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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 $2^{-\Delta\Delta\mathrm{Ct}}$ 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).

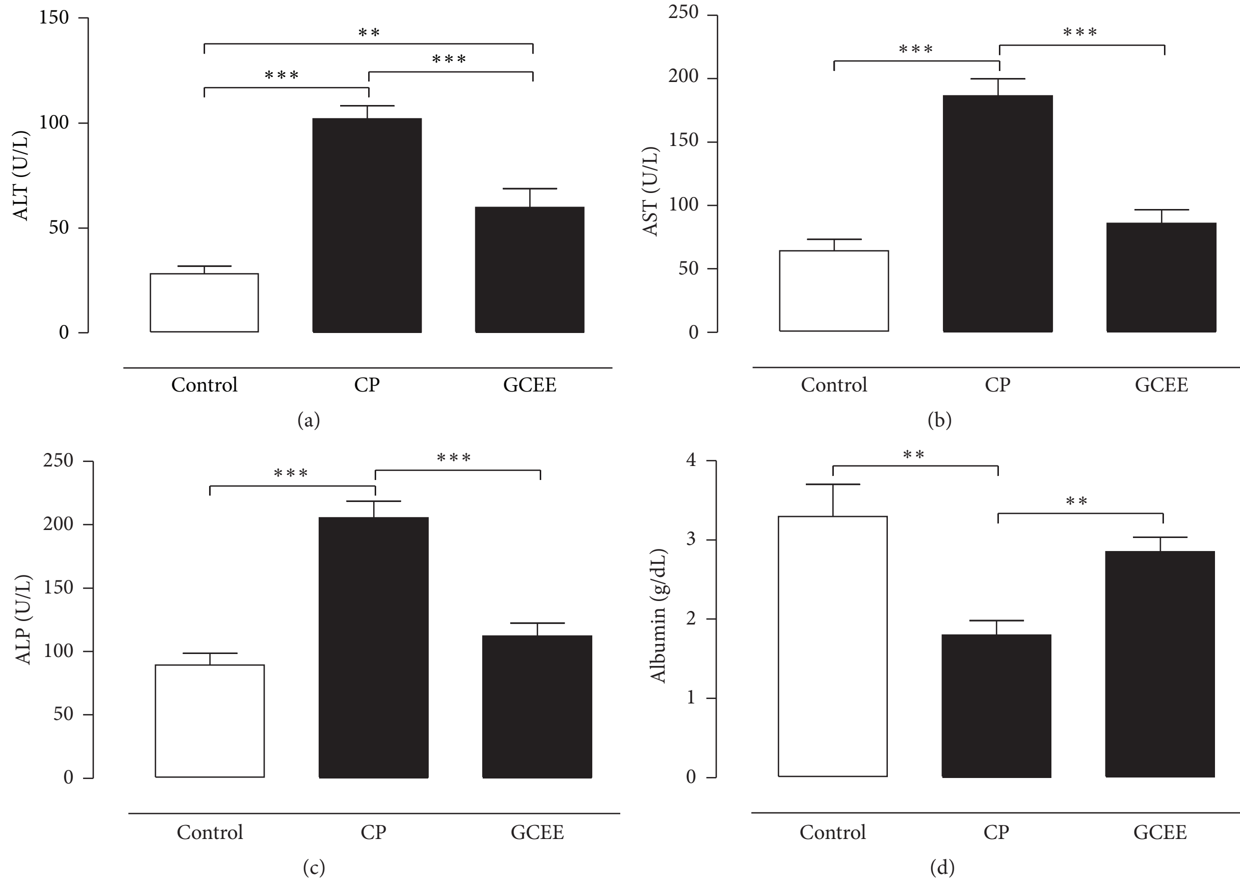

Figure 1: Effect of GCEE on serum (a) ALT, (b) AST, (c) ALP, and (d) albumin in CP-induced rats. Data are expressed as mean ± SEM ($N = 6$). $^{**}P < 0.01$ and $^{***}P < 0.001$. CP, cyclophosphamide; GCEE, gamma-glutamylcysteine ethyl ester; ALT, alanine aminotransferase; AST, aspartate aminotransferase;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

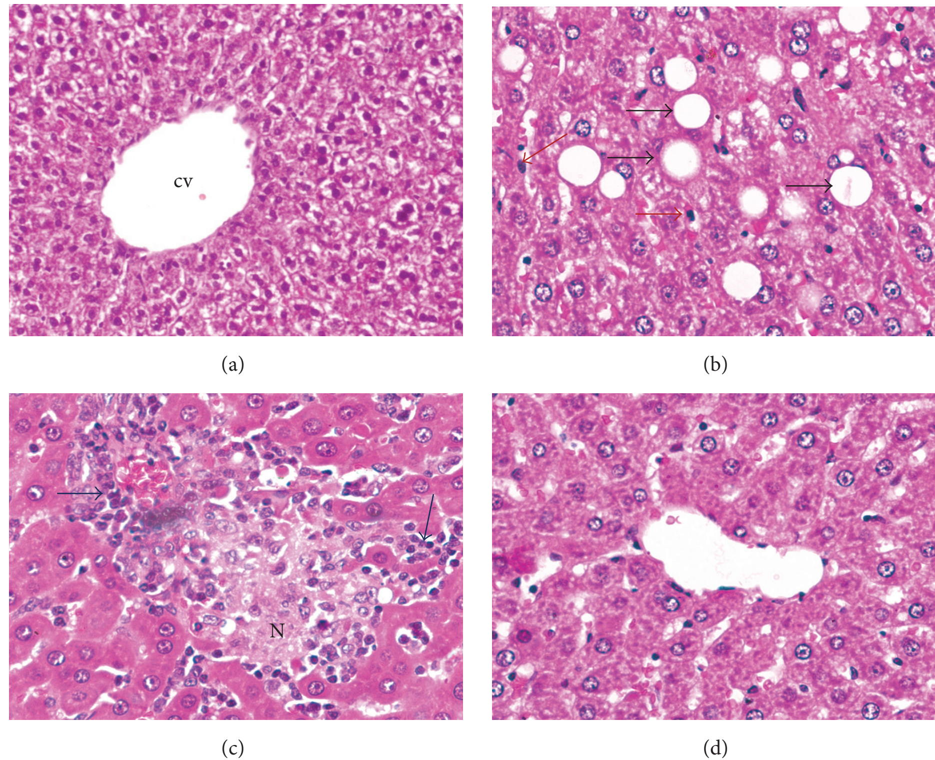

Figure 2: Photomicrographs of H&E-stained liver sections of (a) control rats, (b, c) CP-induced rats revealing activated Kupffer cells (red arrow), hepatic vacuolation of fat type (black arrow), and periportal hepatic necrosis associated with mononuclear inflammatory cells infiltration, mainly macrophages 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

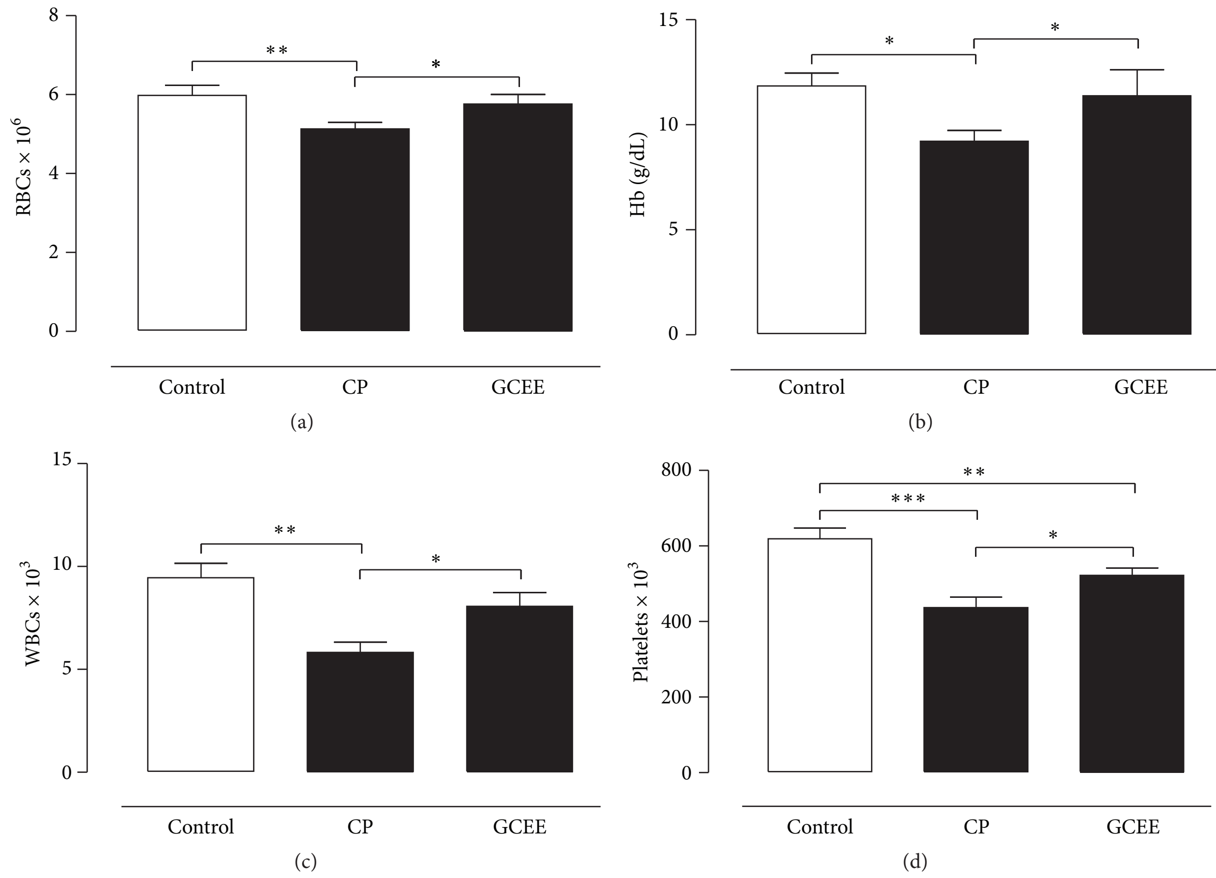

Figure 3: Effect of GCEE on hematopoietic parameters in CP-induced rats. Data are expressed as mean ± SEM ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. CP, cyclophosphamide; GCEE, gamma-glutamylcysteine ethyl ester; RBCs, erythrocytes; Hb, hemoglobin; WBCs, leukocytes.

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

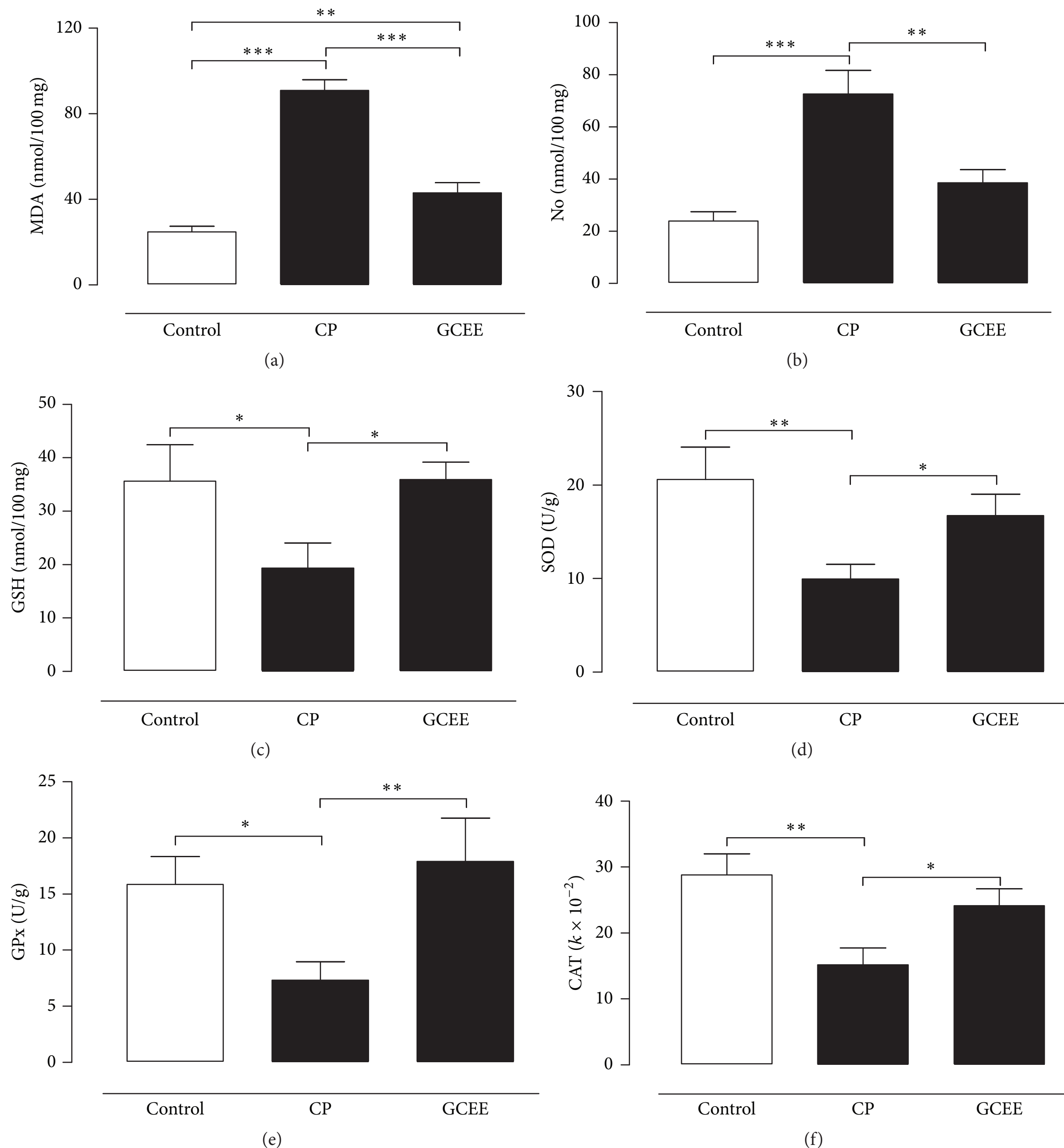

FIGURE 4: Effect of GCEE on (a) lipid peroxidation, (b) nitric oxide, (c) GSH, (d) SOD, (e) GPx, and (f) CAT in liver of CP-induced rats. Data are expressed as mean ± SEM ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. CP, cyclophosphamide; GCEE, gamma-glutamylcysteine ethyl ester; 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

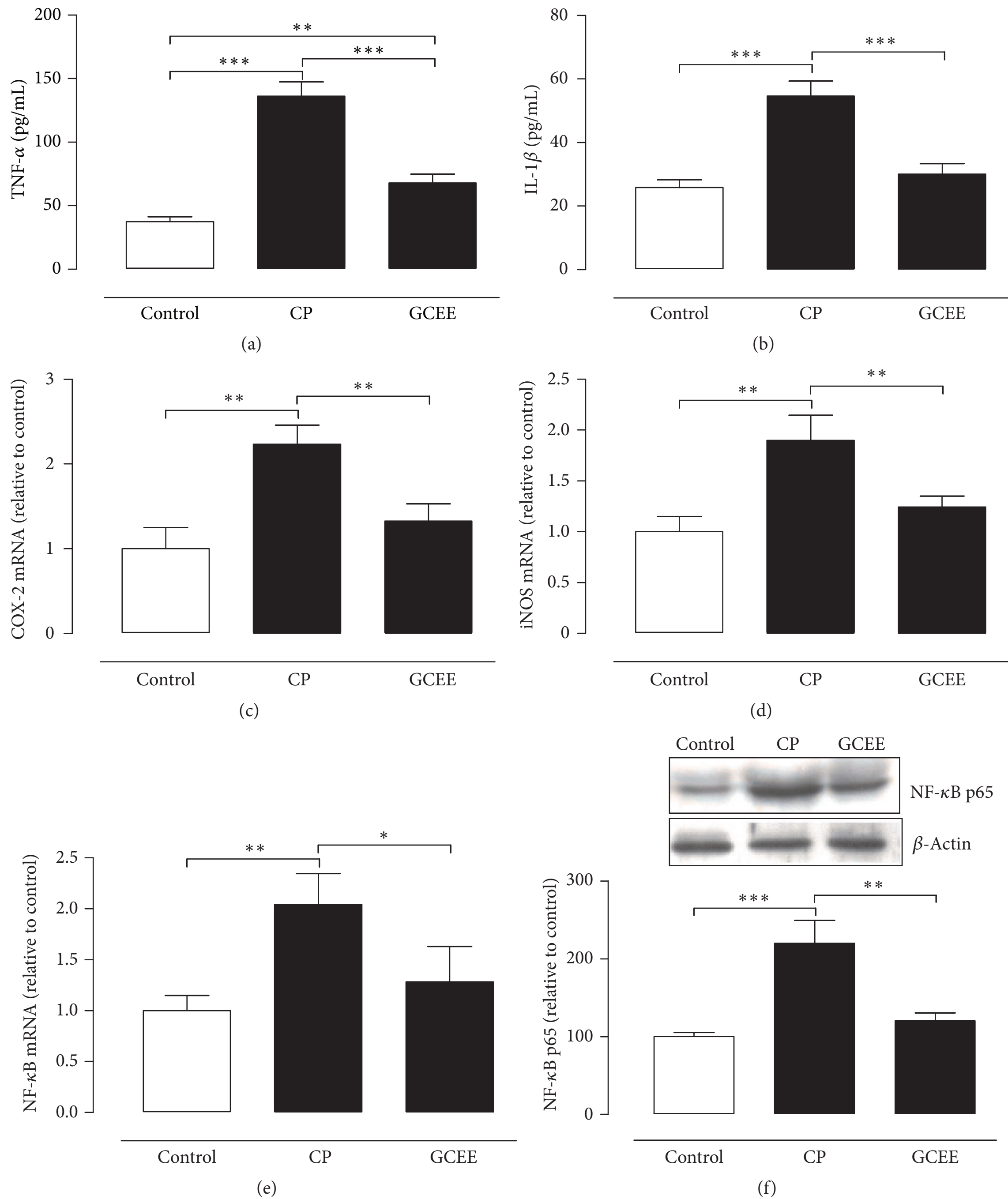

Figure 5: Effect of GCEE on serum TNF-α (a) and IL-1β (b), mRNA expression levels of liver COX-2 (c), iNOS (d), and NF-κB (e), and protein expression of liver NF-κB-p65 (f) in CP-induced rats. Data are expressed as mean ± SEM ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. CP, cyclophosphamide; 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

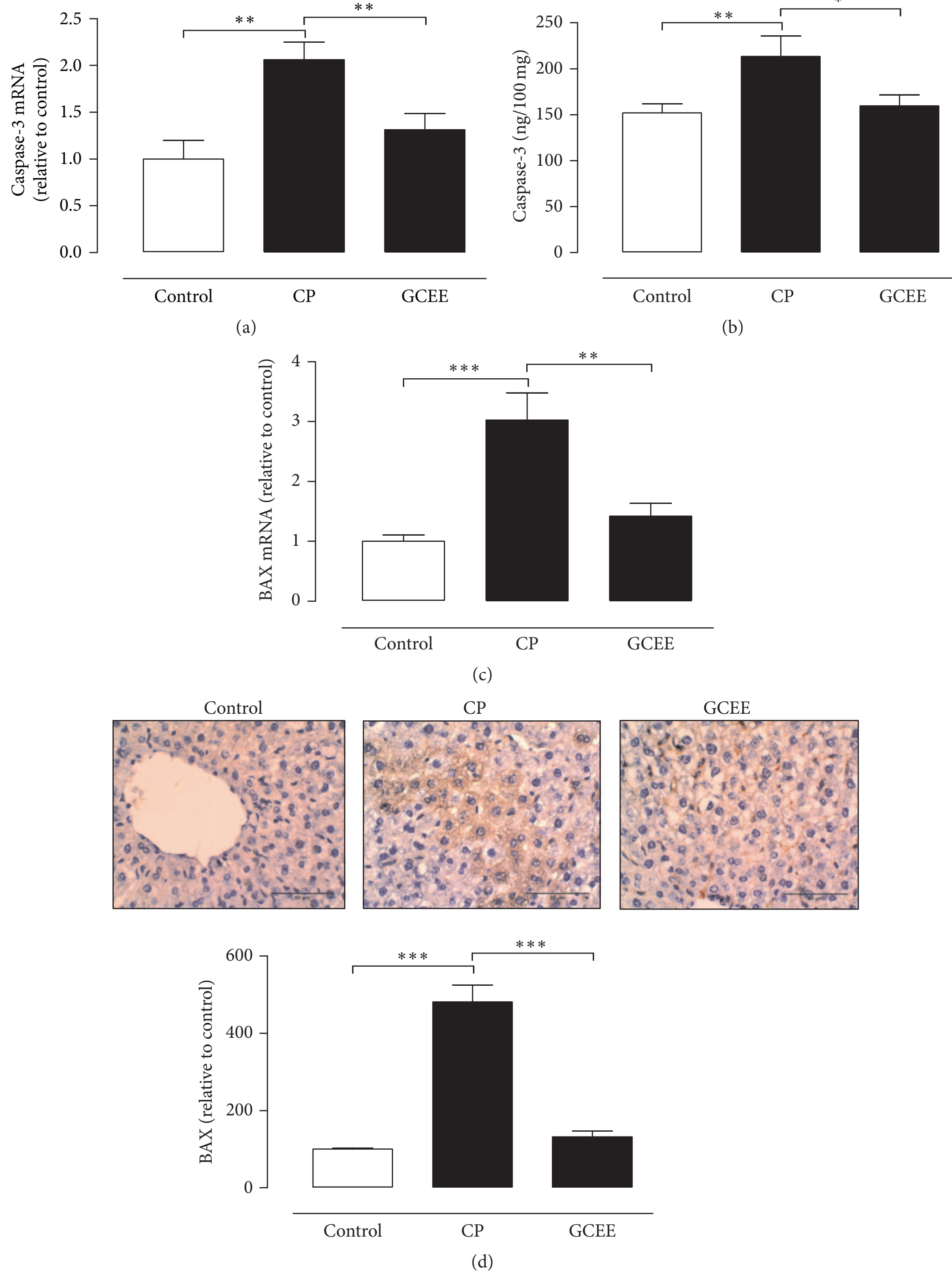

Figure 6: Effect of GCEE on (a) caspase-3 mRNA expression, (b) caspase-3 activity, (c) BAX mRNA expression, and (d) BAX immunohistochemical staining in liver of CP-induced rats. Data are expressed as mean ± SEM ($N = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. CP, cyclophosphamide; GCEE,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

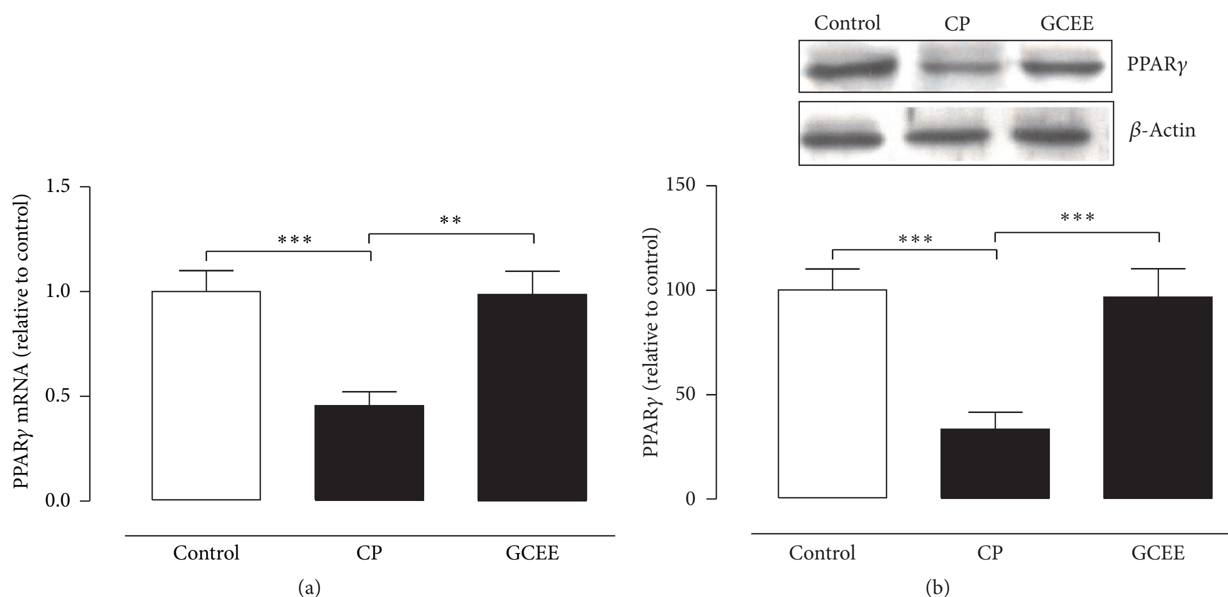

Figure 7: Effect of GCEE on PPARγ (a) mRNA and (b) protein expression in liver of CP-induced rats. Data are expressed as mean ± SEM ($N = 6$). $^{**}P < 0.01$ and $^{***}P < 0.001$. CP, cyclophosphamide; GCEE, gamma-glutamylcysteine ethyl ester; PPARγ, peroxisome proliferator activated receptor gamma.

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

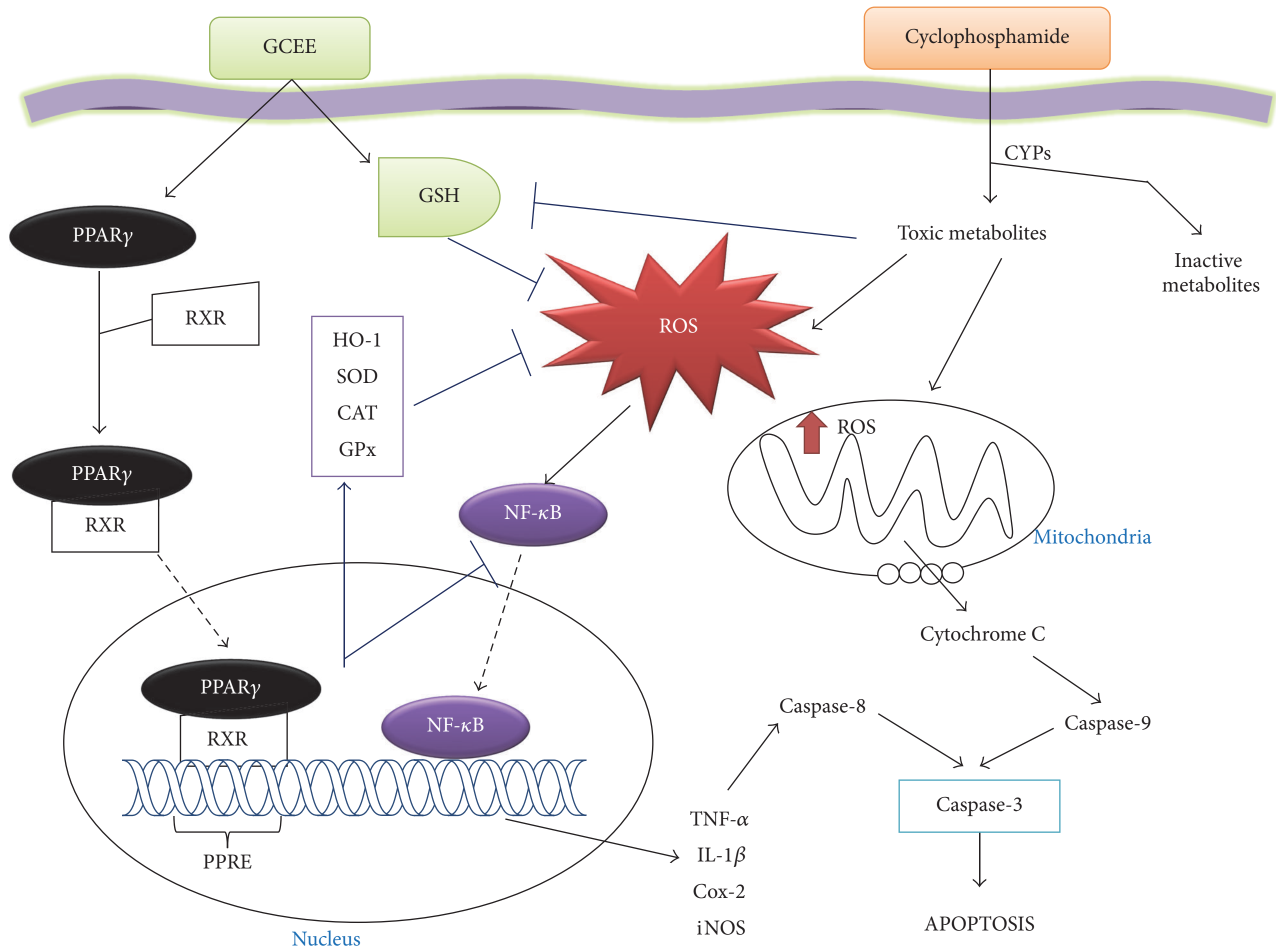

Figure 8: A proposed schematic diagram for the protective mechanisms of GCEE against CP-induced hepatotoxicity. PPARγ, peroxisome proliferator activated receptor gamma; RXR, retinoid X receptor; PPRE, PPAR response element; HO-1, heme oxygenase 1; SOD, superoxide dismutase; GPx, glutathione peroxidase; 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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