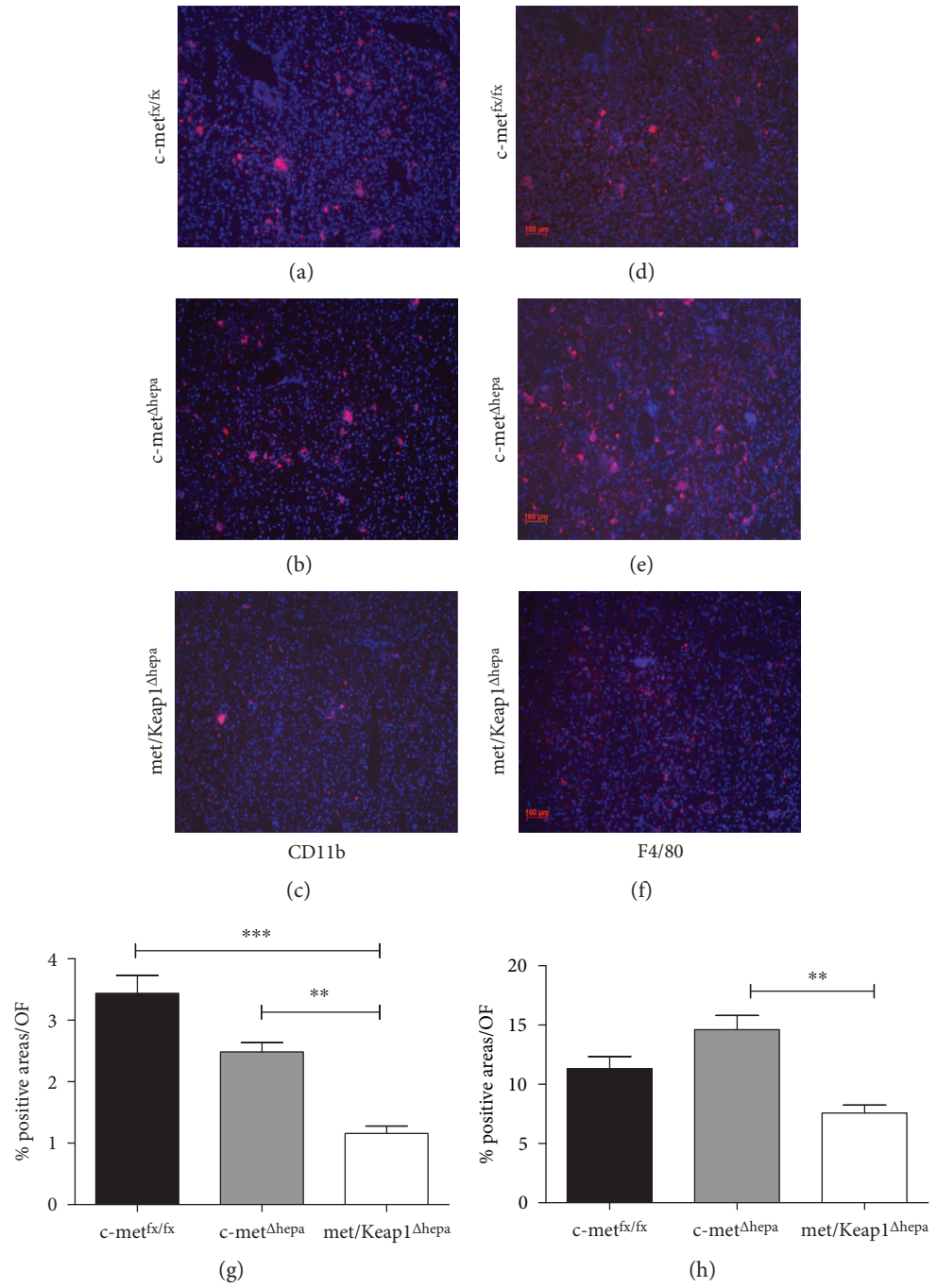

Figure 7: Immunofluorescence staining for neutrophil/monocyte marker CD11b (a, b, c) and macrophage marker F4/80 (d, e, f) of c-met$^{fx/fx}$, c-met$^{\Delta hepa}$, and met/Keap1$^{\Delta hepa}$ liver sections (magnification 100x) after 4 weeks of MCD feeding with relative morphometric analyses (g) $^{***}p$ values < 0.01. (h) $^{**}p$ values < 0.01. *Data are expressed as mean ± SE, ANOVA test with $p < 0.05$ ($N = 3-5$).

seem to be more susceptible to fatty acid-induced ROS cytotoxicity [29]. Consequently, a reduction of CD4$^+$ population results to be deleterious in the progression of fibrosis. This aspect is going to emerge as a reproducible variable in several experimental conditions associated with hepatic Nrf2 overexpression. Further investigations are currently in progress in order to shed light on the nature and functions of this hepatic infiltrate.

Finally, loss of c-met in hepatocytes was previously shown to accelerate the onset of fibrosis not only in several experimental murine models [30] but also in NASH [5], through multiple mechanisms involving increased hepatocyte cell death, altered release of inflammatory mediators, oxidative stress, and cell proliferation. However, production of ROS remains a central player in the pathogenesis of liver fibrosis even if the causal relation in the context of cell

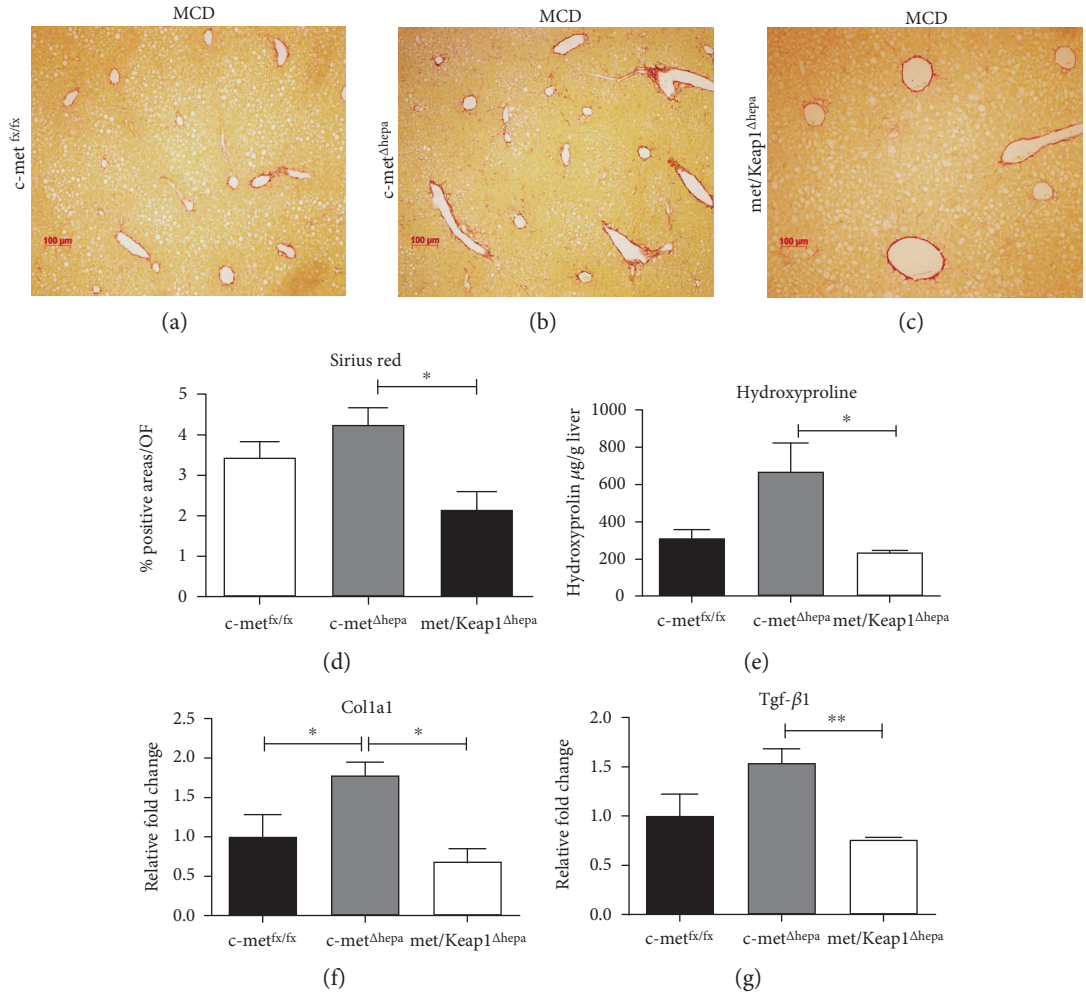

FIGURE 8: Sirius red staining of c-met$^{fx/fx}$ (a), c-met$^{\Delta hepa}$ (b), and met/Keap1$^{\Delta hepa}$ (c) liver sections (magnification 100x) after 4 weeks of MCD feeding with relative morphometric analysis (d). Intrahepatic hydroxyproline content (e) and hepatic gene expression of profibrotic mediators (f) (g) $^{**}p$ values < 0.01, from livers of mice after 4 weeks of MCD feeding. *Data are expressed as mean ± SE, ANOVA test with $p < 0.05$ ($N = 5$).

death and repair is still an argument of intense investigation [31]. Curiously, whereas overexpression of Nrf2 in c-met/Keap1$^{\Delta hepa}$ resulted in less TG accumulation, less oxidative stress, and reduced number of inflammatory cells as compared to controls and single knockouts, the degree of fibrosis in these mice was only moderately dampened to the degree of the control group. These data indicate that genetic enhancement of Nrf2 signaling is sufficient to repress MCD-dependent oxidative stress and cell damage but the efficacy against fibrosis seems to be restricted to a limited spectrum of transcriptional activation. To this end, identification of immune mediators directly regulated through Nrf2 transcriptional activity could shed light on the dark side of chronic overexpression of this transcription factor.

5. Conclusion

The results reported in this work offer further evidence for Nrf2-mediated cytoprotection (partly illustrated also in conference data set [32]). They pinpoint a key role for HGF/c-met signaling in the regulation of redox homeostasis. Actually, genetic Nrf2 overexpression revealed therapeutic effects in c-met-deficient hepatocytes by counteracting oxidative stress thereby attenuating the disease progression. This work highlights critical aspects to be considered for the development of novel therapeutic strategies in the management of NASH.

Conflicts of Interest

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

Acknowledgments

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Research Article

Antioxidant Treatment Induces Hyperactivation of the HPA Axis by Upregulating ACTH Receptor in the Adrenal and Downregulating Glucocorticoid Receptors in the Pituitary

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Glucocorticoid (GC) production is physiologically regulated through a negative feedback loop mediated by the GC, which appear disrupted in several pathological conditions. The inability to perform negative feedback of the hypothalamus-pituitary-adrenal (HPA) axis in several diseases is associated with an overproduction of reactive oxygen species (ROS); however, nothing is known about the effects of ROS on the functionality of the HPA axis during homeostasis. This study analyzed the putative impact of antioxidants on the HPA axis activity and GC-mediated negative feedback upon the HPA cascade. Male Wistar rats were orally treated with N-acetylcysteine (NAC) or vitamin E for 18 consecutive days. NAC-treated rats were then subjected to a daily treatment with dexamethasone, which covered the last 5 days of the antioxidant therapy. We found that NAC and vitamin E induced an increase in plasma corticosterone levels. NAC intensified MC2R and StAR expressions in the adrenal and reduced GR and MR expressions in the pituitary. NAC also prevented the dexamethasone-induced reduction in plasma corticosterone levels. Furthermore, NAC decreased HO-1 and Nrf2 expression in the pituitary. These findings show that antioxidants induce hyperactivity of the HPA axis via upregulation of MC2R expression in the adrenal and downregulation of GR and MR in the pituitary.

1. Introduction

Reactive oxygen species (ROS) are ions or small molecules containing oxygen and an unpaired electron, and this free electron confers high reactivity to oxygen. ROS production in mammals is due to the activity of endogenous pro-oxidant enzymes NADPH oxidase, xanthine oxidase, peroxisomes, and cytochrome P-450. Their product is counterbalanced by endogenous antioxidant enzymes including superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione, and heme oxygenase- (HO-) 1 [1]. These antioxidant defense systems are directly regulated by nuclear factor erythroid 2-related factor 2 (Nrf2). Besides inducing the transcription of endogenous antioxidant enzymes, Nrf2 affects the homeostasis of ROS and reactive nitrogen species (RNS) through regeneration of oxidized cofactors and proteins; synthesis of reducing factors, as GSH and NADPH; and increasing redox transport, including cysteine/glutamate transport by xCT [2]. Redox imbalance is induced by disequilibrium between the production and suppression of ROS. Furthermore, excess of oxidative damage can be controlled by exogenous antioxidants such as vitamins C and E, polyphenols, carotenes, flavonoids, omega-3, and N-acetylcysteine (NAC) [3–5]. These exogenous antioxidants decrease the oxidative damage through distinct mechanisms of action. For instance, vitamin E, a nonenzymatic antioxidant, promotes a lipid

peroxyl radical scavenger and maintains the integrity of long-chain polyunsaturated fatty acids in the membrane of cells [6, 7], while NAC, which is an acetylated cysteine residue, stimulates glutathione synthesis and performs as a scavenger of oxygen free radicals [8].

The HPA axis is a neuroendocrine system regulated by the circadian cycle [9, 10] and stress [11]. After being stimulated, neurons of the paraventricular nucleus of the hypothalamus release corticotropin-releasing hormone (CRH), which will be transported by hypothalamic-pituitary portal circulation and stimulate pituitary corticotroph cells to cleave proopiomelanocortin (POMC) in adrenocorticotrophic hormone (ACTH) [12]. ACTH is released into the bloodstream and acts on melanocortin receptor type 2 (MC2R), situated in the *zona fasciculata* of adrenals, inducing an increase in expression and/or activity of steroidogenic enzymes, including 11β-hydroxysteroid dehydrogenase-type 1 (11β-HSD1) and steroidogenic acute regulatory protein (StAR), and releasing glucocorticoids to the bloodstream [13]. The basal activity of the HPA axis is regulated by a negative feedback which is mediated through activation of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), located in the pituitary and hypothalamus, by glucocorticoids [14, 15].

The HPA axis is the main neuroendocrine system that regulates responses to stress. The production of high levels of ROS into the glands that comprise the HPA axis is associated with the activation of a stress-response system in several models of stress, including social isolation [16] and inflammatory and infectious diseases [17]. HPA axis hyperactivity induced by redox imbalance may occur by a reduction in negative feedback through a decrease in GR translocation to the cellular nucleus in corticotroph cells of the pituitary [17].

Although the free radical theory and the oxidative damage theory describe that accumulation of oxidative damage in cellular macromolecules is immensely toxic, ROS products by normal cell metabolism are vital to cell homeostasis maintenance, especially for its roles in immunocompetence and activation of several signal transduction pathways [18, 19]. Indeed, the antioxidant therapy presents several side effects, which is opposed to the anticipated properties of these substances [3]. Our hypothesis is that any imbalance in the redox system alters the homeostasis of the HPA axis culminating in its hyperactivation, and not just an increase in ROS production as shown in several diseases. Here, we undertook this study to evaluate the putative mechanism underlying the antioxidant-induced hyperactivation of the HPA axis in Wistar rats.

2. Materials and Methods

2.1. Animals and Treatments. Male Wistar rats (250–300 g) were obtained from the Oswaldo Cruz Foundation breeding colony and used in accordance with the guidelines of the Committee on Use of Laboratory Animals of the Oswaldo Cruz Foundation (CEUA-FIOCRUZ, license LW-23/11). Rats were housed in groups of three in a temperature-, humidity-, and light-controlled (12 h light : 12 h darkness cycle) colony room. Rats were given ad libitum access to food and water.

Twelve male rats were randomly assigned into 2 groups as follows: control ($n = 6$) and treated with the antioxidant N-acetyl L-cysteine (NAC) ($n = 6$). In another set of experiments, twelve male rats were randomly assigned into 2 groups as follows: control ($n = 6$) and treated with the antioxidant vitamin E ($n = 6$). In a third set of experiments, twenty male rats were randomly divided into 4 experimental groups: control ($n = 5$), treated with NAC ($n = 5$), treated with the exogenous glucocorticoid dexamethasone ($n = 5$), and treated with NAC ($n = 5$) plus dexamethasone. The rats were treated with NAC (150 mg/kg body weight) [20] or vitamin E (α-tocopherol, 40 mg/kg body weight) [21] by gavage once a day, during 18 consecutive days. Control rats received an equal volume of vehicle (sterile saline 0.9% and DMSO 0.1%, resp.). To analyze corticoid-induced negative feedback sensitivity, a group of animals received dexamethasone (0.02 mg/kg body weight, s.c.) daily, for 5 consecutive days [22], beginning 13 days after the starting of antioxidant treatment.

2.2. Corticosterone Quantification. Animals were euthanized in a CO_2 chamber, during the nadir (08:00 h) of the circadian rhythm as described previously [23], and the blood was immediately collected from the abdominal aorta with heparinized (400 U/ml) saline. Plasma was obtained after sample centrifugation for 10 min at 1000 ×g and stored at −20°C until use. Plasma corticosterone levels were detected by radioimmunoassay (RIA) following manufacturer's guidelines (MP Biomedicals, Solon, OH, USA). Briefly, this is a competitive assay between the hormone presented in the sample and the hormone labelled with radioisotope (I^{125}) to bind to a specific antibody. Thereby, an increase in amount of the hormone in the sample leads to a corresponding decrease in the fraction of labelled hormone bound to the antibody. Radioactivity quantification was carried out using a gamma counter (ICN Isomedic 4/600 HE; ICN Biomedicals Inc., Costa Mesa, CA, USA), and the amount of corticosterone in samples was calculated by interpolation to a standard curve performed in parallel.

2.3. Immunohistochemistry Staining. The adrenals and pituitary glands were immediately dissected after perfusion of rats with 0.9% sterile saline. Adrenals were quickly removed from the rats and cleaned of surrounding fat, while the pituitary glands were gently collected after the decapitation of rats and removal of the brain [24, 25]. Instantly after dissection, the glands were fixed in Milloning and embedded in paraffin. Paraffin-embedded sections of 3 μm of rat pituitary and adrenals were deparaffinized with xylene, rehydrated by a graded series of ethanol washes, and boiled in sodium citrate buffer (10 mM, pH 6.0) at the temperature of 95°C for 15 min to enhance antigen retrieval. Tissue sections were incubated with 3% H_2O_2 in methanol for 20 min to block endogenous peroxidases. To prevent nonspecific binding, sections were then incubated for 3 h with a solution containing 2.5% bovine serum albumin (BSA), 8% fetal bovine serum (FBS), and 1% of nonfat milk dissolved in Tris-buffered saline enriched with 0.1% Tween 20 (TBST). After blocking, sections were incubated with primary specific antibody (polyclonal rabbit

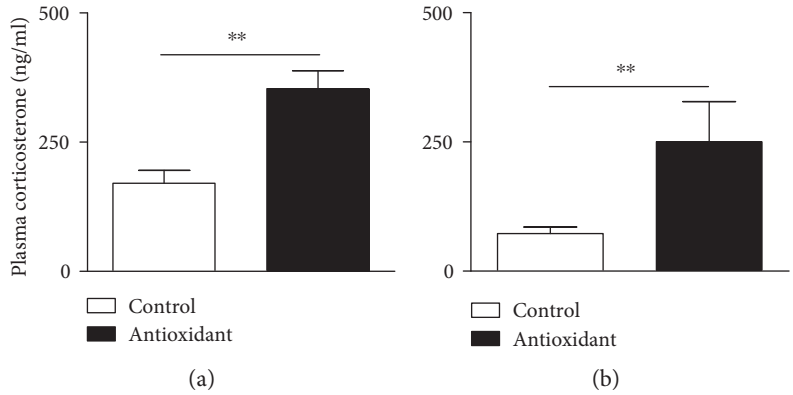

Figure 1: Antioxidant treatment increases circulating levels of plasma corticosterone in Wistar rats. (a) NAC (150 mg/kg, oral route) and (b) vitamin E (40 mg/kg, oral route) were given daily for 18 consecutive days. Untreated animals received an equal amount of vehicle (saline 0.9% or DMSO 0.1%). Data are expressed as the mean ± SEM of 6 animals. This result is a representative of two independent assays. $^{**}p < 0.01$.

anti-rat StAR (1 : 50), GR (1 : 250), or MC2R (1 : 250) and polyclonal goat anti-rat MR (1 : 50), HO-1 (1 : 100), or Nrf2 (1 : 100) from Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in TBST with 1% BSA overnight at 4°C.

Then, primary antibody binding was detected after incubating sections with a horseradish peroxidase conjugated-secondary antibody (polyclonal anti-goat or anti-rabbit IgG HRP, R&D System, Minneapolis, MN, USA) for 2.5 h, followed by a 20 min exposure to the HRP substrate 3-amino-9-ethylcarbazole (AEC). Sections were washed with TBST between all steps and weakly counterstained with hematoxylin for the easy identification of tissue structures. Finally, tissue sections were mounted in aqueous medium and images digitized via scanner microscope (Pannoramic SCAN150, 3D Histech, Budapest, Hungary) using a 20x objective lens. Images obtained from the anterior pituitary or *zona fasciculata* of the adrenal cortex were analyzed with Image Pro Plus 6.2 software (Media Cybernetics). Briefly, red to brown colored pixels associated with a positive immunohistochemistry stain were selected in a model image and applied to the remaining fields. The number of positive pixels was divided by the field area and expressed as pixels/μm^2.

2.4. Chemicals. Sodium citrate, AEC, NAC, vitamin E, dexamethasone, and hydrogen peroxide were purchased from Sigma Chemical Co. (Saint Louis, MO, USA); ethanol, methanol, and xylene from Merck (Rio de Janeiro, RJ, Brazil); and sodium heparin from Roche (São Paulo, SP, Brazil). All solutions were freshly prepared immediately before use.

2.5. Statistical Analysis. The data are reported as the mean ± standard error of the mean (SEM). All data were evaluated to ensure normal distribution. The assay of corticoid-induced negative feedback sensitivity was analyzed by one-way ANOVA followed by a Student-Newman-Keuls post hoc test, while all the other results were statistically analyzed by unpaired *t*-test, with Graphpad Prism 5.0. Probability values (p) of 0.05 or less were considered significant.

3. Results

3.1. Antioxidant Therapy Increases Plasma Corticosterone Levels in Wistar Rats. Initially, we investigated the impact of antioxidant therapy on circulating corticosterone levels. We observed that rats treated with either NAC (Figure 1(a)) or vitamin E (Figure 1(b)), for 18 consecutive days, presented a significant increase in plasma corticosterone levels compared to controls (mean ± SEM, $n = 6$; $p < 0.01$; two-tailed *t*-test).

3.2. NAC Induces Adrenal Hypertrophy and Upregulation of ACTH Receptor and StAR in the Adrenal Cortex of Wistar Rats. We hypothesized that the high corticosterone levels were due to increased stimulation of the adrenal cortex. We noted that NAC induced adrenal hypertrophy, as evidenced by the ratio between adrenal weight (mg) and body weight (g). The values of adrenal/body weight ratio increased from 0.066 ± 0.003 in control rats to 0.095 ± 0.008 (mean ± SEM, $n = 6$; $p < 0.01$; two-tailed *t*-test) in NAC-treated rats. The absolute adrenal weights were 24 ± 1.8 mg and 30 ± 1.5 mg (mean ± SEM, $n = 6$; $p < 0.05$; two-tailed *t*-test) to control and NAC-treated rats, respectively. In parallel, we showed that treatment with NAC increased the expression of ACTH receptor (MC2R) (Figures 2(b) and 2(e)) and steroidogenic enzyme StAR (Figures 2(d) and 2(f)) in the *zona fasciculata* of the adrenal cortex compared to that of control rats (Figures 2(a) and 2(c), resp.) (mean ± SEM, $n = 6$; $p < 0.05$ and $p < 0.01$, resp.; two-tailed *t*-test). The expression of MC2R is located in the membrane and cytoplasm of cells (Figures 2(a) and 2(b)), while StAR is expressed only in the cytoplasm of the cells (Figures 2(c) and 2(d)).

3.3. NAC Decreases Nrf2 and HO-1 Expression in the Anterior Pituitary of Wistar Rats. Our next approach was to determine if the treatment with NAC could interfere with the expression of antioxidant arsenal in the anterior pituitary, an important component of the HPA axis which regulates corticosterone production by adrenals. Treatment with NAC reduces the expression of transcription factor Nrf2 (Figures 3(b) and 3(e)) and the antioxidant enzyme HO-1

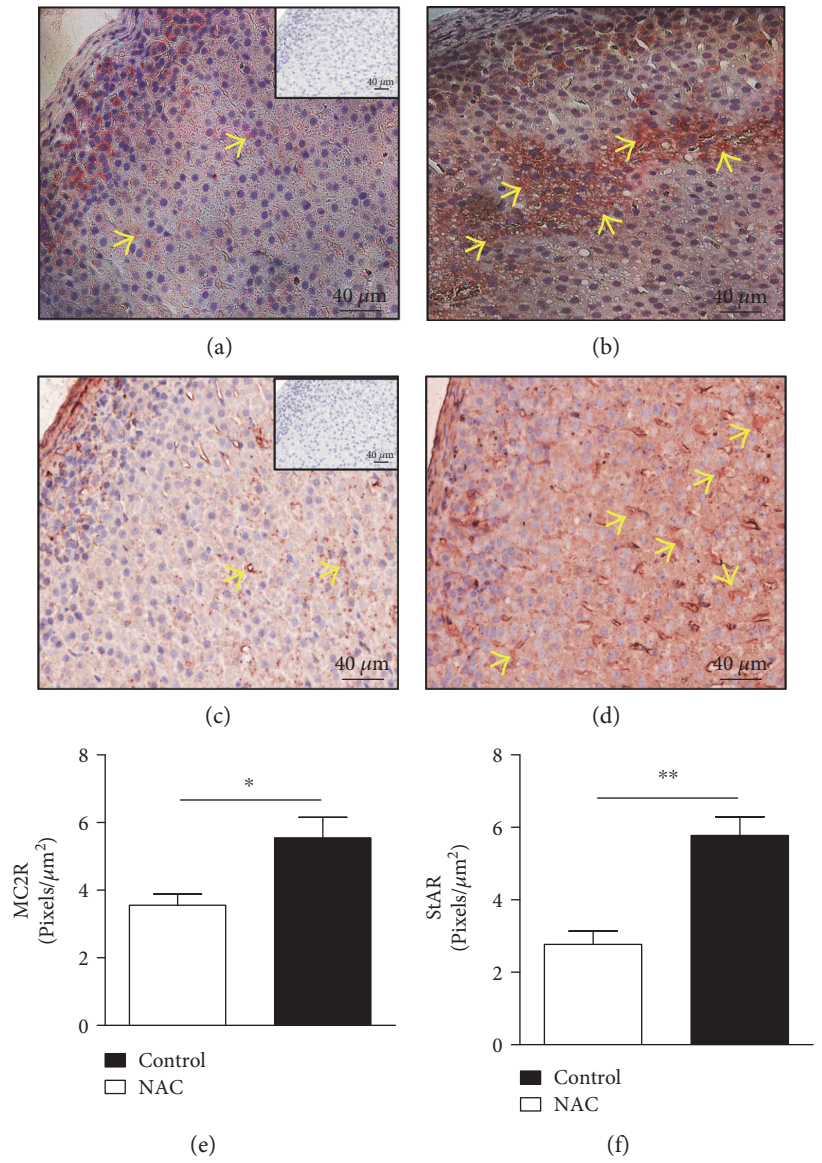

Figure 2: NAC induces an upregulation of MC2R and StAR expression in the *zona fasciculata* of the adrenal of Wistar rats. NAC (150 mg/kg, oral route) was given daily for 18 consecutive days and the analysis was made by immunohistochemistry. The panels show representative photomicrographs of adrenal expression of MC2R in the (a) control and (b) NAC-treated rats and StAR in (c) control and (d) NAC-treated rats. The quantification of pixels associated with MC2R and StAR expression is shown in (e) and (f), respectively. Inserts represent negative controls. Yellow arrows indicate immunolabelling of MC2R (a, b) and StAR (c, d) in the *zona fasciculata* of adrenals. Data are expressed as the mean ± SEM of 6 animals. This result is a representative of two independent assays. $^{*}p < 0.05$ and $^{**}p < 0.01$.

(Figures 3(d) and 3(f)) in the anterior pituitary compared to that of control rats (Figures 3(a) and 3(c), resp.) (mean ± SEM, $n = 6$; $p < 0.05$ and $p < 0.05$, resp.; two-tailed t-test). The expression of Nrf2 is located in the nucleus and cytoplasm of cells (Figures 3(a) and 3(b)), while HO-1 is expressed only in the cytoplasm of the cells (Figures 3(c) and 3(d)).

3.4. NAC Reduces Glucocorticoid Receptor Expression in the Anterior Pituitary and Impaired Negative Feedback of the HPA Axis in Wistar Rats. High levels of plasma corticosterone induced by antioxidants can also be associated with a failure in the negative feedback of the HPA axis. Treatment with NAC decreases the expression of both glucocorticoid receptors GR (Figures 4(b) and 4(e)) and MR (Figures 4(d) and 4(f)) in the anterior pituitary compared to that of control rats (Figures 4(a) and 4(c), resp.) (mean ± SEM, $n = 6$; $p < 0.05$ and $p < 0.001$, resp.; two-tailed t-test). The expressions of GR (Figures 4(a) and 4(b)) and MR (Figures 4(c) and 4(d)) are located in the nucleus and cytoplasm of cells.

Thus, we treated rats with a low dose of dexamethasone (0.02 mg/kg, s.c.) and analyzed the circulating levels of corticosterone. We showed that dexamethasone induced a strong negative feedback response and reduced the plasma corticosterone levels in control rats (mean ± SEM, $n = 6$; $p < 0.05$; one-way ANOVA followed by a Student-Newman-Keuls post hoc test t-test); however, although treatment with

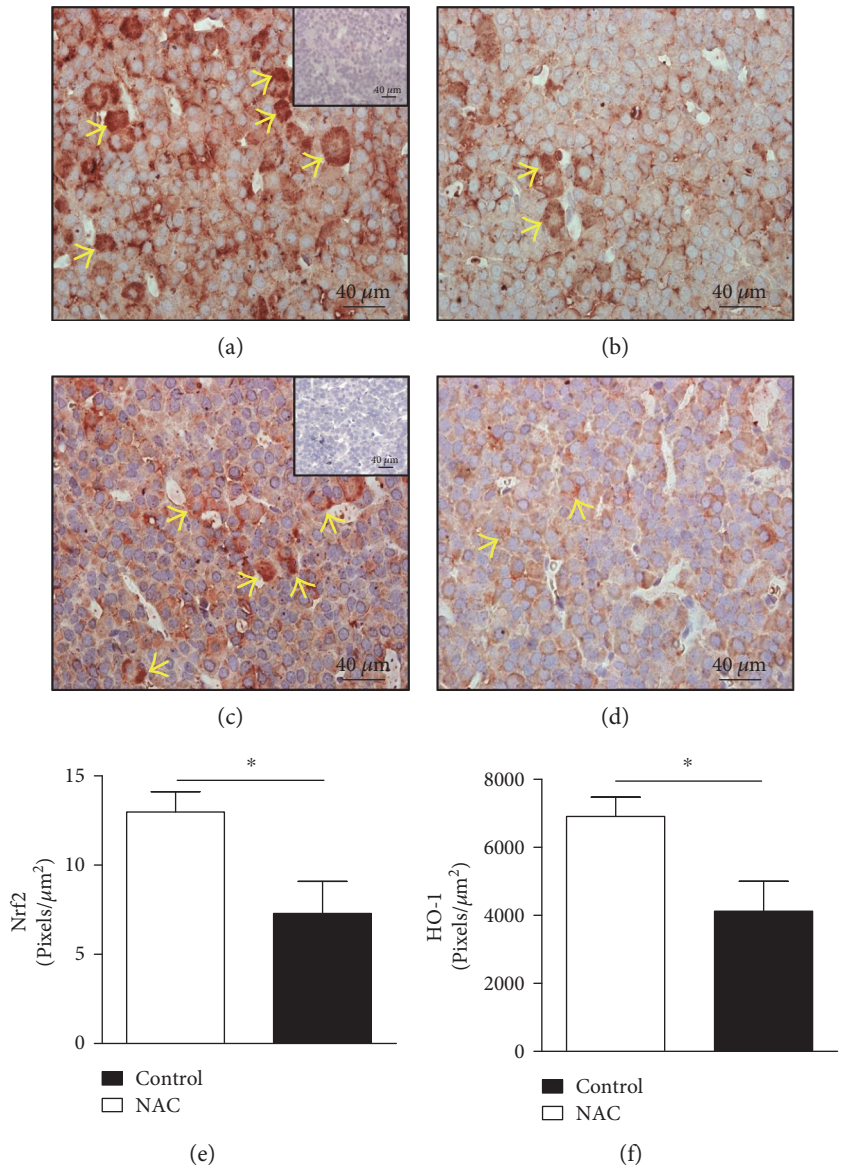

Figure 3: NAC reduces Nrf2 and HO-1 expression in the anterior pituitary of Wistar rats. NAC (150 mg/kg, oral route) was given daily for 18 consecutive days and analysis was made by immunohistochemistry. The panels show representative photomicrographs of pituitary expression of Nrf2 in (a) control and (b) NAC-treated rats and HO-1 in (c) control and (d) NAC-treated rats. The quantification of pixels associated with Nrf2 and HO-1 expression is shown in (e) and (f), respectively. Inserts represent negative controls. Yellow arrows indicate immunolabelling of Nrf2 (a, b) and HO-1 (c, d) in the anterior pituitary. Data are expressed as the mean ± SEM of 6 animals. This result is a representative of two independent assays. $^{*}p < 0.05$.

NAC induced an increase in plasma corticosterone levels compared to controls (mean ± SEM, $n = 6$; $p < 0.01$; one-way ANOVA followed by a Student-Newman-Keuls post hoc test), dexamethasone did not alter the levels of corticosterone in NAC-treated rats (Figure 5).

4. Discussion

This study investigated the role of antioxidants on the modulation of endogenous glucocorticoid levels. We found that treatment with antioxidants either NAC or vitamin E increases the plasma levels of corticosterone in rats, in association with an overexpression of ACTH receptor and the steroidogenic enzyme StAR in the adrenal glands. NAC also induces a drop in HO-1 and Nrf2 expression in the pituitary and blocked the ability of dexamethasone to perform negative feedback of the HPA axis by decreasing the expression of glucocorticoid receptors in the pituitary. Our findings suggest that antioxidants cause a hyperactivation of the HPA axis with a clear dependency of upregulation of ACTH receptor in adrenals and downregulation of glucocorticoid receptors in the pituitary.

In this study, we showed that both NAC and vitamin E increase circulating levels of corticosterone in rats. NAC and vitamin E are antioxidants that act through distinct mechanisms of action. While NAC provides cysteine, which

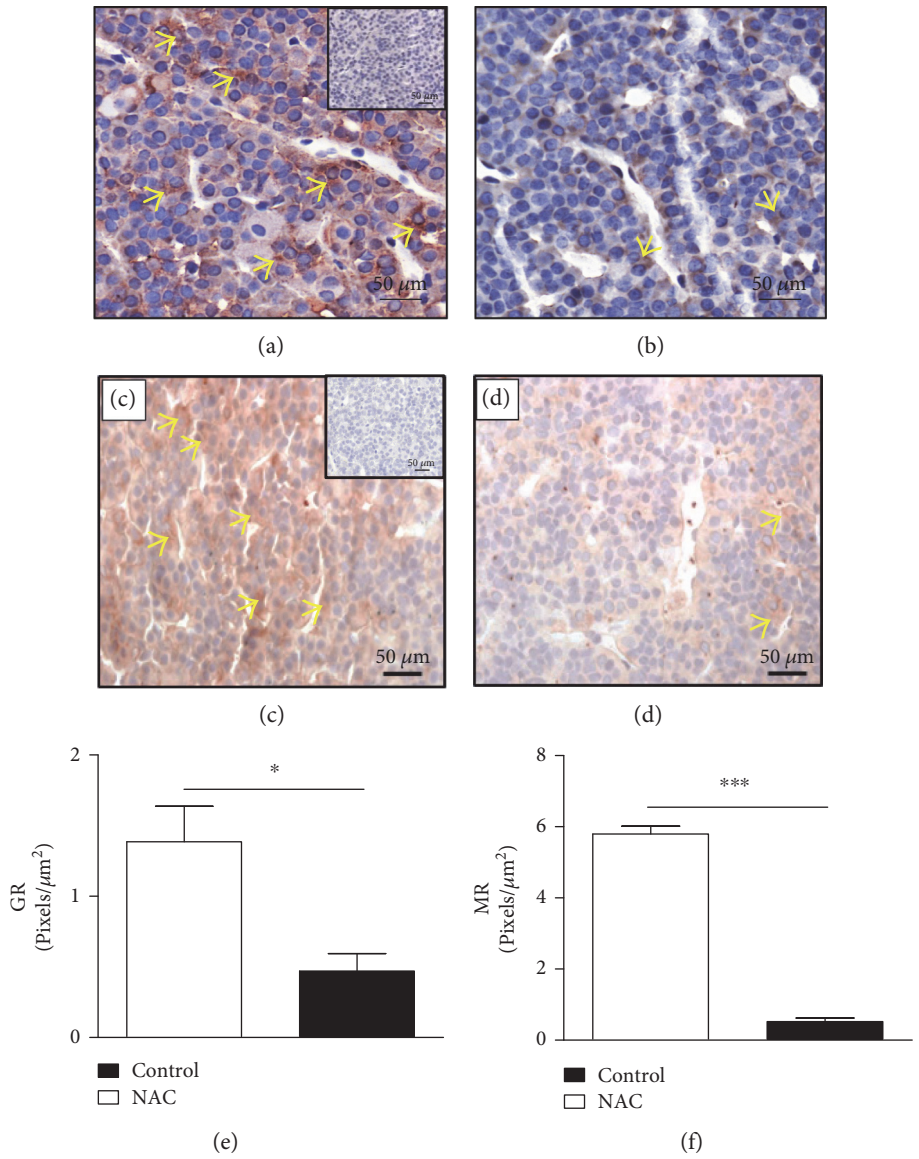

Figure 4: NAC decreases GR and MR expression in the anterior pituitary of Wistar rats. NAC (150 mg/kg, oral route) was given daily for 18 consecutive days and analysis was made by immunohistochemistry. The panels show representative photomicrographs of pituitary expression of GR in (a) control and (b) NAC-treated rats and MR in (c) control and (d) NAC-treated rats. The quantification of pixels associated with GR and MR expression is shown in (e) and (f), respectively. Inserts represent negative controls. Yellow arrows indicate immunolabelling of GR (a, b) and MR (c, d) in the anterior pituitary. Data are expressed as the mean ± SEM of 6 animals. This result is a representative of two independent assays. $^{*}p < 0.05$ and $^{***}p < 0.001$.

is a precursor for reduced glutathione production, and scavenges oxidants directly, including hydroxyl radical, $^{-}$OH, and hypochlorous acid [8]; vitamin E is a peroxyl radical scavenger and due to its lipid solubility plays an important role in maintaining integrity of long-chain polyunsaturated fatty acids in the membranes of cells [6, 7]. The fact that prolonged treatment with two antioxidants with different mechanisms of action can increase circulating levels of corticosterone indicates that this is not an epiphenomenon, but suggests that inhibition of physiological levels of ROS in the HPA axis is responsible for its hyperactivity.

The HPA axis is the main neuroendocrine system that regulates responses to stress [8]. It is well known that the production of high levels of ROS into the glands that comprise the HPA axis is associated with the activation of a stress-response system [13, 14]. Therewith, antioxidant treatment reduces corticosterone levels in several models of diseases, including brain oxidative stress induced by lipopolysaccharide [26]. However, although accumulation of oxidative damage in cellular macromolecules is immensely toxic, ROS products by normal cell metabolism are vital to cell homeostasis maintenance [15, 16]. Our hypothesis is that physiological levels of ROS have a fundamental role in maintaining the homeostasis of the HPA axis. In fact, treatment with NAC in normal rats effectively reduced ROS levels in chondrocytes and Lin^{-}CD45^{+}AnV^{-} marrow cells [27], suggesting that in our model, the antioxidant therapy probably reduces intracellular ROS content in the adrenal and pituitary glands.

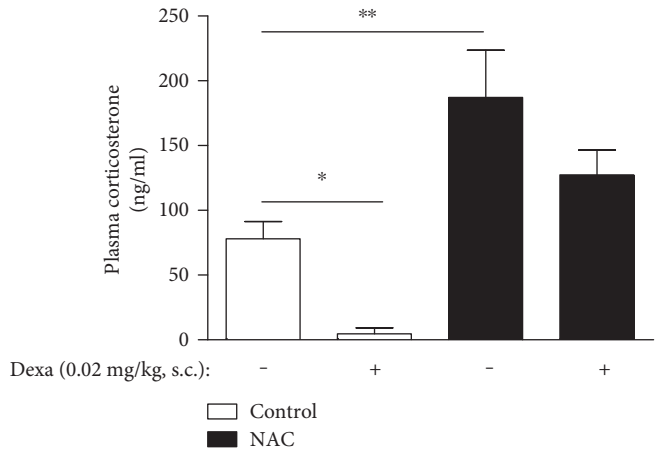

Figure 5: NAC impairs dexamethasone-induced negative feedback of the HPA axis in Wistar rats. NAC (150 mg/kg, oral route) was given daily for 18 consecutive days. Some groups of animals were injected with dexamethasone (0.02 mg/kg, s.c.) starting 13 days after the beginning of NAC treatment, daily during five consecutive days. Data are expressed as the mean ± SEM of 5 animals. This result is a representative of two independent assays. $^{*}p < 0.05$ and $^{**}p < 0.01$.

Thereby, we strongly suggest that any imbalance in the redox system in glands which comprise the HPA axis culminates in its hyperactivation.

In an attempt to elucidate how antioxidants induce the production of glucocorticoids by the HPA axis, we analyzed the expression of adrenal MC2R. Prolonged treatment with NAC increases adrenal MC2R expression. This higher adrenal MC2R expression after treatment can explain, at least partially, the capacity of NAC to increase circulating glucocorticoid levels. Increased expression of MC2R may lead to high activation of this receptor by ACTH and induction of the transcription of several key genes of enzymes involved in steroidogenesis, including StAR [28]. In fact, we showed that NAC induces an upregulation in the expression of StAR into the adrenal glands. StAR rapidly transports cholesterol to the inner mitochondrial membrane, where the conversion of this steroid precursor into pregnenolone, a precursor of steroid hormones, occurs [29]. This metabolic step is crucial to rapid glucocorticoid production into the adrenals in a stress stimulus, once steroidogenic cells store very little amount of glucocorticoids [10].

Although the increased expression of MC2R and StAR alone may explain the increase in glucocorticoids levels, other molecular alterations can also participate in the HPA axis hyperactivity noted after antioxidant treatment. Once the HPA axis is finely regulated by a negative feedback response on the hypothalamus and/or pituitary that normalizes circulating corticosterone levels, we hypothesized that antioxidants could induce a defect in the negative feedback regulation in the HPA axis. We observed that treatment with NAC downregulated the expression of Nrf2 in the anterior pituitary gland of rats. Nrf2 is a transcription factor that regulates expression of several antioxidant enzymes, including superoxide dismutase, catalase, glutathione peroxidase, and HO-1 [30]. Although NAC can induce upregulation of Nrf2 expression in phosgene-induced acute lung injury [31], our data is in accordance with others which described that NAC inhibited Nrf2 expression in lymphoid malignant cell lines stimulated with honokiol [32], suggesting that the effect of antioxidant NAC on Nrf2 expression depends on the cell type and condition of the study. Furthermore, we noted that NAC also decreased the expression of HO-1 in the anterior pituitary of rats. The drop in HO-1 levels after treatment with NAC indicates that the low content of Nrf2 is associated with a reduced ability of this transcription factor to induce production of antioxidant enzymes by pituitary cells. We suppose that the downregulation of Nrf2 expression is a strategy of the organism to maintain homeostasis in rats treated for several days with NAC. In fact, it has been shown that exogenous antioxidants can reduce the expression and/or activity of endogenous antioxidant enzymes [33, 34]. These data indicate that the pituitary as well as adrenals is also a direct target of antioxidant drug effects.

Our next approach was to investigate the sensitivity of the HPA axis to negative feedback induced by synthetic glucocorticoid in NAC-treated rats. Dexamethasone decreased plasma corticosterone levels in control rats; however, it did not alter circulating glucocorticoid amount in rats treated with NAC, showing that antioxidants abolish the ability of glucocorticoids to perform negative feedback of the HPA axis. NAC treatment also decreased expression of both GR and MR in the pituitary of rats, indicating that a reduction in glucocorticoid receptor expression in the pituitary of rats can explain the inability of dexamethasone to induce negative feedback of the HPA axis in NAC-treated animals. Our data confirmed the capacity of NAC in inhibiting GR expression, once NAC decreases GR protein levels in the hypothalamus of mice fed with a high-cholesterol diet [35].

Currently, many people consume dietary supplementation with antioxidants to combat diseases associated with aging [36]; however, several clinical trials testing benefits and harms of antioxidant supplements found that antioxidants have been unable to demonstrate beneficial effects and pointed that they seemed to cause an increase in all-

cause mortality [37–40]. Once antioxidants induce a HPA axis dysfunction with concomitant increased levels of circulating glucocorticoids, this food supplement is shown as a risk to human health. This occurs because the hyperactivation of the HPA axis, and consequently the glucocorticoid signaling system, may alter the epigenetic landscape and influence genomic regulation and function conducting to the development of aging-related diseases [41]. Some harmful effects of hypercorticoidism, which can culminate with aging-related diseases, are deleterious effects on the central nervous system (CNS), including neuroinflammatory environment, loss of neuronal function, and apoptosis of neuronal cells, causing a decrease in hippocampal neurogenesis and an increase in neuroinflammation and neurodegeneration [42–44]. These deleterious effects of hypercorticoidism on the CNS can lead to the development of a variety of progressive neurodegenerative and psychiatric diseases, including schizophrenia, dementia, depression, Huntington's disease, and Alzheimer's disease [45–49]. Although we showed that antioxidants can induce high production of glucocorticoids, it is well known that chronic stress promotes redox imbalance throughout the body [50], as in blood of humans [51–53], such as in several structures of the CNS of rats including the frontal cortex, hypothalamus, and hippocampus [54]. These data suggest that chronic stress accelerates cellular aging through inducing increased levels of ROS [50]. These observations are conflicting with ours, once we show that antioxidants decreased the expression of Nrf2 and HO-1. However, the induction of ROS production is not the only mechanism related to stress-induced cellular aging. Chronic stress reduces brain-derived neurotrophic factor (BDNF) in the hippocampus and prefrontal cortex and increases neuroinflammation, an alteration noted in the formation of depression [44]. Furthermore, chronic stress induces prolonged periods of glutamate release in the hippocampus and decreases the ability to clear extracellular glutamate. These alterations in the glutamate transmission may be related to the impairments in the spatial and contextual memory performance and stress-associated psychiatric disorders, including mood and anxiety [55].

In addition, hypercorticoidism can also increase susceptibility to cancer [56], one of the most important aging-related diseases. Although ROS can cause oncogenic mutations and activate oncogenic pathways [57], dietary supplementation with antioxidants promotes increased incidence and death from lung and prostate cancer [58]. Furthermore, antioxidants induce melanoma progression by promoting metastasis [59]. One possibility is that the high incidence of cancer in people which use dietary supplementation with antioxidants can be related to hyperactivation of the HPA axis.

In summary, our results indicate that antioxidant therapy can induce an activation of the HPA axis, with an increase in the levels of systemic glucocorticoids by upregulating ACTH receptor in the adrenal and downregulating glucocorticoid receptors in the pituitary. Thereby, indiscriminate use of antioxidant supplements can be a risk to develop several morbidities related to persistent hypercorticoidism, as observed in Cushing's disease.

Conflicts of Interest

The authors declare that they have no conflict of interest. Furthermore, the mentioned received funding in the "Acknowledgments" section did not lead to any conflict of interests regarding the publication of this manuscript.

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Research Article

Activation of the Nrf2-Keap 1 Pathway in Short-Term Iodide Excess in Thyroid in Rats

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Wistar rats were randomly divided into groups of varying iodide intake: normal iodide; 10 times high iodide; and 100 times high iodide on Days 7, 14, and 28. Insignificant changes were observed in thyroid hormone levels ($p > 0.05$). Urinary iodine concentration and iodine content in the thyroid glands increased after high consumption of iodide from NI to 100 HI ($p < 0.05$). The urinary iodine concentration of the 100 HI group on Days 7, 14, and 28 was 60–80 times that of the NI group. The mitochondrial superoxide production and expressions of Nrf2, Srx, and Prx 3 all significantly increased, while Keap 1 significantly decreased in the 100 HI group when compared to the NI or 10 HI group on Days 7, 14, and 28 ($p < 0.05$). Immunofluorescence staining results showed that Nrf2 was localized in the cytoplasm in NI group. Although Nrf2 was detected in both cytoplasm and nucleus in 10 HI and 100 HI groups, a stronger positive staining was found in the nucleus. We conclude that the activation of the Nrf2-Keap 1 antioxidative defense mechanism may play a crucial role in protecting thyroid function from short-term iodide excess in rats.

1. Introduction

Iodine being a critical constituent of thyroid hormones is essential for normal growth and development in all vertebrates [1, 2]. During thyroid hormone synthesis, there is a constant production of reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2), which is subsequently utilized for the oxidation of iodide [3]. Although the basal level of ROS production is important for maintaining thyroid hormone biosynthesis, iodide excess may increase the production of ROS in thyrocytes [4, 5]. Higher amounts of ROS can cause oxidative stress by damaging the cellular components and affecting organelle integrity [5]. The increased generation of ROS triggered by iodide excess is responsible for its cytotoxic effect on thyrocytes [6, 7].

Thomopoulos reported that hyperthyroidism may develop in around 10% of patients with excess iodine and that it may occur several years after the initiation of iodine excess [8]. Wolff reported that chronic ingestion of more than ten times the daily requirement of iodide or iodide-generating organic compounds could lead to iodide goiter in certain subjects [9]. In the thyroid slices of several species, excess iodide is known to stimulate the generation of H_2O_2 [10]. This occurs when the latter is in the presence of either 300 μM KI in dog thyroid slices or 100 μM KI in bovine thyroid slices [10].

Possible molecular mechanisms responsible for excess iodide-induced ROS production are described as below. When iodide is in excess as compared with tyrosine residues, it reacts with the iodonium cation formed by iodide oxidation to give molecular iodine. Excess molecular iodine induces apoptosis through an increased generation of free radicals [11]. Various types of iodolipids are produced when iodine binds to membrane lipids, which is considered to be the main mechanism of free radical-induced damage. The mitochondria contain specific receptors for the thyroid hormones,

and much of the ROS production occurs here via oxidative phosphorylation [3, 12]. ROS include free radicals, such as superoxide anions, hydroxyl radicals, and H_2O_2.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor, which is vital in regulating the expression of some antioxidative enzymes, such as hemeoxygenase-1, thioredoxin, peroxiredoxins (Prxs), and Sulfiredoxin (Srx) [13]. Nrf2 is released from the Nrf2-Keap 1 (Kelch-like ECH-associated protein 1) complex and translocated to the nucleus after the initiation of oxidative stress [14]. Srx, a recently discovered member of the oxidoreductases family, contributes to cellular redox balance. Previous studies have shown Srx to be the only enzyme which catalyses ATP-dependent reduction of the hyperoxidized form of Prxs [15, 16]. Prxs are important peroxidases that reduce peroxides [17, 18]. Peroxiredoxin 3 (Prx 3) is a critical scavenger for mitochondrial H_2O_2. Also, mitochondria contain 30 times more Prx 3 than glutathione peroxidase [19].

In the present study, we aim to investigate the effect of normal iodide intake (NI), 10 times high iodide intake (10 HI), and 100 times high iodide intake (100 HI) on Days 7, 14, and 28 on the antioxidative action of Srx and Prx 3 via Nrf2-Keap 1 pathway in the thyroid of rats.

2. Methods

2.1. Animals and Diet. A total of 216 Wistar rats (eight weeks old) at SPF level, weighing 296.36 ± 8.53 g, were randomly assigned to NI, 10 HI, and 100 HI groups. Along with the normal diet, the NI group (with the addition of deionized water), 10 HI and 100 HI groups received different dosages of potassium iodide in the deionized water, resulting in the following daily iodide intake: 7.5 μg/d, 75 μg/d, and 750 μg/d, respectively [20]. The rats were sacrificed after iodide intake for a week, two weeks, and four weeks at ages of 9, 10, and 12 weeks, respectively (they are collectively referred to as Day 7, Day 14, and Day 28). In this study, a total of two rats died in the NI group, and none died in any other group. Animal procedures were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University (the number is SYXK (Jin): 2014-0004), which is in accordance with the NIH Guide.

2.2. Reagent. Anti-Peroxiredoxin-3 antibody (ab16751) and anti-Keap 1 antibody (ab66620) were purchased from Abcam (Abcam, Cambridge, MA, USA). Nrf2 (H-300): sc-13032, Sulfiredoxin (FL-137): sc-99076, goat anti-rabbit IgG-HRP: sc-2004, goat anti-mouse IgG-HRP: sc-2005, and goat anti-rabbit IgG-PE: sc-3739 were bought from Santa Cruz (Santa Cruz Biotechnology, Inc., CA, USA). MitoSOX Red (3,8-phenanthridinediamine,5-(6′-triphenyl-phosphoniumhexyl)-5,6-dihydro-6-phenyl) mitochondrial superoxide indicator (M36008) was purchased from Invitrogen (Invitrogen Life Technologies, CA, USA). β-Actin (AA128) was purchased from Beyotime (Beyotime Institute of Biotechnology, Jiangsu, China). RPMI-1640 and fetal bovine serum (FBS) were purchased from GE Healthcare Life Sciences (HyClone, UT, USA). Immobilon Western Chemiluminescent HRP Substrate (WBKLS0100) was purchased from Millipore (Merck Millipore, MA, USA). All the other chemicals made in China were of analytic grade [21].

2.3. Thyroid Weight Measurement. Following the intake of NI, 10 HI, and 100 HI, the body weight and the thyroid weight were measured when the rats were sacrificed on Days 7, 14, and 28. We calculated the ratio of the thyroid weight/body weight (milligrams per 100 gram body) according to the ratio of the viscera (viscera/body weight) [21, 22]. The rats were anesthetized at appropriate concentrations (10% chloral hydrate, 0.3 mL/100 g). Skin, subcutaneous tissue, fascia, and muscles of the anterior neck were removed and the thyroid gland was exposed. In order to ensure the integrity of the thyroid gland, it was removed carefully with a trachea ring. The fascia covering thyroid gland was stripped and the thyroid gland was collected under stereoscopic microscope carefully.

2.4. Measurement of Urinary Iodine Concentration and Iodine Content in the Thyroid Glands. Urine samples were collected using metabolic cages for 24 hours the day before the rats were sacrificed. Thyroid tissue homogenates were prepared. Urinary iodine concentration and iodine content in the thyroid glands were measured by As-Ce catalytic spectrophotometry in the Key Lab of Hormones and Development Ministry of Health, Institute of Endocrinology, Tianjin Medical University [23]. The iodine standard solution and samples were added to the test tubes (15 mm × 150 mm), respectively. 1 mL ammonium persulfate was added and then mixed and digested for 60 minutes at 100°C. After the test tubes were cooled down, 2.5 mL of arsenious acid solution was added and mixed. Consequently, 0.3 mL cerium sulfate solution was added and mixed every 30 s. The absorbance at 400 nm was measured with a spectrophotometer.

2.5. Serum Thyroid Hormones Levels Measurement. Blood samples were taken from the carotid artery and then centrifuged for 10 min at 2000 r/min to obtain the samples of the serum. After that, the samples were stored at −80°C for further analysis. Levels of serum total thyroxine (TT4), total triiodothyronine (TT3), free thyroxine (FT4), and free triiodothyronine (FT3) were all determined using a chemiluminescent immunoassay technique. All kits for thyroid function were purchased from Siemens (Siemens Healthcare Diagnostics Products Limited, Llanberis, UK).

2.6. Flow Cytometry. MitoSOX Red was used to measure mitochondrial superoxide production by flow cytometry. The thyroid cell suspension of Wistar rats was prepared. 5 μM of MitoSOX Red was added and the suspension was incubated for 10 min at 37°C in the dark. Flow cytometry was carried out using a FACSCalibur (BD Bioscience, San Jose, CA). Collecting FL2 channel forward scattering (forward scatter, FSC) and lateral scattering (side scatter, SSC) data, 10000 cells were collected for each sample. The control group without MitoSOX was regarded as the blank zero group for standardization [24].

Table 1: Changes of the median urinary iodine concentration (μg/L) of Wistar rats following the intake of NI, 10 HI, and 100 HI on Days 7, 14, and 28 ($N = 6$ for each group). All data is presented as the median (range).

Group	The median urinary iodine concentration (μg/L)		
	Day 7	Day 14	Day 28
NI	321.2 (150.0–351.7)	358.2 (300.2–373.8)	333.0 (188.6–413.5)
10 HI	3052.5* (1592.8–3798.0)	3532.5* (1487.0–4056.0)	2628.0* (1651.0–3404.5)
100 HI	26489.2*# (5856.6–42170.4)	24461.0*# (17607.5–29874.8)	22663.2*# (8342.5–29816.2)

*Compared to the NI group ($p < 0.05$).
#Compared to the 10 HI group ($p < 0.05$).

2.7. Western Blot Analysis. The bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) was used to detect the concentration of proteins. 50 μg proteins were separated by SDS-PAGE and transferred to the PVDF membrane. Subsequently, the membrane was blocked for 1 hour at room temperature using 5% nonfat milk. Then the membrane was incubated overnight at 4°C with primary antibodies followed by horseradish peroxidase conjugated secondary antibodies. The proteins were detected by Immobilon Western Chemiluminescent HRP Substrate. β-Actin was used as a loading control. Blots were scanned as gray scale images and quantified using Image J software (NIH). All the blot intensities were normalized with that of the loading control β-actin.

2.8. Immunofluorescence Staining. The thyroid tissues were first embedded with an Optimal Cutting Temperature (OCT) compound at −80°C. Following which the tissues were frozen in a cryostat machine and cut into frozen sections for 5 μm at −20°C. The slices were incubated with 5% FBS for 60 min at room temperature. Subsequently, they were incubated with a primary antibody [Nrf2 (1 : 100) or Srx (1 : 100)] at 4°C, overnight. After three washes with PBS, the second antibody was linked to fluorophores (goat anti-rabbit IgG-PE). The nucleus was stained for 5 min with Hoechst 33258 (50 μL) and washed 3 times with PBS. MitoSOX Red was used to measure mitochondrial superoxide production. Using a Zeiss LSM 510 confocal microscope, fluorescent images of the prepared slides were obtained.

2.9. Statistics. The data of urinary iodine concentration (Table 1) showed a skewed distribution and were expressed as the median. Differences between groups were evaluated by nonparametric Kruskal–Wallis test. If the latter test showed significant differences between groups, the individual groups were compared with the control group by the Nemenyi tests using SPSS 22.0. A p value of <0.05 was considered statistically significant [25].

The other data was expressed as mean ± SD. Differences between groups were evaluated by one-way analysis of variance (ANOVA); if this test showed significant differences between groups, the individual groups were compared with the control group by Least Significant Difference (LSD) test using SPSS 22.0. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Effects of the Iodide Intake (NI, 10 HI, and 100 HI) on the Ratio of Thyroid Weight/Body Weight on Days 7, 14, and 28. The parameters body weight, thyroid weight, and the ratio of the thyroid weight/body weight were not significantly altered following the intake of NI, 10 HI, and 100 HI on Days 7, 14, and 28 ($p > 0.05$) (Figure 1).

3.2. Effects of the Iodide Intake (NI, 10 HI, and 100 HI) on Urinary Iodine Concentration and Iodine Content in the Thyroid Glands on Days 7, 14, and 28. The median urinary iodine concentration and their ranges for NI, 10 HI, and 100 HI on Days 7, 14, and 28 are illustrated in Table 1. In all the different time periods, there was a significant increase in the urinary iodine concentration among 10 HI and 100 HI when compared to the NI group ($p < 0.05$). In addition, the urinary iodine concentration of the 100 HI group also increased significantly when compared to 10 HI ($p < 0.05$). Between any of the iodide intake groups (NI, 10 HI, and 100 HI), there was no significant difference in the urinary iodine concentration on Day 14 and Day 28 when compared to Day 7 ($p > 0.05$). Furthermore, there was no significant difference between Day 14 and Day 28 ($p > 0.05$). When the intake of iodide was increased from NI to 10 HI and further to 100 HI, a simultaneous increase in the urinary iodine concentration was also observed on Days 7, 14, and 28. The urinary iodine concentration in the 10 HI group was approximately 10 times that of the NI group, whereas the urinary iodine concentration in the 100 HI group was about 60–80 times that of the NI group on Days 7, 14, and 28.

In our study, we demonstrated that the iodine content in the thyroid glands was significantly increased in the 100 HI group when compared to the NI group on Days 7, 14, and 28 ($p < 0.05$). Between any of the iodide intake groups (NI, 10 HI, and 100 HI), there was no significant difference found in the iodine content in the thyroid glands on Days 7, 14, and 28 ($p > 0.05$). When the intake of iodide was increased from NI to 10 HI and further to 100 HI, the iodine content in the thyroid glands increased gradually on Days 7, 14, and 28 (Table 2).

3.3. Effects of the Iodide Intake (NI, 10 HI, and 100 HI) on the Changes of Serum Thyroid Hormones Levels on Days 7, 14, and 28. There were no significant alterations in TT3, TT4, FT3,

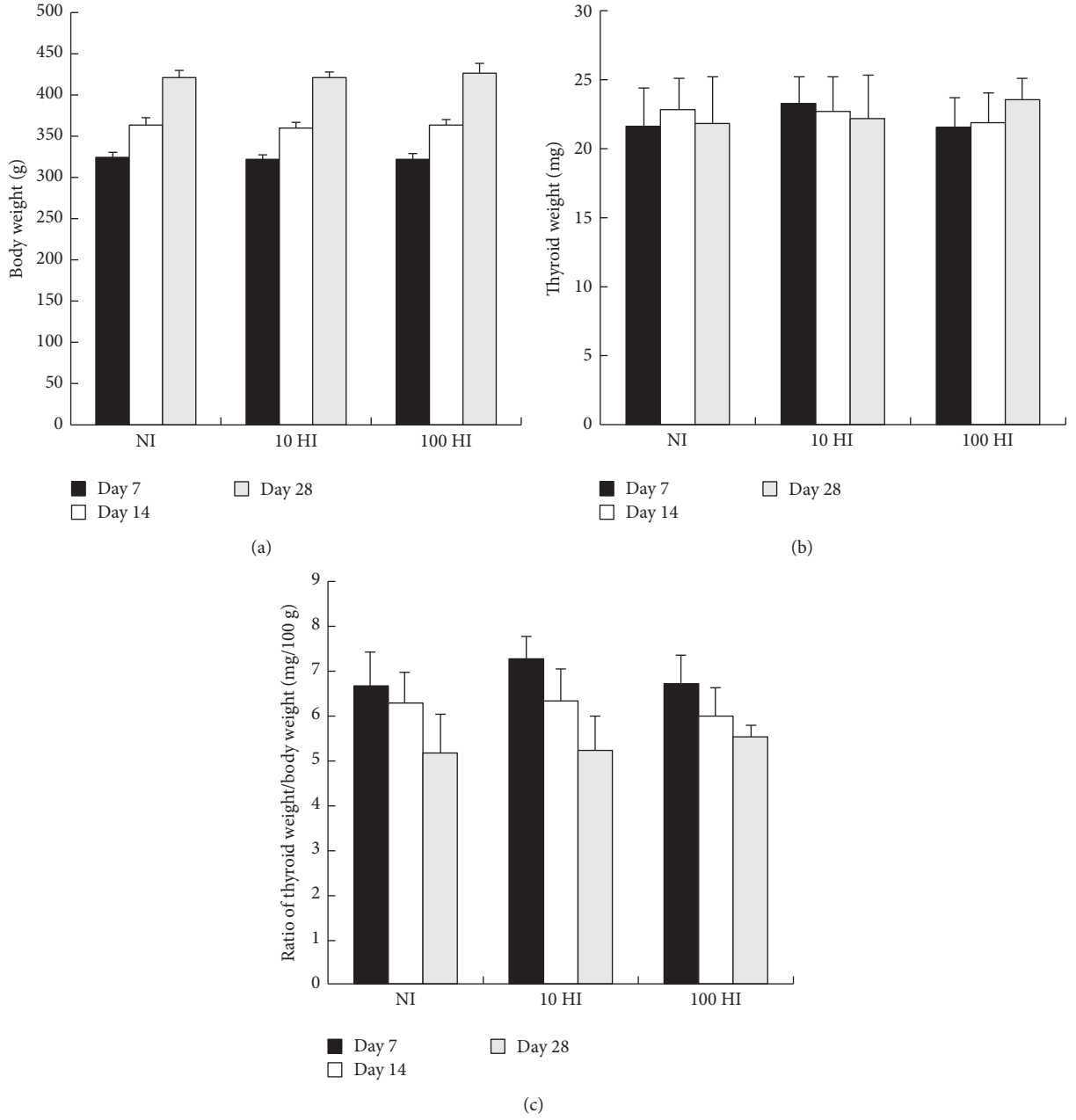


Figure 1: Effects of the iodide intake (NI, 10 HI, and 100 HI) on (a) the body weight; (b) the thyroid weight; and (c) the ratio of the thyroid weight/body weight on Days 7, 14, and 28. All data is presented as mean ± SD ($N = 6$ for each group). Statistical analyses were performed by one-way analysis of variance (ANOVA) with the Least Significant Difference (LSD) test.

and FT4 levels following the intake of NI, 10 HI, and 100 HI on Days 7, 14, and 28 ($p > 0.05$). Moreover, for all three dosages of iodide intake, there were no significant differences in any of the serum thyroid hormones levels on Day 14 and Day 28 when compared to Day 7 ($p > 0.05$). In addition, there were no significant changes between Day 14 and Day 28 in serum thyroid hormones levels (TT3, TT4, FT3, and FT4) ($p > 0.05$) (Table 3).

3.4. Effects of the Iodide Intake (NI, 10 HI, and 100 HI) on the Changes of Mitochondrial Superoxide Production on Days 7, 14, and 28. On Days 7, 14, and 28, when compared to the NI group, the mitochondrial superoxide production in the 10 HI group showed no significant increase ($p > 0.05$). However, there was a significant increase in the mitochondrial superoxide production in the 100 HI group ($p < 0.05$). When compared to the 10 HI, there was a significant increase in the mitochondrial superoxide production in the 100 HI group ($p < 0.05$). In the NI and 10 HI groups, the mitochondrial superoxide production on Days 7, 14, and 28 showed no significant difference ($p > 0.05$). However, in the 100 HI group, compared to Day 7, there was a significant increase in

Table 2: Changes of iodine content in the thyroid glands (μg/100 mg) of Wistar rats following the intake of NI, 10 HI, and 100 HI on Days 7, 14, and 28 ($N = 6$ for each group). All data is presented as the mean ± SD.

Group	Iodine content in the thyroid glands (μg/100 mg)		
	Day 7	Day 14	Day 28
NI	62.19 ± 5.94	60.12 ± 8.13	63.37 ± 9.08
10 HI	121.25 ± 10.48	126.45 ± 8.93	152.33 ± 10.77
100 HI	133.53 ± 8.61*	136.36 ± 9.64*	178.45 ± 8.74*

*Compared with NI group ($p < 0.05$).

Table 3: Changes of serum thyroid hormones levels of Wistar rats following the intake of NI, 10 HI, and 100 HI on Days 7, 14, and 28 ($N = 6$ for each group). All data is presented as the mean ± SD.

Group	The levels of serum thyroid hormones			
	TT3 (nmol/L)	TT4 (nmol/L)	FT3 (pmol/L)	FT4 (pmol/L)
Day 7				
NI	1.18 ± 0.15	90.77 ± 5.52	3.96 ± 0.10	22.03 ± 1.70
10 HI	1.19 ± 0.08	91.54 ± 7.74	3.77 ± 0.26	23.02 ± 2.06
100 HI	1.07 ± 0.11	89.17 ± 9.03	3.78 ± 0.11	20.08 ± 5.43
Day 14				
NI	1.15 ± 0.07	95.83 ± 5.20	3.92 ± 0.16	22.34 ± 3.71
10 HI	1.12 ± 0.08	86.15 ± 2.52	3.63 ± 0.48	20.19 ± 1.92
100 HI	1.19 ± 0.04	91.54 ± 3.31	3.84 ± 0.46	20.98 ± 2.50
Day 28				
NI	1.13 ± 0.06	94.17 ± 2.38	3.89 ± 0.13	20.25 ± 5.72
10 HI	1.18 ± 0.08	90.77 ± 3.78	3.98 ± 0.57	21.15 ± 5.34
100 HI	1.14 ± 0.22	87.69 ± 6.35	3.79 ± 0.46	21.17 ± 5.43

mitochondrial superoxide production on Day 28 ($p < 0.05$). Similarly, compared to Day 14, there was also a significant increase in mitochondrial superoxide production on Day 28 ($p < 0.05$). Accordingly, the fluorescent intensity of MitoSOX Red on Day 28 gradually increased after the increased dosages of iodide intake from NI to 100 HI, which was consistent with our results of flow cytometry (Figure 2).

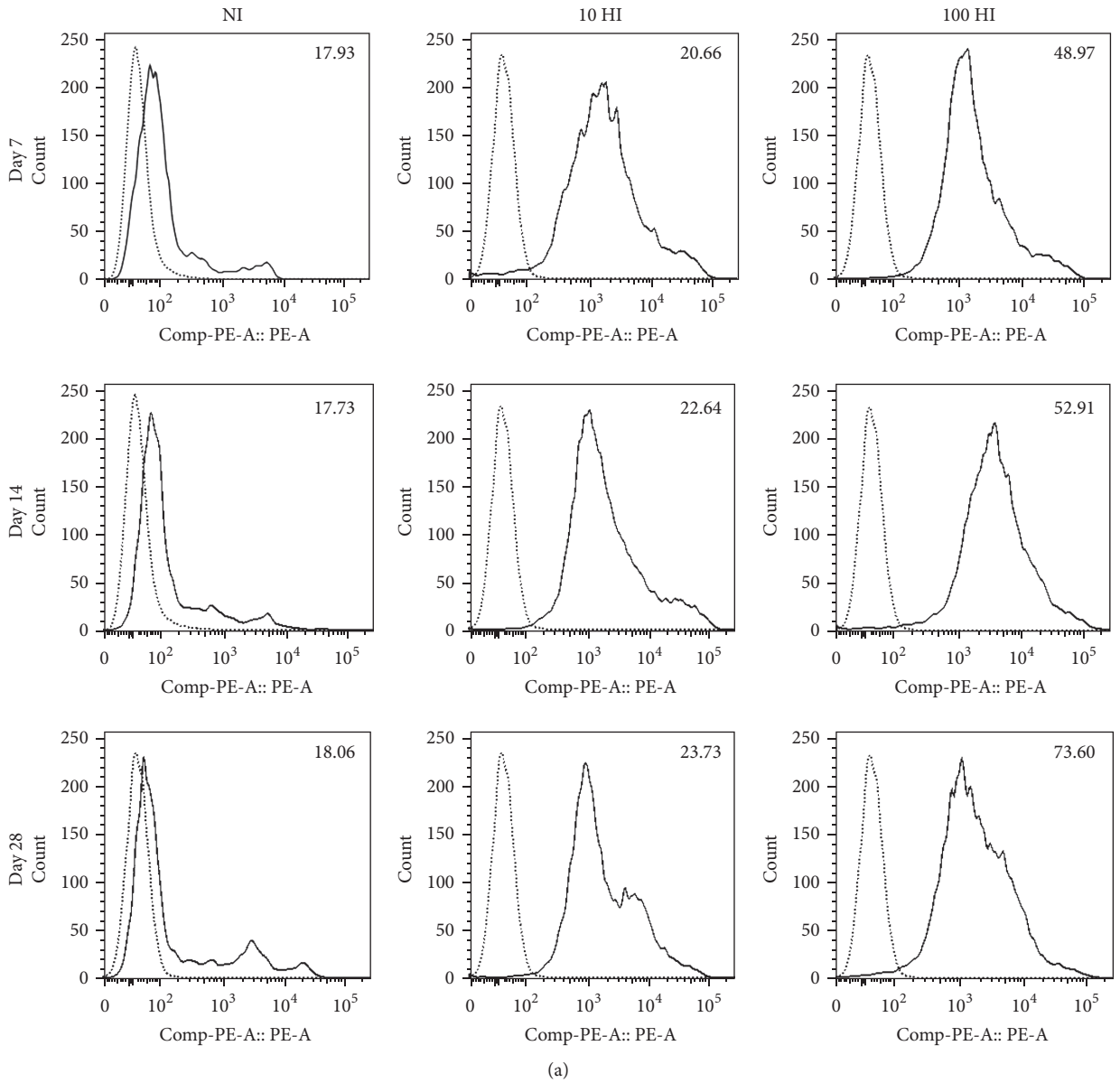
3.5. Effects of the Iodide Intake (NI, 10 HI, and 100 HI) on the Changes of Nrf2, Keap 1, Srx, and Prx 3 Expressions on Days 7, 14, and 28. On Days 7, 14, and 28, when compared to the NI group, the expressions of Nrf2, Keap 1, Srx and Prx 3 showed no significant differences in the 10 HI group ($p > 0.05$); the expressions of Nrf2, Srx, and Prx 3 were significantly increased while Keap 1 was notably decreased in the 100 HI group ($p < 0.05$). On Days 7, 14, and 28, when compared to the 10 HI group, the expressions of Nrf2, Srx, and Prx 3 were significantly increased while Keap 1 was significantly decreased in the 100 HI group ($p < 0.05$) (Figure 3).

3.6. Effects of the Iodide Intake (NI, 10 HI, and 100 HI) on the Changes of Immunofluorescence Staining on Days 7, 14, and 28. Following the increased iodide intake from NI to 10 HI and further to 100 HI, the expressions of Nrf2 and Srx intensified. In the NI group, Nrf2 was localized in the cytoplasm on Days 7, 14, and 28. In the 10 HI group, the positive staining of Nrf2 can be observed in both the nucleus and the cytoplasm. Moreover, in the 100 HI group, a stronger positive staining of Nrf2 can be detected in the nucleus on Days 7, 14, and 28 (Figure 4(a)). Srx positive staining was only located in the cytoplasm on Days 7, 14, and 28 (Figure 4(b)).

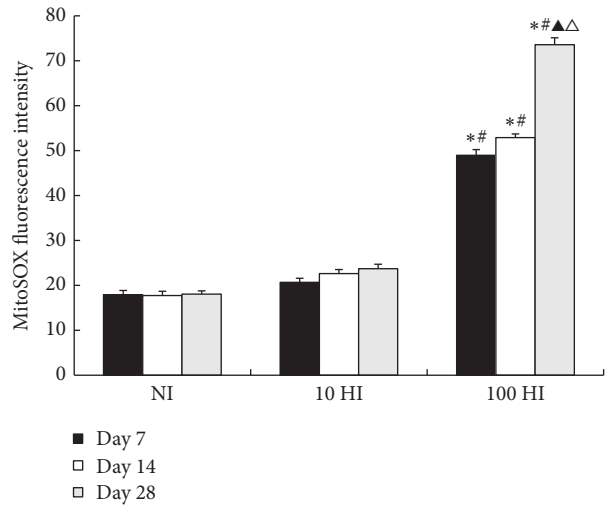
4. Discussion

In our study, we found that there were no significant alterations in TT3, TT4, FT3, and FT4 levels following the intake of NI, 10 HI, and 100 HI on Days 7, 14, and 28 ($p > 0.05$). There are very efficient homeostatic mechanisms that resist changes in circulating T3 and T4 levels in response to iodide excess. Due to the compensatory mechanisms, such as the Wolff-Chaikoff effect, the changes in T3 and T4 levels following an increase in iodide intake are minimal and usually transient in nature [26–28]. The Wolff-Chaikoff effect relies on a high ($\geq 10^{-3}$ molar) intracellular concentration of iodide. During initial exposure, excess iodide is transported by the sodium-iodide symporter (NIS) into the cells. When intracellular concentration reaches at least 10^{-3} molar, iodide organification is blocked [27, 29]. Moreover, the regulatory mechanisms include modulation of blood flow, enzyme activity, gene expression, and transport proteins in signaling pathways [2, 30, 31]. In a study conducted by Eng et al., 16 Wistar rats were given 2000 μg of iodide acutely; it was observed that there was a significant decrease in serum T4 and T3 levels on Day 1 of the study. Subsequently, on Day 6, both serum T4 and T3 levels returned to normal ranges. However, in both cases, the serum TSH levels remained unchanged [32]. Mooij et al. observed no significant changes in serum thyroid hormones levels when female Wistar rats were given 100 μg iodide daily for 18 weeks [33]. Paul et al. demonstrated that when normal volunteers received 1500 μg of supplemental iodine daily for 14 days, a small decrease in serum T3 and T4 concentrations with compensatory increase of TSH was detected, although all values remained within the normal ranges [34]. However, the presence of handicaps such as an increased autoimmune susceptibility, fetal period, extremes of age, pregnancy, lactation, or an active pathological entity significantly impair these mechanisms [1, 35–37].

Our study demonstrated that both the urinary iodine concentration and the iodide intake increased simultaneously from NI to 10 HI and further to 100 HI. Interestingly, we found that the urinary iodine concentration of the 10 HI group was approximately 10 times that of the NI group, whereas the urinary iodine concentration of the 100 HI group was about 60–80 times that of the NI group on Days 7, 14, and 28. The urinary iodine concentration is regarded as a sensitive indicator of iodine status because approximately 90% of ingested iodide is excreted in the urine [38, 39]. There is an increase for the maintenance of thyroid homeostasis as well as the steady state of the internal environment of the body. The thyroid gland has adaptation mechanisms that reduce iodide metabolism when the supply is abundant, thus

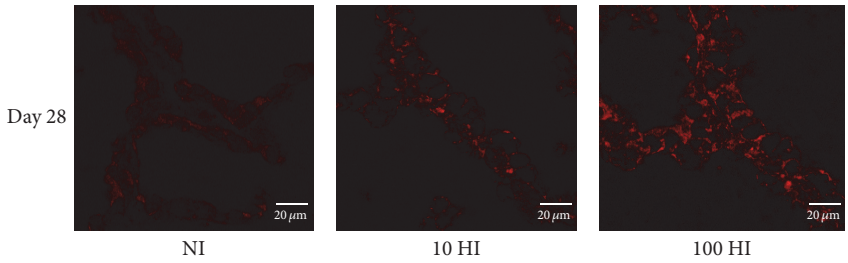


(a)



(b)

Figure 2: Continued.



(c)

Figure 2: Effects of the iodide intake (NI, 10 HI, and 100 HI) on the changes of mitochondrial superoxide production on Days 7, 14, and 28. (a) There was a significant increase in the mitochondrial superoxide production in the 100 HI group on Days 7, 14, and 28 ($p < 0.05$). The difference was more significant on Day 28 when compared to Day 7 or Day 14. (b) Histogram analysis was performed on the mean fluorescence intensity of MitoSOX Red. All data is presented as the mean ± SD ($N = 6$ for each group). Statistical analyses were performed by one-way analysis of variance (ANOVA) with the Least Significant Difference (LSD) test. $^{*}p < 0.05$ versus the NI group on Days 7, 14, and 28, respectively. $^{\#}p < 0.05$ versus the 10 HI group on Days 7, 14, and 28, respectively. $^{\blacktriangle}p < 0.05$ versus the Day 7 group in the 100 HI group. $^{\triangle}p < 0.05$ versus the Day 14 in the 100 HI group. Experiments were repeated 3 times with similar results. (c) There was a significant increase in the mitochondrial superoxide production in the 100 HI group on Day 28 and was observed by confocal microscopy. Scale bar: 20 μm.

avoiding thyrotoxicosis. There are several mechanisms which include a direct inhibitory effect of iodide in the thyroid itself and inhibition by iodide of its own organification (Wolff-Chaikoff effect), its transport, thyroid hormones secretion, cAMP formation in response to TSH, and several other metabolic steps [40]. We suggest that all the protective mechanisms may ensure the excessive iodide intake of the 10 HI group on Days 7, 14, and 28 be eliminated from urine. The urinary iodine concentration of the 100 HI group on Days 7, 14, and 28 was about 60–80 times that of the NI group, although the thyroid function was normal. This leads us to propose the idea that excessive iodide accumulated in the body may trigger the oxidative and antioxidative signaling pathway to maintain the normal thyroid function.

We demonstrated that the production of mitochondrial superoxide significantly increased on Days 7, 14, and 28 in the 100 HI group. This is consistent with our previous study on metallothionein-I/II knockout mice [24]. Joanta et al. reported that the initiation of free radical production was observed after giving a high dose of iodide [3]. Serrano-Nascimento et al. demonstrated that an increased mitochondrial superoxide production was shown in response to NaI (10^{-6} M to 10^{-3} M) treatment in PCCl3 thyroid cells by using MitoSOX Red [41]. Mitochondria are potent producers of superoxide, from complexes I and III of the electron transport chain. Mitochondrial superoxide production is a major cause of cellular oxidative damage [42]. Physiologically, ROS are not necessarily harmful because they are continuously balanced by the process of hormone synthesis and the endogenous antioxidant system [1]. Excess ROS are generated during the trapping, oxidation, and organification of excessive iodine in thyrocytes, which could lead to increased oxidative stress [1].

The Nrf2-Keap 1 pathway is the chief cytoprotective mechanism in response to oxidative stress caused by ROS. During normal and balanced redox homeostasis, the Nrf2 function is inhibited because of constant proteasomal degradation after ubiquitination of the protein. This is regulated through the binding of the inhibitor protein Keap 1 [13, 14]. It is reported that Srx activation via Nrf2 dependent pathway protects from oxidative liver injury through Pyrazole [43] and alcohol in mice [44]. Similar findings in lung tissues have shown that there is a marked increase in the expressions of Srx and Prx 3 in human squamous cell carcinoma [45]. This suggests that these proteins may play a protective role against oxidative injury. Also, the pathway including Keap 1, Nrf2, and ARE-mediated protein expression plays a very critical role in protecting cells from oxidative stress [46, 47]. Focusing on the pathway, we demonstrated that the expression of Nrf2 was significantly increased, while Keap 1 was significantly decreased in the 100 HI group when compared to the NI or 10 HI group on Days 7, 14, and 28. Similarly, Ajiboye et al. showed that rats treated with Chalcone dimers not only increased the expression of Nrf2, but also suppressed cytoplasmic Keap 1 expression [48]. Yang et al. also showed that the downregulation of Keap 1 level may be responsible for the overactivation of Nrf2 [49]. Pang et al. showed that caffeic acid prevents acetaminophen-induced liver injury by activating the Nrf2-Keap 1 antioxidative defense system [50].

In order to verify whether high levels of expression of Nrf2 and its nuclear translocation can upregulate some antioxidant enzymes in the thyroid gland, the expressions of Prx 3 and Srx following the intake of NI, 10 HI, and 100 HI on Days 7, 14, and 28 were measured. We demonstrated that the expressions of Srx and Prx 3 in the 100 HI group were significantly increased when compared to the NI group or 10 HI group on Days 7, 14, and 28. The possible explanations are described as below. Firstly, Srx is a cytosolic protein that is able to translocate to sites where hyperoxidized (inactivated) Prx 3 is located. Therefore, it engages itself in the reactivation of Prx 3 under oxidative conditions [51]. Secondly, Prx 3 is a typical 2-Cys Peroxiredoxin located exclusively in the mitochondrial matrix; it is the principal peroxidase responsible for protecting cells from oxidative damage by reducing peroxides such as H_2O_2 [52]. Finally, mitochondria contain 30 times more Prx 3 than glutathione

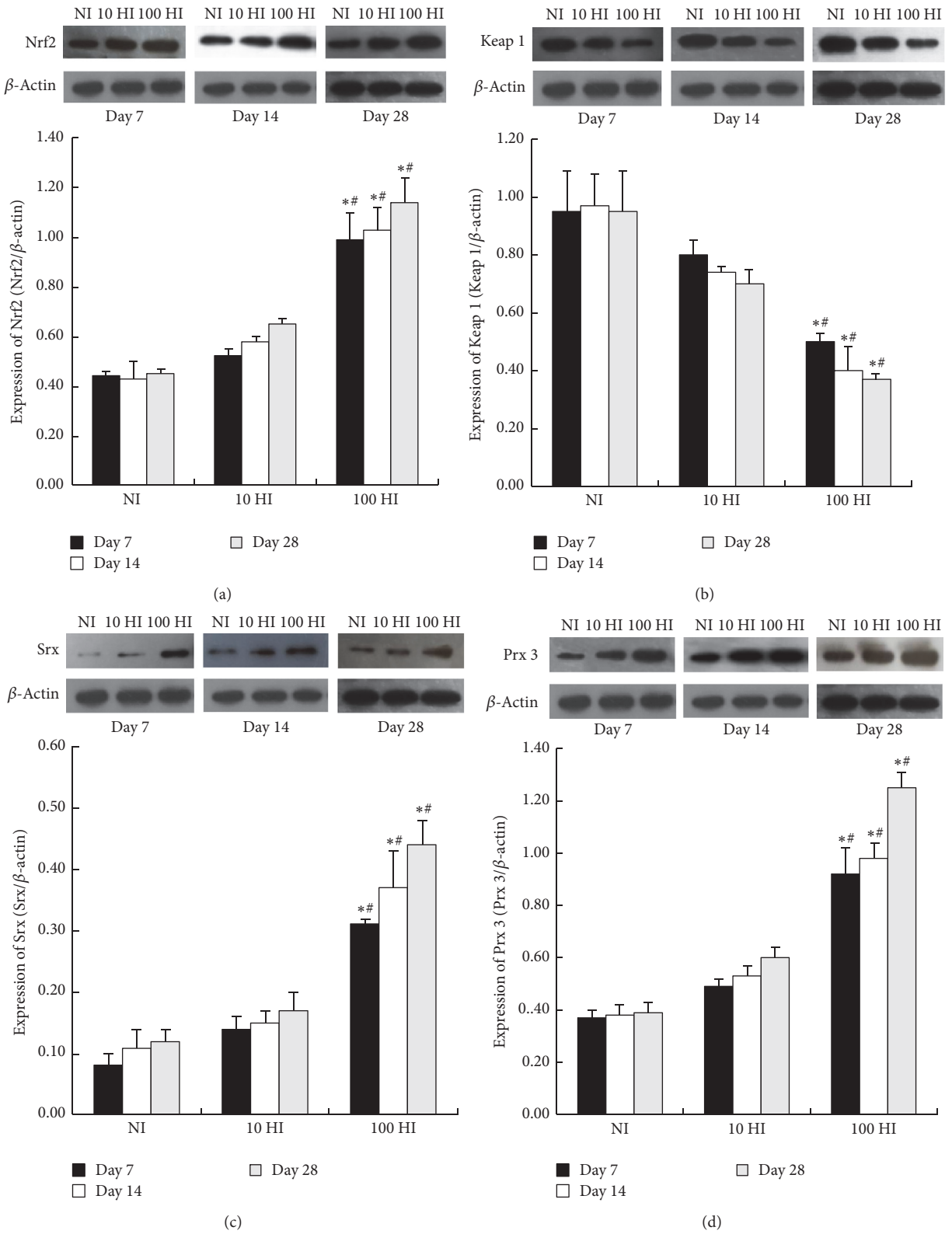


Figure 3: Effects of the iodide intake (NI, 10 HI, and 100 HI) on the changes of (a) Nrf2; (b) Keap 1; (c) Srx; and (d) Prx 3 expressions on Days 7, 14, and 28. Representative western blot and histograms of densitometric analyses normalized for the relative β-actin content. All data is presented as the mean ± SD ($N = 6$ for each group). Statistical analyses were performed by one-way analysis of variance (ANOVA) with the Least Significant Difference (LSD) test. $^{*}p < 0.05$ versus the NI group on Days 7, 14, and 28, respectively. $^{\#}p < 0.05$ versus the 10 HI group on Days 7, 14, and 28, respectively. Experiments were repeated 3 times with similar results.

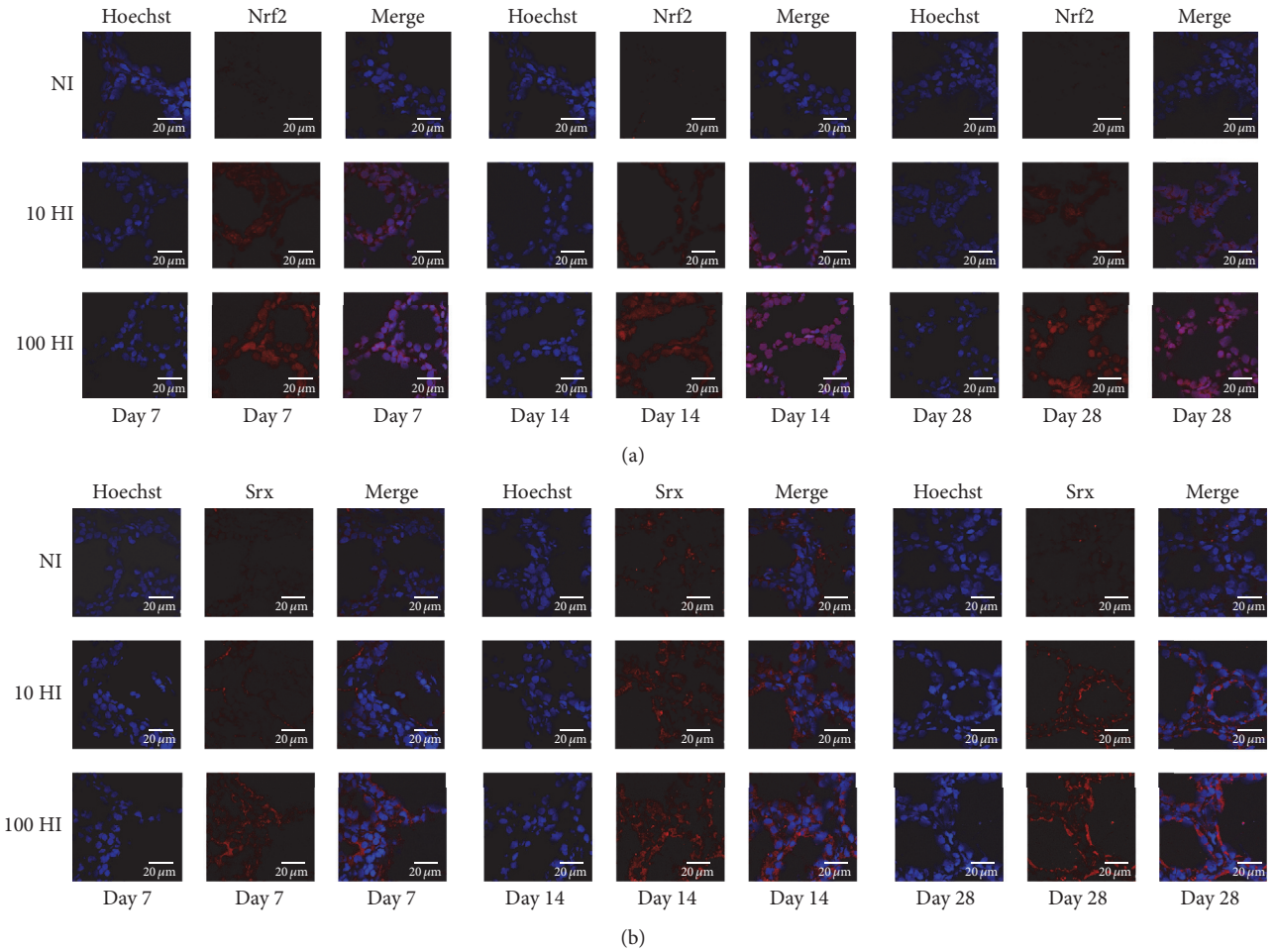


Figure 4: Effect of the iodide intake (NI, 10 HI, and 100 HI) on the changes of immunofluorescence staining on Days 7, 14, and 28. (a) In the NI group, Nrf2 (red) was localized in the cytoplasm on Days 7, 14, and 28. In the 10 HI group, the positive staining of Nrf2 can be observed in both the nucleus and the cytoplasm. Moreover, in the 100 HI group, a stronger positive staining of Nrf2 can be detected in the nucleus on Days 7, 14, and 28; the nucleus was dyed with Hoechst (blue). (b) Srx (red) positive staining was located in the cytoplasm; the nucleus was dyed with Hoechst (blue). Scale bar: 20 μm.

peroxidase; Prx 3 can be classified as an important regulator of mitochondrial H_2O_2 [22]. The elevated expression of Prx 3 is associated with the blockage of apoptosis, increasing cell proliferation, and is related to adaptive responses, which are all required to maintain mitochondrial function [53, 54]. Bae et al. have suggested that Prx 3 and Srx jointly protect mice from Pyrazole-induced oxidative liver injury in a Nrf2-dependent manner [43].

The novelty we demonstrated in the present study is that iodide excess induced both oxidative stress and antioxidative defense increases through Nrf2-Keap 1 pathway in the thyroid gland from rats. We extended our established mechanisms by applying the Nrf2-Keap 1 pathway to set up a bridge between oxidative stress and antioxidative defense induced by iodide excess in the thyroid gland. In our previous study, we have established that oxidative stress induced by acute high concentrations of iodide in FRTL cells significantly increases mitochondrial superoxide production [55]. The inhibitors of the mitochondrial respiratory chain complexes I and III are involved in mitochondrial superoxide production. We demonstrated that exposure to 100 μM KI for 2 hours significantly increased mitochondrial superoxide production, enhanced by either 0.5 μM Rotenone (an inhibitor of mitochondrial complex I) or 10 μM Antimycin A (an inhibitor of complex III) [56]. We illustrated that 300 μM PTU (an inhibitor of TPO) attenuated the excessive iodide-induced mitochondrial superoxide production. We showed that 30 μM $KClO_4$ (a competitive inhibitor of iodide transport) relieved the production the mitochondrial superoxide induced by iodide excess. We displayed that 10 mU/mL TSH can inhibit excessive iodide-induced strong mitochondrial superoxide production [55]. MT-I and MT-II are mainly involved in the protection of tissue against oxidative stresses; we indicated that metallothionein-I/II knockout mice aggravated mitochondrial superoxide production in thyroid after excessive iodide exposure [24]. In addition, we demonstrated that both the oxidative stress and the antioxidative defense increased simultaneously after high dosages of iodide intake.

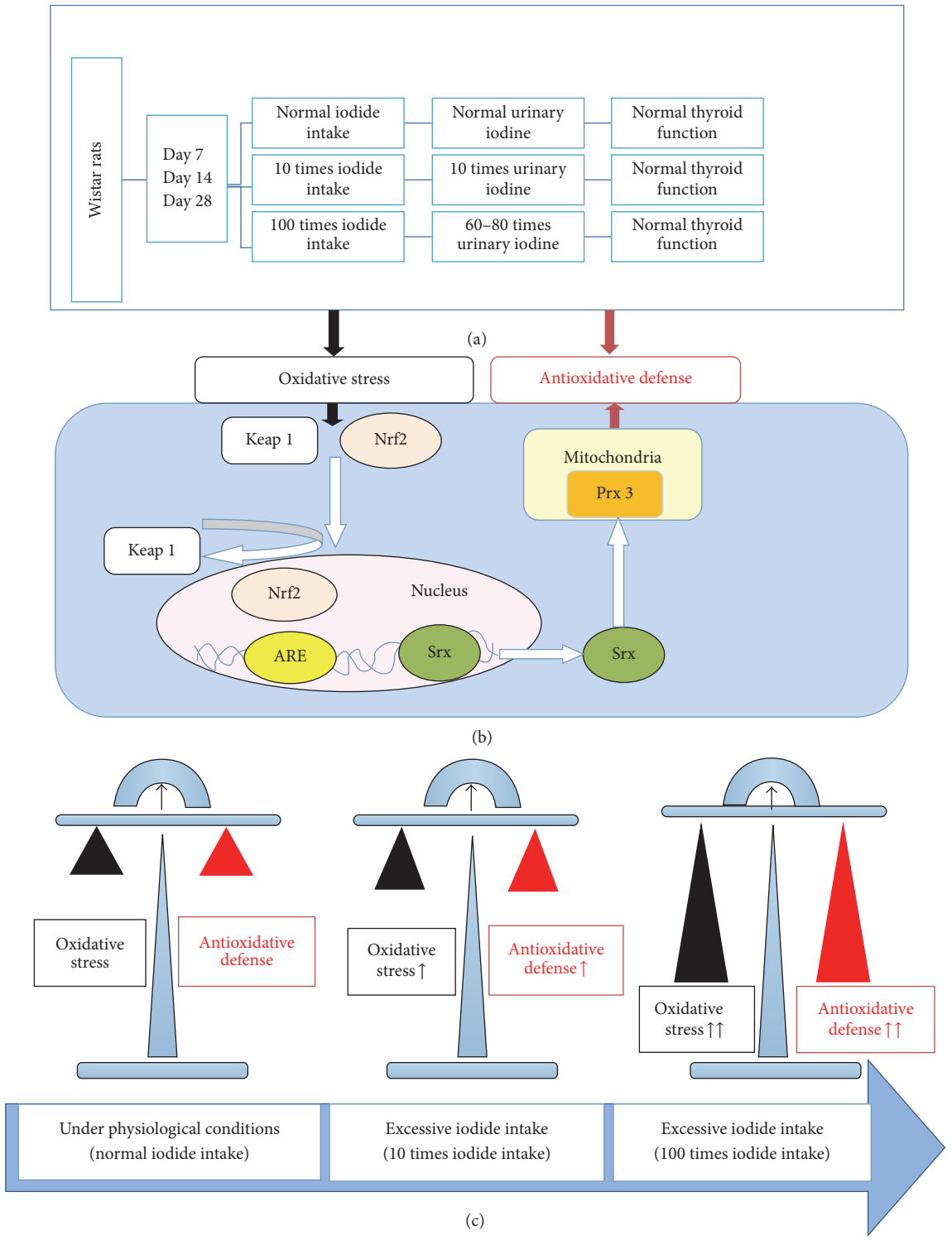


Figure 5: Proposed mechanisms in the present study. (a) The urinary iodine concentration and thyroid function of Wistar rats were detected. (b) The activation of the Nrf2-Keap 1 pathway induced by iodide excess in the thyroid. (c) The balance between oxidative stress and antioxidative defense under physiological conditions and excessive iodide intake.

We suggested that the Nrf2-Keap 1 pathway is vital for the balance between oxidative stress and antioxidative defense induced by iodide excess in the thyroid gland.

Excessive iodide stimulated the Nrf2-Keap 1 pathway and enhanced the antioxidative defense. We found that the urinary iodine concentration of the 100 HI group was about 60–80 times that of the NI group on Days 7, 14, and 28; however the thyroid functions were normal. We proposed that the excessive iodide accumulated in the body may trigger the Nrf2-Keap 1 pathway to maintain a normal thyroid function. We demonstrated that the Nrf2 moves from the cytoplasm to the nucleus under the microscope, with significantly increased expressions of Nrf2, Srx, and Prx 3 and notably decreased Keap 1 when exposed to high iodide. This suggests that excessive iodide stimulates the disassociation of Nrf2 from Keap 1 and assists Nrf2 to penetrate the nucleus. Then, Nrf2 attaches to the antioxidant response element (ARE) to activate the expression of the antioxidative genes, Srx and Prx 3, resulting in an enhanced antioxidative defense induced by high iodide. By activation of the Nrf2-Keap 1 pathway, there is a proportional increase in oxidative stress and antioxidative defense in response to iodide excess. It is to be noted that the thyroid function was normal in the 10 HI group and 100 HI in present study. Inspired by the report by Poncin et al. [6], we proposed that there should be a balance between oxidative stress and antioxidative defense in response to iodide excess in the 10 HI and the 100 HI groups.

5. Conclusion

In conclusion, our results highlight that the activation of Nrf2-Keap 1, Srx, and Prx 3 antioxidative defense mechanisms may play a crucial role in protecting the thyroid from iodide excess induced oxidative stress on Days 7, 14, and 28 (Figure 5).

Abbreviations

NI: Normal iodide intake
HI: High iodide intake
ROS: Reactive oxygen species
H_2O_2: Hydrogen peroxide
Nrf2: Nuclear factor erythroid 2-related factor 2
Keap 1: Kelch-like ECH-associated protein 1
ARE: Antioxidant response element
Prx: Peroxiredoxin
Srx: Sulfiredoxin
Prx 3: Peroxiredoxin 3
TT4: Total thyroxine
TT3: Total triiodothyronine
FT4: Free thyroxine
FT3: Free triiodothyronine
TSH: Thyrotropin
LSD: Least significant difference.

Disclosure

This manuscript has been edited by Umar Iqbal, Iruni Roshanie Abeysekera, Gargi Naha, and Tejas Bharadwaj, all of whom are currently studying at the International School of Medicine, Tianjin Medical University, China.

Competing Interests

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

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Research Article

Gamma-Glutamylcysteine Ethyl Ester Protects against Cyclophosphamide-Induced Liver Injury and Hematologic Alterations via Upregulation of PPARγ and Attenuation of Oxidative Stress, Inflammation, and Apoptosis

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Gamma-glutamylcysteine ethyl ester (GCEE) is a precursor of glutathione (GSH) with promising hepatoprotective effects. This investigation aimed to evaluate the hepatoprotective effects of GCEE against cyclophosphamide- (CP-) induced toxicity, pointing to the possible role of peroxisome proliferator activated receptor gamma (PPARγ). Wistar rats were given GCEE two weeks prior to CP. Five days after CP administration, animals were sacrificed and samples were collected. Pretreatment with GCEE significantly alleviated CP-induced liver injury by reducing serum aminotransferases, increasing albumin, and preventing histopathological and hematological alterations. GCEE suppressed lipid peroxidation and nitric oxide production and restored GSH and enzymatic antioxidants in the liver, which were associated with downregulation of COX-2, iNOS, and NF-κB. In addition, CP administration significantly increased serum proinflammatory cytokines and the expression of liver caspase-3 and BAX, an effect that was reversed by GCEE. CP-induced rats showed significant downregulation of PPARγ which was markedly upregulated by GCEE treatment. These data demonstrated that pretreatment with GCEE protected against CP-induced hepatotoxicity, possibly by activating PPARγ, preventing GSH depletion, and attenuating oxidative stress, inflammation, and apoptosis. Our findings point to the role of PPARγ and suggest that GCEE might be a promising agent for the prevention of CP-induced liver injury.

1. Introduction

Drug-induced liver injury (DILI) refers to abnormalities in liver function tests related to the intake of medicinal compounds [1]. DILI has been the single most frequent reason for drug withdrawal from the market [2, 3]. The potential of a drug to cause hepatotoxicity is often realized after release onto the market [2] and it has been estimated that more than a thousand drugs have been associated with liver injury and hepatotoxicity [4, 5]. Cyclophosphamide (CP) is an alkylating agent commonly used in the treatment of different cancers [6]. The therapeutic applications of CP have been associated with different side effects and organ toxicity [7, 8]. CP cytotoxicity has been attributed to the toxic metabolites, acrolein, and phosphoramide produced during its metabolism [9]. Acrolein can bind to reduced glutathione (GSH) leading to increased production of reactive oxygen species (ROS) and subsequently oxidative stress and lipid peroxidation [10, 11]. Therefore, agents with free radical scavenging and antioxidant properties can offer protection against CP-induced oxidative stress and hepatotoxicity.

Peroxisome proliferator activated receptor gamma (PPARγ) is a ligand-inducible transcription factor known to have roles in normal cell function [12]. When activated, PPARγ heterodimerizes with retinoid X receptor (RXR), binds to specific response elements (PPREs), and promotes

the expression of target genes [13]. PPARγ is induced during preadipocytes differentiation and plays a central role in lipid metabolism, glucose homeostasis, inflammation, and cell proliferation [14]. In the liver, disruption of PPARs has been associated with different disorders [15]. On the other hand, activation of PPARγ inhibited the fibrogenic response to liver injury [16] and protected against drug-induced hepatotoxicity as we recently reported [3, 17, 18].

Attenuation of oxidative stress through restoring GSH levels is a well-known strategy to combat drug-induced toxicity. For example, administration of N-acetylcysteine (NAC), a precursor of GSH, protected the liver against carbon tetrachloride [19] and methotrexate-induced toxicity [20]. Gamma-glutamylcysteine ethyl ester (GCEE), a synthetic GSH precursor, has been demonstrated to boost endogenous GSH levels and block oxidative stress in neurons [21, 22] as well as cerebral endothelial cells [23]. We believe that nothing has yet been reported on the possible protective effects of GCEE against CP-induced hepatotoxicity. In the present study, we asked whether GCEE can attenuate CP-induced oxidative stress, apoptosis, and inflammation in the liver of rats, pointing to the role of PPARγ.

2. Materials and Methods

2.1. Chemicals. Gamma-glutamyl cysteine ethyl ester (GCEE) and cyclophosphamide (CP; Endoxan) were purchased from Bachem (Torrance, CA, USA) and Baxter Oncology (Dusseldorf, Germany), respectively. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and albumin assay kits were supplied by Spinreact (Spain). PPARγ, nuclear factor-κB (NF-κB), and Bcl-2-associated X protein (BAX) antibodies were obtained from Santa Cruz Biotechnology (USA). Cytokines assay kits were purchased from R&D Systems (USA). All other chemicals were obtained from Sigma (USA) and other standard commercial supplies.

2.2. Experimental Animals and Treatments. Male albino Wistar rats (10 weeks old) from the Institute of Ophthalmology (Giza, Egypt) were included in the present study. They were maintained on a 12 h dark/light cycle at 22 ± 2°C with ad libitum access to standard laboratory diet and water. All animal procedures related to care, treatments, and sampling were in accordance with the guidelines of the Institutional Animal Ethics Committee of Beni-Suef University (Egypt).

Twenty-four rats were divided randomly into three groups of 8 rats each and allowed to adapt for 1 week prior to the experiment. Group I (Control) received normal saline solution for 16 days, Group II (CP) received saline for 15 days and 150 mg/kg b.wt. CP on day 16 [18], and Group III (GCEE + CP) received 100 mg/kg b.wt. GCEE for 15 days and 150 mg/kg b.wt. CP on day 16.

The dose, route, and day of CP administration were selected based on our previous studies [18, 24]. Since GCEE has been proven to be effective in vivo at doses of 10 mg/kg [25] and 150 mg/kg b.wt. [21], we selected a dose of 100 mg/kg to be tested in our study. All experimental solutions were administered intraperitoneally.

At day 21, the animals were sacrificed by cervical dislocation and various samples were collected. Blood samples were either collected on heparinized tubes for hematological analysis or left to coagulate for serum separation. Livers were immediately excised, washed in cold phosphate buffered saline (PBS), and weighed. Samples from the liver were fixed in 10% neutral buffered formalin for histological and immunohistochemical processing. Other samples were homogenized (10% w/v) in cold PBS for biochemical assays or kept frozen at −80°C for gene and protein expression analysis.

2.3. Biochemical Assays

2.3.1. Determination of Liver Function Markers. Serum aminotransferases were assayed using Spinreact (Spain) reagent kits according to the method of Schumann and Klauke [26]. Serum ALP activity and albumin concentration were measured using Spinreact (Spain) reagent kit according to the methods of Wenger et al. [27] and Webster [28], respectively.

2.3.2. Determination of Oxidative Stress and Antioxidant Defenses. Liver malondialdehyde (MDA) and GSH levels were determined according to the methods of Preuss et al. [29] and Beutler et al. [30], respectively. Liver nitric oxide (NO) was determined as nitrite using Griess reagent. Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) were determined according to the methods of S. Marklund and G. Marklund [31], Matkovics et al. [32], and Cohen et al. [33], respectively.

2.3.3. Determination of Proinflammatory Cytokines. Tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) were determined in serum samples using specific rats ELISA kits (R&D Systems, USA) according to the manufacturer's instructions.

2.3.4. Determination of Caspase-3 Activity. Liver caspase-3 activity was measured using the CaspACE assay system (Promega, Madison, WI, USA) following the manufacturer's instructions. The assay is based on the action of caspase-3 on the substrate Ac-DEVD-pNA releasing yellow chromophore p-nitroaniline. The activity of caspase-3 activity was presented as percentage of corresponding control.

2.4. Determination of Hematological Parameters. Samples of blood from all animals were collected into heparinized tubes and red blood corpuscles (RBCs), total white blood cells (WBCs), platelet count, and hemoglobin (Hb) content were determined using an automated hematoanalyzer.

2.5. Histopathology and Immunohistochemistry. Samples from the liver were immediately washed in cold PBS and fixed for histological processing and hematoxylin and eosin (H&E) staining.

Liver sections were immunohistochemically stained with anti-BAX antibody. Briefly, the slides were deparaffinized, rehydrated, and incubated in 3% hydrogen peroxide (H_2O_2) for 5 min. The slides were washed in Tris-buffered saline (pH 7.6), blocked with protein block (Novocastra), and incubated

Table 1: Primers used for qRT-PCR.

Gene	GenBank accession number	Sequence (5′-3′)
Pparg	NM_001145367	F: GGACGCTGAAGAAGAGACCTG
		R: CCGGGTCCTGTCTGAGTATG
Casp3	NM_012922	F: GGAGCTTGGAACGCGAAGAA
		R: ACACAAGCCCATTTCAGGGT
BAX	NM_017059	F: AGGACGCATCCACCAAGAAG
		R: CAGTTGAAGTTGCCGTCTGC
NF-κB	AF079314	F: TCTCAGCTGCGACCCCG
		R: TGGGCTGCTCAATGATCTCC
COX2	NM_017232	F: TGATCTACCCTCCCCACGTC
		R: ACACACTCTGTTGTGCTCCC
iNOS	U03699	F: ATTCCCAGCCCAACAACACA
		R: GCAGCTTGTCCAGGGATTCT
β-Actin	NM_031144	F: AGGAGTACGATGAGTCCGGC
		R: CGCAGCTCAGTAACAGTCCG

with rabbit polyclonal anti-BAX. The sections were incubated with the secondary antibody and then horseradish peroxidase conjugated with streptavidin. Sections were then washed, counterstained with hematoxylin, mounted in DPX, and examined by light microscopy.

2.6. Gene Expression Study. To study the effect of GCEE on the mRNA expression levels of caspase-3, BAX, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), NF-κB, and PPARγ in the liver of CP-induced rats, quantitative RT-PCR was used as we previously reported [3]. In brief, total RNA was isolated from liver tissue samples using Invitrogen (USA) TrIzol reagent. RNA was treated with RNase-free DNase, purified using RNeasy purification kit (Qiagen, Germany), and quantified at 260 nm. RNA integrity was further confirmed using formaldehyde-agarose gel electrophoresis. 2 μg RNA was reverse transcribed into first strand cDNA using AMV reverse transcriptase. DNA was amplified using SYBR Green master mix purchased from Fermentas. The primers used to specifically amplify caspase-3, BAX, COX-2, iNOS, NF-κB, PPARγ, and β-actin are listed in Table 1. The $2^{-\Delta\Delta Ct}$ method [34] was used to analyze the obtained amplification data and the results were normalized to β-actin.

2.7. Western Blot. Total liver tissue protein was extracted using RIPA buffer supplemented with proteinase inhibitors and Bradford reagent was used to determine protein concentration. Aliquots of the lysate containing 50 μg proteins were separated on SDS-PAGE, electrotransferred onto PVDF membranes followed by blocking. The membranes were probed with PPARγ, NF-κB p65, and β-actin primary antibodies, washed, and then incubated with the proper secondary antibodies. The blots were developed by enhanced chemiluminescence kit (BIO-RAD, USA). The intensity of obtained bands was quantified using ImageJ, normalized to β-actin, and presented as percent of control.

2.8. Statistical Analysis. Results were analyzed using GraphPad Prism 5 (La Jolla, CA, USA) and were expressed as means ± standard error of the mean (SEM). The statistical comparisons were made using one-way analysis of variance (ANOVA) followed by Tukey's test post hoc analysis to judge the difference between various groups. A P value < 0.05 was considered to be statistically significant.

3. Results

3.1. GCEE Protects against CP-Induced Liver Injury. To test the protective effect of GCEE on CP-induced hepatocellular injury, we assayed serum markers of liver function and performed histological examination.

Administration of CP induced hepatotoxicity evidenced by the significantly ($P < 0.001$) increased serum ALT (Figure 1(a)), AST (Figure 1(b)), and ALP (Figure 1(c)) activities when compared with the control group. Pretreatment of the CP-induced rats with GCEE produced significant ($P < 0.001$) reduction in serum aminotransferases and ALP activities. On the other hand, CP-administered rats showed a significant ($P < 0.01$) decline in serum albumin levels when compared with the corresponding control rats as depicted in Figure 1(d). Supplementation of GCEE prior to CP produced a significant ($P < 0.01$) amelioration of serum albumin levels in CP-intoxicated rats.

Microscopic examination of the liver sections stained with H&E revealed normal hepatic strands, hepatocytes, and sinusoids in control rats (Figure 2(a)). CP administration to rats produced several histological alterations in the liver sections such as activated Kupffer cells and hepatic vacuolation of fat type as most of vacuoles were with clear lumen and round borders, indicating hepatic steatosis (Figure 2(b)). In addition, CP induced periportal hepatic necrosis with mononuclear inflammatory cells infiltration, mainly macrophages and histiocytes (Figure 2(c)). Liver sections from GCEE pretreated rats showed noticeable amelioration of the liver histological architecture as depicted in Figure 2(d).

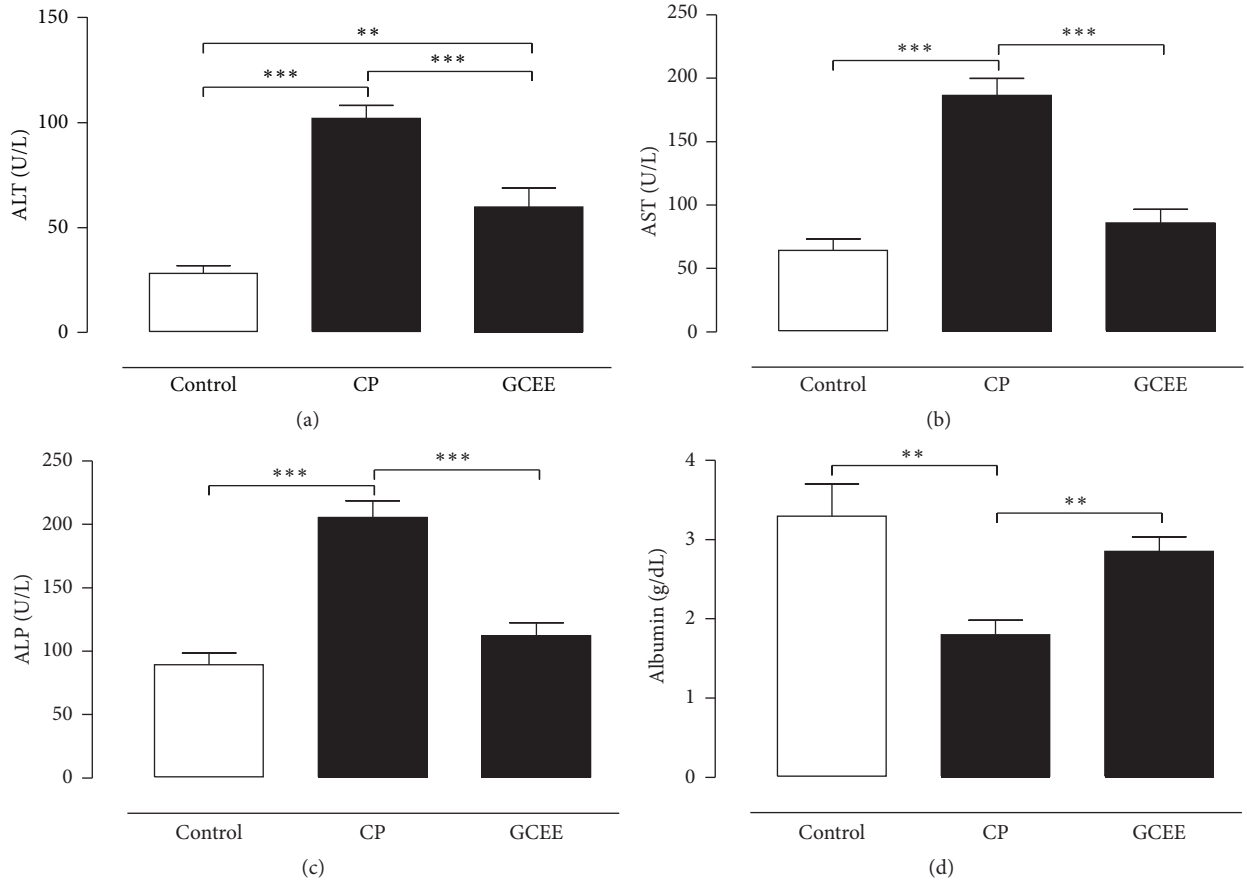

Figure 1: Effect of GCEE on serum (a) ALT, (b) AST, (c) ALP, and (d) albumin in CP-induced rats. Data are expressed as mean ± SEM ($N = 6$). $^{**}P < 0.01$ and $^{***}P < 0.001$. CP, cyclophosphamide; GCEE, gamma-glutamylcysteine ethyl ester; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

3.2. GCEE Mitigates CP-Induced Hematological Alterations in Rats. CP-induced rats showed significant ($P < 0.01$) decrease in RBCs number when compared with the control rats as represented in Figure 3(a). This effect was significantly ($P < 0.05$) reversed in CP-induced rats pretreated with GCEE. HB content as well was significantly ($P < 0.05$) declined in the blood of CP-induced rats (Figure 3(b)). Pretreatment of the rats with GCEE significantly ($P < 0.05$) prevented CP-induced Hb decline.

Concerning WBCs count, CP-induced rats showed significant ($P < 0.01$) leukopenia when compared with the control rats. Platelets exhibited nearly similar pattern where their number was significantly ($P < 0.001$) declined in the blood of CP-induced rats. Pretreatment of the CP-induced rats with GCEE significantly prevented leukopenia ($P < 0.05$) and thrombocytopenia ($P < 0.05$) as depicted in Figures 3(c) and 3(d), respectively.

3.3. GCEE Attenuates CP-Induced Oxidative Stress in the Liver of Rats. The protective effect of GCEE against CP-induced oxidative stress was determined through assessment of lipid peroxidation and NO as well as antioxidant defenses. Intraperitoneal administration of CP produced a significant ($P < 0.001$) increase in lipid peroxidation (Figure 4(a)) and NO (Figure 4(b)) in the liver of rats when compared with the control group. Pretreatment of the CP-induced rats with GCEE significantly ($P < 0.001$) decreased lipid peroxidation levels in the liver of rats. Similarly, GCEE pretreatment produced a significant ($P < 0.01$) decline in liver NO levels.

On the other hand, CP-induced rats showed a significant ($P < 0.05$) decline in liver GSH content when compared with the corresponding control rats (Figure 4(c)). GCEE administration prior to CP produced a significant ($P < 0.05$) improvement in liver GSH content. The enzymatic antioxidants exhibited a similar pattern where CP-induced rats exhibited significant decrease in the activity of liver SOD ($P < 0.01$; Figure 4(d)), GPx ($P < 0.05$ Figure 4(e)), and CAT ($P < 0.01$ Figure 4(f)) when compared with the control rats. GCEE administration produced significant amelioration in the activity of SOD ($P < 0.05$), GPx ($P < 0.01$), and CAT ($P < 0.05$) in the liver of CP-induced rats.

3.4. GCEE Reduces CP-Induced Inflammation in the Liver of Rats. Circulating levels of the proinflammatory cytokine TNF-α showed significant ($P < 0.001$) increase in CP-induced rats when compared with control rats (Figure 5(a)). Pretreatment of the CP-induced rats with GCEE for 15 days produced significant ($P < 0.001$) decrease in serum

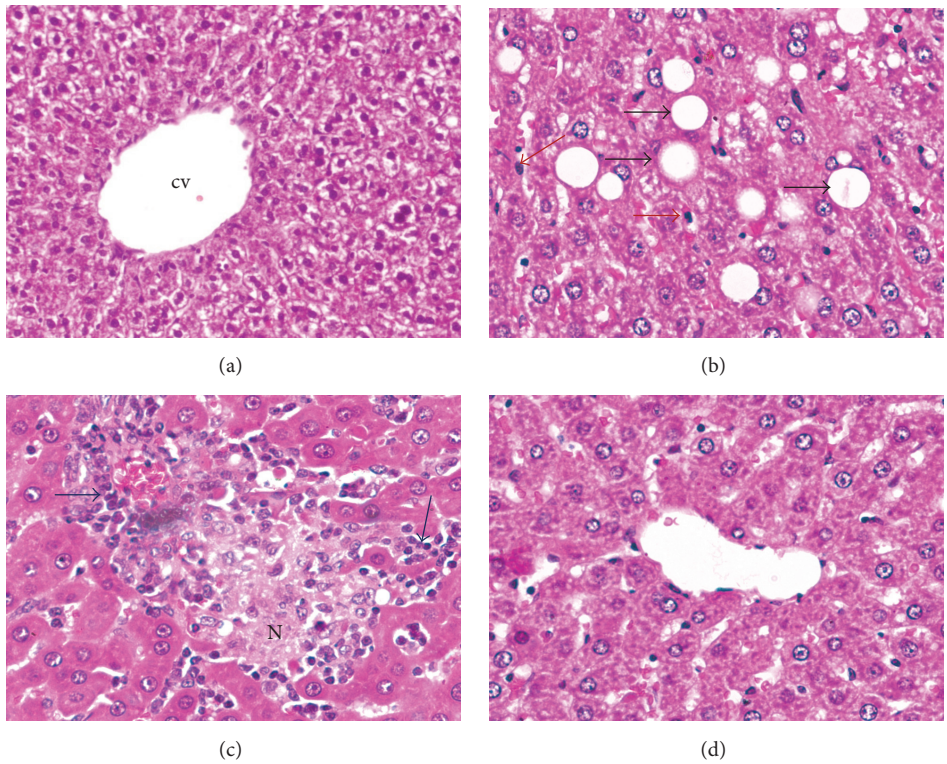

Figure 2: Photomicrographs of H&E-stained liver sections of (a) control rats, (b, c) CP-induced rats revealing activated Kupffer cells (red arrow), hepatic vacuolation of fat type (black arrow), and periportal hepatic necrosis associated with mononuclear inflammatory cells infiltration, mainly macrophages and histiocytes (blue arrow), and (d) CP-administered rats pretreated with GCEE showing noticeable amelioration of the liver histological architecture. CV, central vein; GCEE, gamma-glutamylcysteine ethyl ester.

TNF-α levels. IL-1β levels were significantly ($P < 0.001$) increased in serum of CP-induced rats when compared with the control group, an effect that was reversed by GCEE treatment (Figure 5(b)).

To further confirm the anti-inflammatory effect of GCEE, the expression of COX-2, iNOS, and NF-κB was assayed in the liver of CP-induced rats. COX-2 mRNA expression showed a significant ($P < 0.01$) upregulation in the liver of CP-induced rats when compared with the control rats (Figure 5(c)). Pretreatment of the CP-induced rats with GCEE significantly ($P < 0.01$) downregulated liver COX-2 mRNA expression.

iNOS mRNA expression revealed significant ($P < 0.01$) upregulation in the liver of CP-induced rats when compared with the control group as represented in Figure 5(d). GCEE produced a significant ($P < 0.01$) downregulation of iNOS mRNA expression in the liver of CP-induced rats.

Liver NF-κB expression showed a significant upregulation in CP-induced rats at both gene ($P < 0.01$; Figure 5(e)) and protein levels ($P < 0.001$; Figure 5(f)) when compared with the control rats. GCEE administered prior to CP significantly decreased NF-κB both mRNA ($P < 0.05$) and protein ($P < 0.01$) expression.

3.5. GCEE Prevents CP-Induced Apoptosis in the Liver of Rats. To study the effect of GCEE on CP-induced apoptosis, we determined both gene and protein expression levels of the proapoptotic factors caspase-3 and BAX. As represented in Figure 6(a), the liver of CP-induced rats showed a significant ($P < 0.01$) increase in mRNA abundance of caspase-3 when compared with the control rats. Caspase-3 protein levels showed a similar significant ($P < 0.01$) increase in liver of CP-induced rats. Pretreatment of the CP-induced rats with GCEE significantly decreased both caspase-3 mRNA expression ($P < 0.01$) and protein levels ($P < 0.05$).

Similarly, BAX mRNA expression levels showed significant ($P < 0.001$) increase in the liver of CP-induced rats when compared with the control group (Figure 6(c)). Pretreatment with GCEE produced a marked ($P < 0.01$) decrease in BAX mRNA expression levels in liver of the CP-induced rats. BAX protein expression levels, determined by immunohistochemistry, showed a significant ($P < 0.001$) increase in the liver of CP-induced rats when compared with the control rats (Figure 6(d)). GCEE administered prior to CP produced marked ($P < 0.001$) decrease in the expression of BAX protein in the liver of rats.

3.6. GCEE Upregulates PPARγ in the Liver of CP-Induced Rats. PPARγ mRNA abundance, determined by qRT-PCR, showed a significant ($P < 0.001$) decrease in the liver of CP-induced rats, as depicted in Figure 7(a). Conversely, GCEE supplementation produced a significant ($P < 0.01$) upregulation of PPARγ mRNA expression in the liver of CP-induced rats. PPARγ protein expression followed a similar pattern where

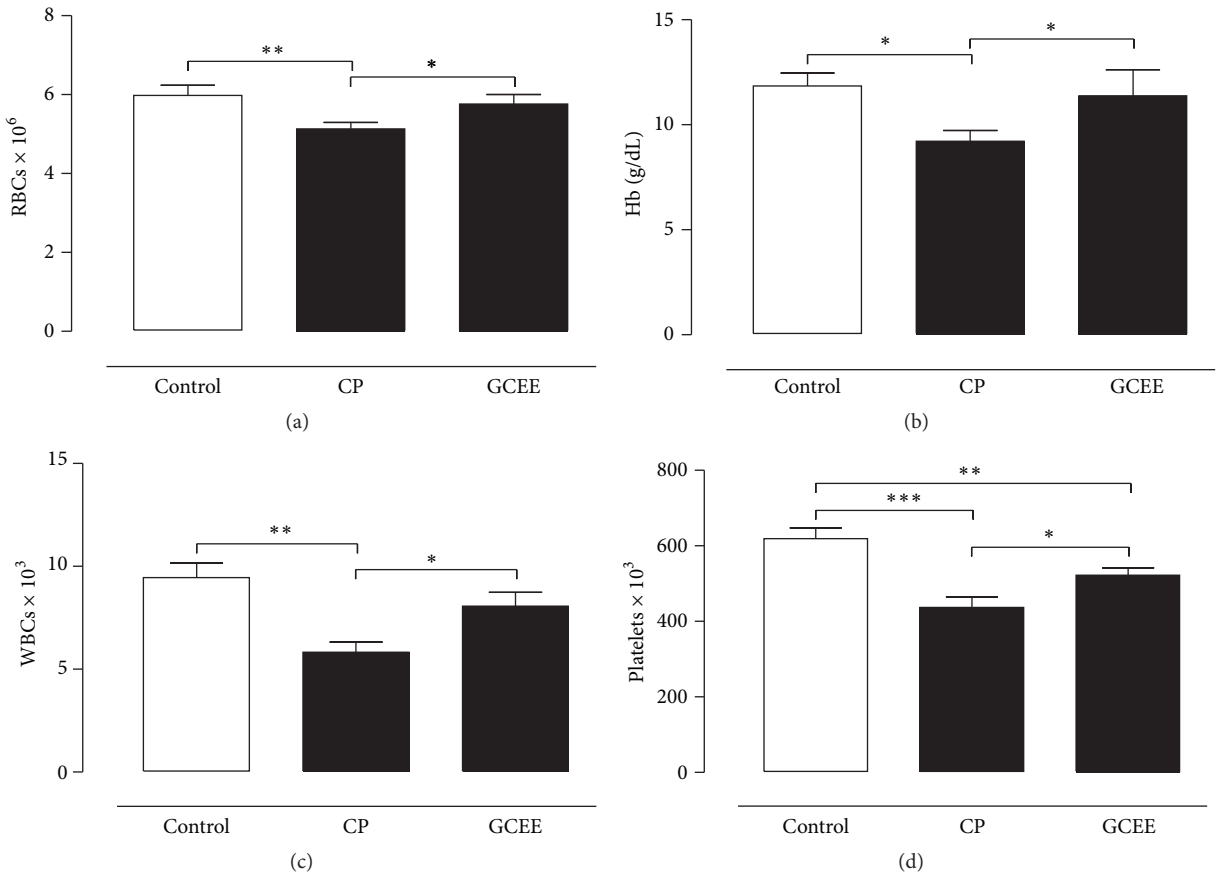

Figure 3: Effect of GCEE on hematopoietic parameters in CP-induced rats. Data are expressed as mean ± SEM ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. CP, cyclophosphamide; GCEE, gamma-glutamylcysteine ethyl ester; RBCs, erythrocytes; Hb, hemoglobin; WBCs, leukocytes.

it was significantly ($P < 0.001$) downregulated in the liver of CP-induced rats when compared with the control group (Figure 7(b)). CP-induced rats pretreated with GCEE exhibited marked ($P < 0.01$) upregulation of liver PPARγ protein expression.

4. Discussion

Gamma-glutamylcysteine is the limiting substrate in GSH synthesis and thus encourages product formation when present. In the present study, we showed for the first time that the GSH mimetic GCEE can protect against CP-induced hepatotoxicity. We assumed that this hepatoprotective activity of GCEE is mediated, at least in part, through its ability to upregulate PPARγ expression.

CP is an alkylating agent used for treatment of several types of cancer [6, 35]; however, its use has been limited due to severe toxicity [7, 8]. Our studies have demonstrated that hepatotoxicity is one of the major side effects of CP [3, 18, 24, 36]. Here, CP administration induced liver injury confirmed by increased circulating levels of liver function marker enzymes, declined serum albumin levels, and marked histopathological changes of liver structures. Accordingly, we have previously demonstrated increased serum ALT, AST, and ALP in CP-intoxicated rats [3, 18, 24, 36]. These enzymes are used as reliable markers for the assessment of liver function [37]. Elevated circulating levels of these enzymes indicate hepatocellular damage induced by CP as previously reported [3, 18, 38]. In addition, CP-induced rats showed leukopenia, anemia, and thrombocytopenia, indicating hematopoietic dysfunction due to CP-induced bone marrow toxicity [39, 40]. Similar findings have been reported in mice received CP at doses of 125 mg/kg [41].

Interestingly, GCEE supplementation significantly alleviated circulating levels of hepatic enzymes suggesting its membrane stabilizing potential. The hepatoprotective effect of GCEE against CP was further confirmed by the improved histological structures of the liver and increased serum levels of albumin. Rats treated with CP developed liver damage characterized histologically by activated Kupffer cells, hepatic vacuolation of fat type, periportal hepatic necrosis, and mononuclear cells infiltration, mainly macrophages and histiocytes. These findings were consistent with our previous study [18]. The decreased serum albumin in drug-induced hepatotoxicity could be attributed to the provoked inflammation and oxidative stress [42]. During inflammation, declined production of albumin has been linked to its function as a negative acute phase protein [43]. GCEE markedly prevented

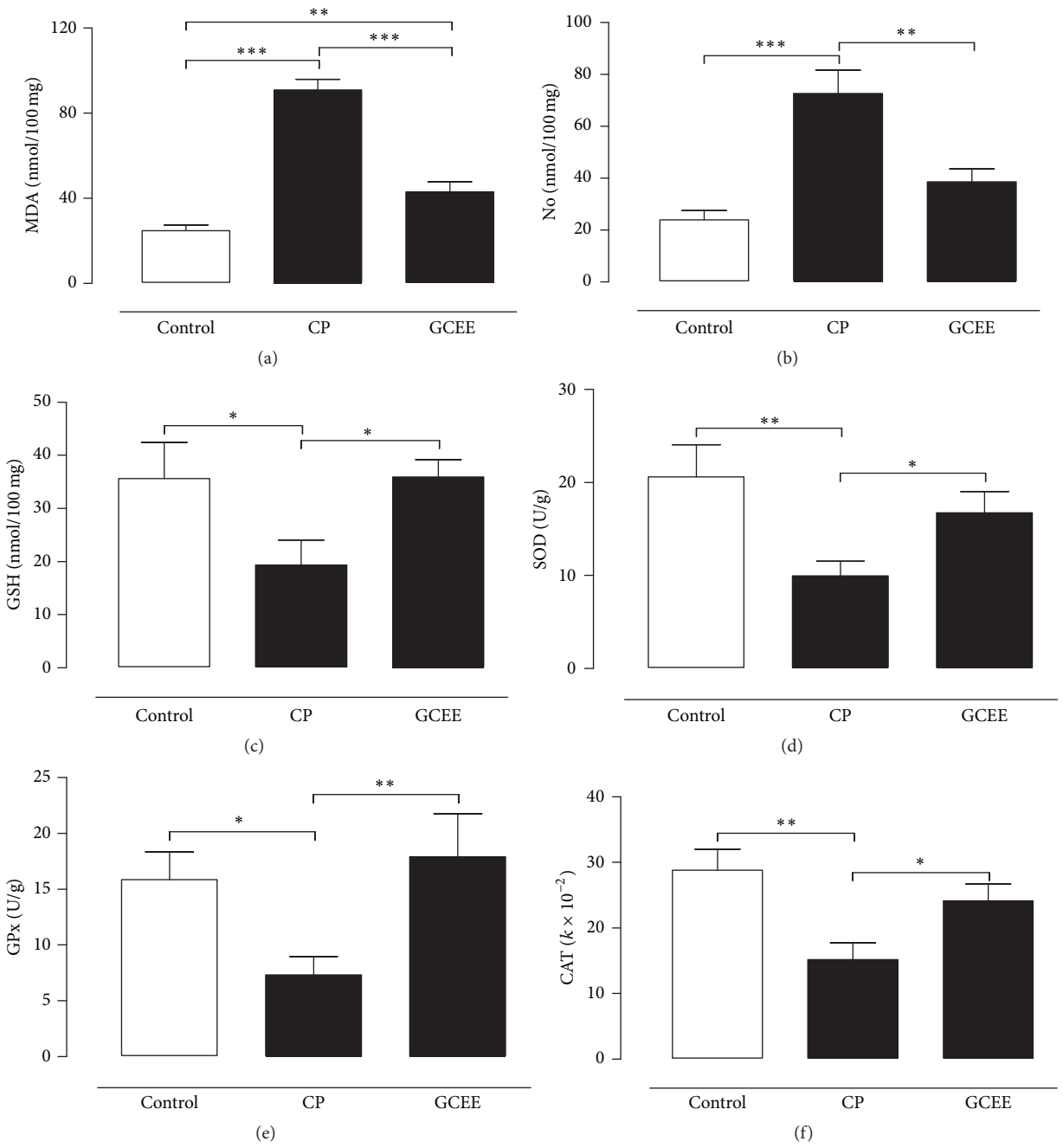

Figure 4: Effect of GCEE on (a) lipid peroxidation, (b) nitric oxide, (c) GSH, (d) SOD, (e) GPx, and (f) CAT in liver of CP-induced rats. Data are expressed as mean ± SEM ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. CP, cyclophosphamide; GCEE, gamma-glutamylcysteine ethyl ester; MDA, malondialdehyde; NO, nitric oxide; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

histological alterations and increased serum albumin levels, confirming its hepatoprotective activity. In addition, GCEE ameliorates the hematopoietic parameters and hence protects the bone marrow against CP-induced suppression.

Oxidative stress has been implicated in the hepatotoxic effect of CP [18, 24]. Therefore, finding a strategy to attenuate oxidative stress might grasp a key to alleviate the CP-induced hepatotoxicity. The present study showed increased levels of lipid peroxidation in the liver of CP-intoxicated rats. Excessive ROS production induced by CP can attack membrane lipids leading to lipid peroxidation [3, 7, 18, 24]. In addition, liver NO was significantly increased as a result of CP administration. NO has been reported to be involved in CP-induced hepatotoxicity [44]. It can combine with superoxide anions producing the versatile oxidant peroxynitrite ($ONOO^{-}$) [45]. $ONOO^{-}$ activates NF-κB in Kupffer cells and

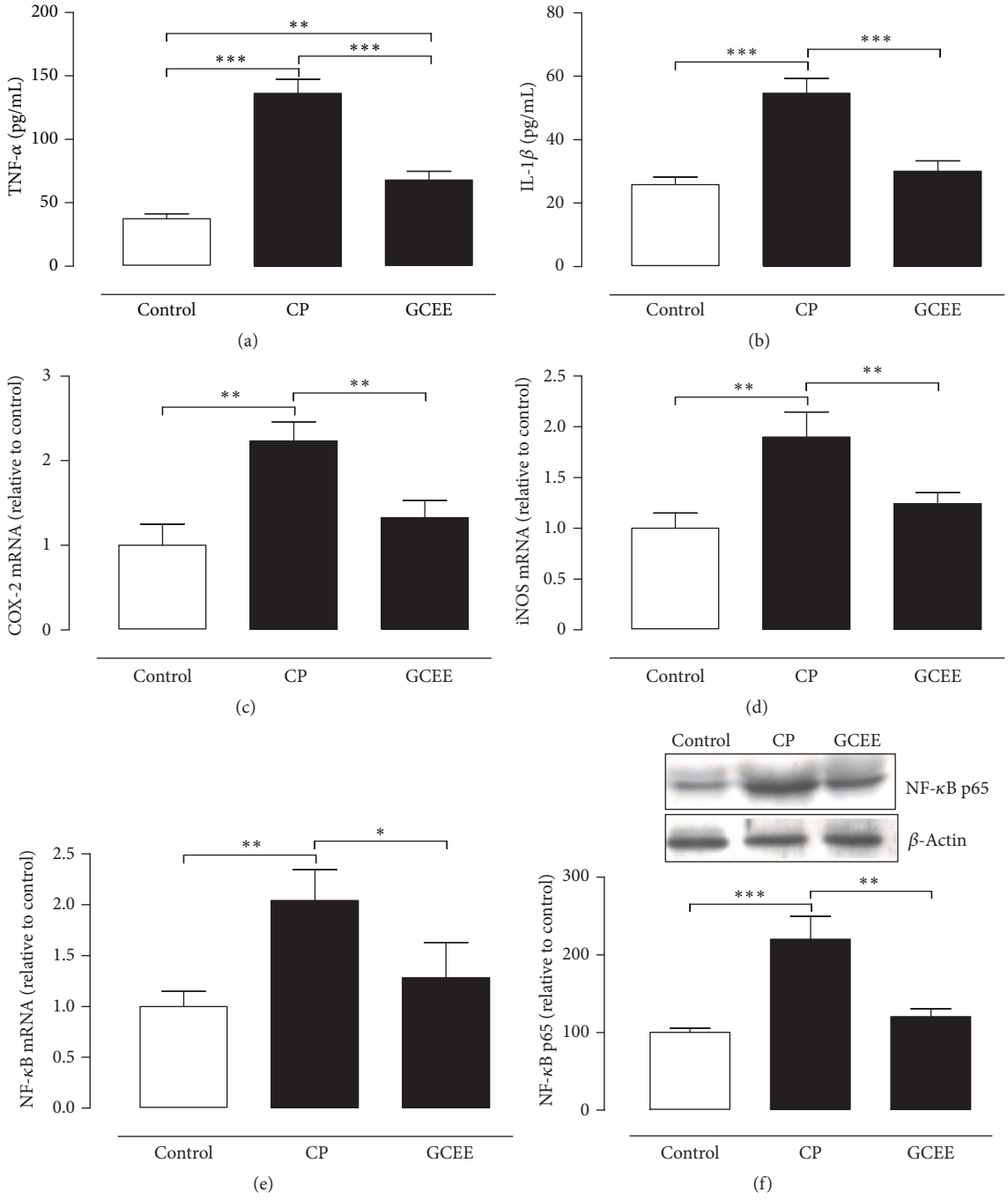

Figure 5: Effect of GCEE on serum TNF-α (a) and IL-1β (b), mRNA expression levels of liver COX-2 (c), iNOS (d), and NF-κB (e), and protein expression of liver NF-κB-p65 (f) in CP-induced rats. Data are expressed as mean ± SEM ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. CP, cyclophosphamide; GCEE, gamma-glutamylcysteine ethyl ester; TNFα, tumor necrosis factor alpha; IL-1β, interleukin-1beta; NF-κB, nuclear factor-kappaB; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

subsequently increased production of the proinflammatory cytokines [46]. The increased production of liver NO is a direct result of upregulated expression of iNOS as we previously reported in CP-induced rats [18]. Moreover, CP-induced rats exhibited declined liver GSH as well as activities of the antioxidant enzymes. GSH depletion is a result of its direct conjugation with CP metabolites [47], leading to declined cellular defenses and necrotic cell death [48].

GCEE prevented the CP-induced lipid peroxidation, NO production, depletion of GSH, and suppression of SOD, CAT, and GPx activities in the liver of rats. These findings indicate clearly that GCEE protected against CP-induced

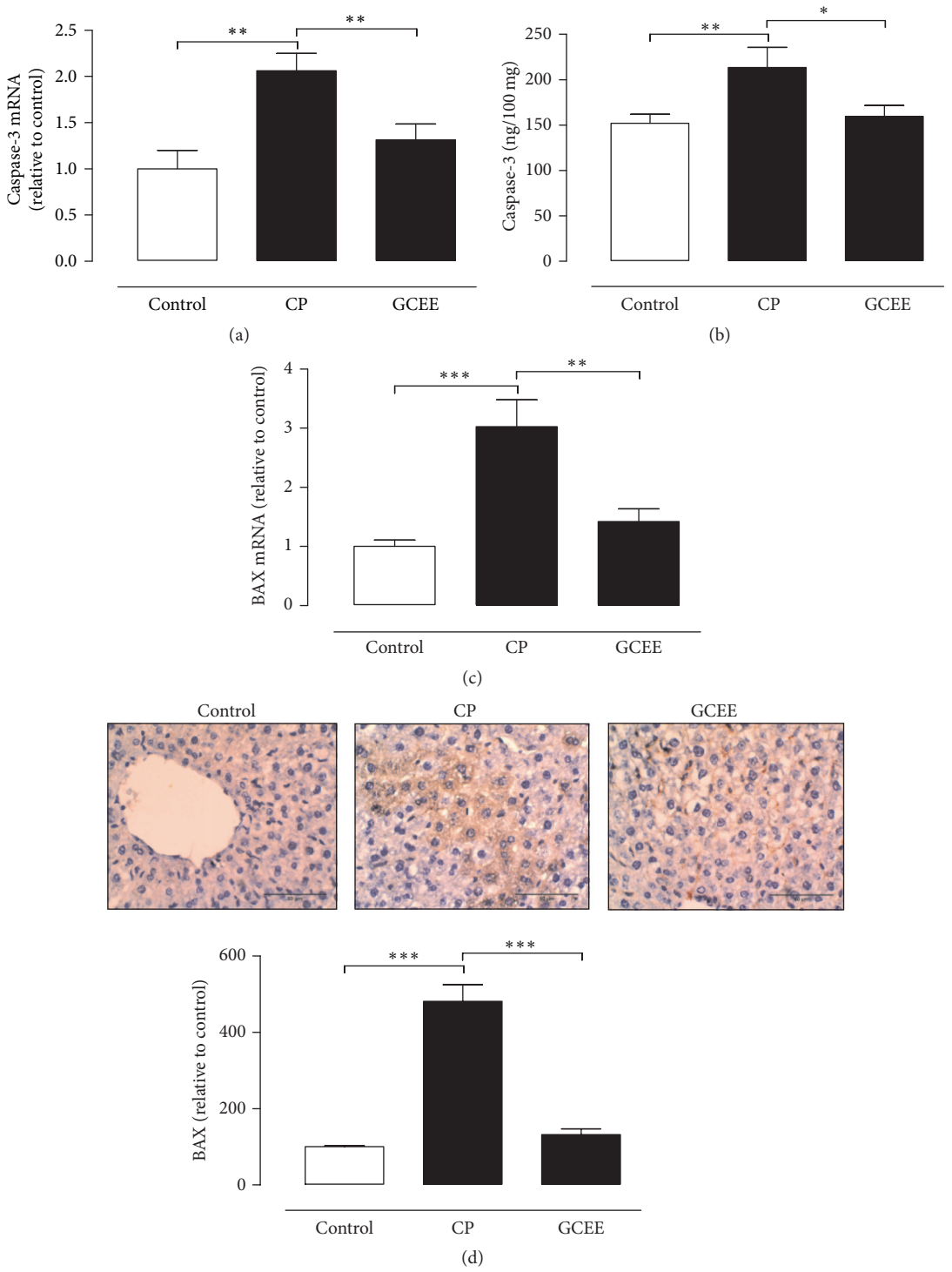

Figure 6: Effect of GCEE on (a) caspase-3 mRNA expression, (b) caspase-3 activity, (c) BAX mRNA expression, and (d) BAX immunohistochemical staining in liver of CP-induced rats. Data are expressed as mean ± SEM ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. CP, cyclophosphamide; GCEE, gamma-glutamylcysteine ethyl ester; BAX, BCL2-associated X protein.

oxidative stress through preventing GSH depletion and enhancing the enzymatic antioxidants. In the same context, Kobayashi et al. [49] reported that GCEE protects against ischemia/reperfusion-induced liver injury through preventing GSH depletion. More recently, the study of Salama et al. [50] showed similar findings in iron-overload rat model supplemented with glutamyl cysteine dipeptide.

In conjunction with oxidative stress, increased production of inflammatory cytokines has been reported in CP-administered rats. Previous studies from our laboratory

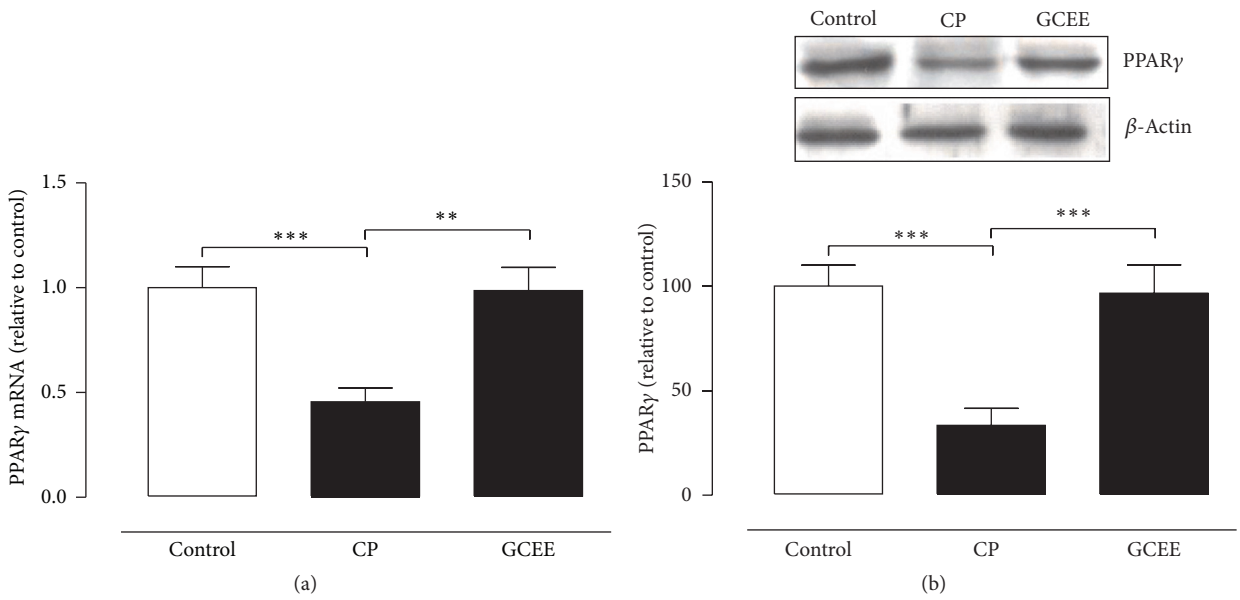

Figure 7: Effect of GCEE on PPARγ (a) mRNA and (b) protein expression in liver of CP-induced rats. Data are expressed as mean ± SEM ($N = 6$). $^{**}P < 0.01$ and $^{***}P < 0.001$. CP, cyclophosphamide; GCEE, gamma-glutamylcysteine ethyl ester; PPARγ, peroxisome proliferator activated receptor gamma.

showed increased production and/or expression of inflammatory cytokines following CP administration [3, 18, 24, 36]. Akcay et al. [51] revealed that DILI is associated with increased production of inflammatory mediators produced by injured or immune cells-induced infiltration of leukocytes into the site of injury. In addition, studies have demonstrated that ROS augment gene expression of inflammatory mediators and NF-κB [52, 53] and increase production of TNF-α from Kupffer cells [54]. Here, CP-induced rats showed significant increase in serum TNF-α and IL-1β and liver COX-2 and iNOS. This inflammatory response could be directly connected to the CP-induced upregulation of NF-κB expression. Similar findings were showed in our previous studies [3, 18, 24, 36]. Oral administration of GCEE potentially decreased serum proinflammatory cytokines and COX-2 and iNOS mRNA expression in the liver of CP-induced rats. This anti-inflammatory effect is a direct result of downregulated NF-κB expression and attenuated ROS production.

Oxidative stress together with inflammation induces apoptotic cell death in the liver [53]. Under cell stress conditions, hepatocytes become more susceptible to the lethal effects of TNFα and Fas ligand (FasL) which bind to intracellular death receptors and subsequently activate caspase-8 [55]. Within the mitochondria, drugs or their metabolites can cause ATP depletion, excessive ROS production, DNA damage, and increase permeability of the mitochondrial membrane. The resultant mitochondrial membrane permeabilization leads to the release of cytochrome C and activation of procaspase-9. These events activate executioner caspase-3 resulting in apoptotic cell death [56, 57]. Here, CP-induced rats showed significant increase in expression of the apoptotic markers caspase-3 and BAX. A recent study by Germoush [58] showed significant increase in liver BAX mRNA and protein expression in CP-induced rats. These findings might be explained in terms of the CP-induced inflammation and oxidative stress in the liver of rats. GCEE supplementation markedly prevented CP-induced apoptosis which is a direct result of its ability to attenuate inflammation and oxidative stress. In agreement with our findings, Salama et al. [50] reported decreased caspase-3 activity in liver of iron-overload rat model following treatment with glutamyl cysteine.

To further explore how GCEE prevented CP-induced oxidative stress, inflammation, and apoptosis, expression levels of PPARγ were determined. PPARγ is a nuclear receptor we hypothesized to have a role in mediating the protective effect of GCEE against CP-induced hepatotoxicity. Previous work from our laboratory showed declined PPARγ expression in the liver of CP-induced rats [3, 18]. Interestingly, we have found a marked upregulation of liver PPARγ expression in GCEE-treated rats.

PPARγ is emerging as an important regulator of the response to oxidative stress and inflammation. This notion has been supported by the findings of several studies using the PPARγ-specific agonists thiazolidinediones (TZDs). Together with other agonists, TZDs showed beneficial therapeutic effects in oxidative stress-related diseases [59, 60]. As an example, rosiglitazone induces the antioxidant enzyme heme oxygenase 1 (HO-1) in hepatocytes [61] and pioglitazone protects against CP-induced oxidative stress in rats [60]. In response to oxidative stress, activation of PPARγ has been reported to directly modulate the expression of several antioxidant genes. Human, mouse and rat CAT is transcriptionally regulated by PPARγ through PPREs containing the canonical direct repeat 1 [62] located 12kb far from the transcription initiation site [63]. Furthermore, PPARγ activation promotes the expression of GPx3 [64], manganese SOD

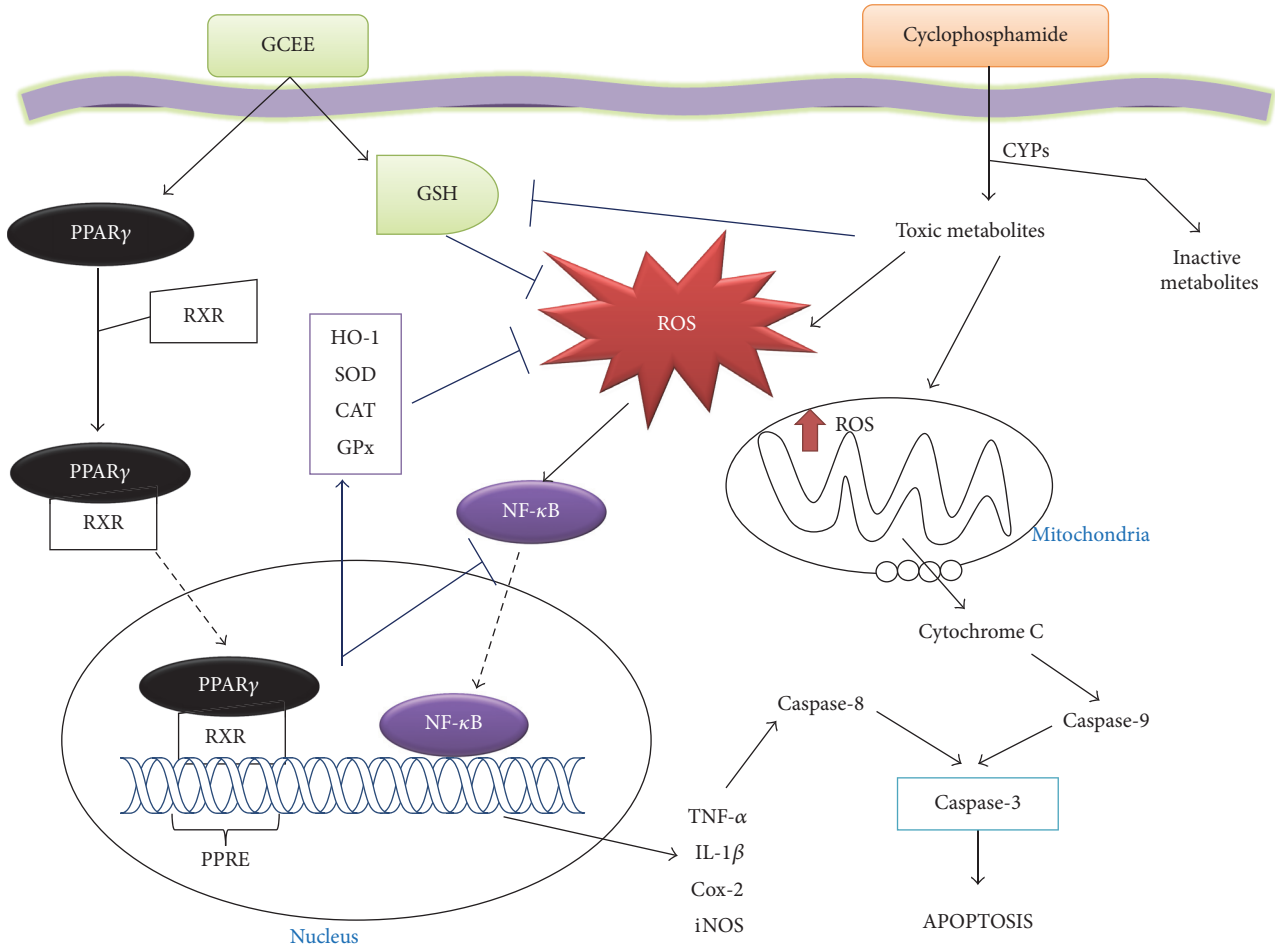

Figure 8: A proposed schematic diagram for the protective mechanisms of GCEE against CP-induced hepatotoxicity. PPARγ, peroxisome proliferator activated receptor gamma; RXR, retinoid X receptor; PPRE, PPAR response element; HO-1, heme oxygenase 1; SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; CYP, cytochrome P-450; GSH, glutathione; GCEE, gamma-glutamylcysteine ethyl ester; NF-κB, nuclear factor-kappaB; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

[65], the mitochondrial uncoupling protein 2 (UCP2) [66], and HO-1 [61].

PPARγ has also been shown to induce anti-inflammatory responses through inhibiting the activation of NF-κB resulting in attenuation of proinflammatory cytokines production [67]. PPARγ can transrepress NF-κB activation via direct binding or formation of a repressor complex in the promoter of its target genes [68, 69]. Studies have also showed that PPARγ downregulates COX-2 and iNOS [70].

Furthermore, new experimental evidences suggested the possible interaction and/or coactivation of PPARγ and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) can protect against CP-induced hepatotoxicity [18]. Upon activation, Nrf2 translocates into the nucleus and promotes expression of antioxidant and cytoprotective proteins [71]. In addition, Nrf2 pathway has been regarded to have a central role in the control of inflammation [72] and studies have shown several anti-inflammatory agents which upregulate Nrf2 pathway and suppress NF-κB [18, 73]. Recently, we have reported that simultaneous activation of PPARγ and Nrf2 in CP-induced rats significantly enhanced antioxidant defenses, downregulated NF-κB and iNOS, and prevented the production of proinflammatory cytokines [18]. Through preventing oxidative stress and inflammation, PPARγ is therefore able to protect against apoptosis. Our findings were supported by the studies of Fuenzalida et al. [74] and Ren et al. [75] who showed that PPARγ has a prosurvival action and protects glial cells and cardiomyocytes from oxidative stress-induced apoptosis. These antiapoptotic effects were mediated by induction of B-cell lymphoma 2 (Bcl-2) independently of the protein kinase B and mitogen-activated protein kinase pathways [74, 75].

In conclusion, our study shows, for the first time that GCEE, a GSH precursor, confers protection against CP-induced hepatotoxicity in rats. The hepatoprotective mechanisms of GCEE are associated with activation of PPARγ resulting in enhancement of antioxidant defenses, prevention of GSH depletion, and attenuation of excessive inflammatory response and apoptosis (summarized mechanistic pathways are represented in Figure 8). Therefore, GCEE has the potential to provide cellular protection against CP-induced hepatotoxicity.

Abbreviations

GCEE: Gamma-glutamylcysteine ethyl ester
PPARγ: Peroxisome proliferator activated receptor gamma
CP: Cyclophosphamide
Nrf2: Nuclear factor erythroid 2-related factor 2
iNOS: Inducible nitric oxide synthase
NF-κB: Nuclear factor-kappaB
HO-1: Heme oxygenase 1
ROS: Reactive oxygen species
RXR: Retinoid X receptor
ARE: Antioxidant response element
ALP: Alkaline phosphatase
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
MDA: Malondialdehyde
NO: Nitric oxide
GSH: Reduced glutathione
SOD: Superoxide dismutase
CAT: Catalase
GPx: Glutathione peroxidase
RBCs: Erythrocytes
Hb: Hemoglobin
WBCs: Leukocytes
BAX: BCL2-associated X protein
TNF-α: Tumor necrosis factor alpha
IL-1β: Interleukin-1beta
PBS: Phosphate buffered saline
qRT-PCR: Quantitative reverse transcription-polymerase chain reaction
ANOVA: One-way analysis of variance.

Disclosure

Both authors participated as first author.

Competing Interests

The authors have declared that no competing interests exist.

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