Original Research

Post-treatment with glycyrrhizin can attenuate hepatic mitochondrial damage induced by acetaminophen in mice

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Impact statement

At present, N-acetylcysteine is still an only drug approved by FDA to treat APAP poisoning. Due to high cost of new drug discovery and development, re-purposing an existing drug may be the best choice for a new antidote development to treat APAP hepatotoxicity. Glycyrrhizin, an active ingredient of liquorice, has been clinically used to ameliorate various chronic liver diseases in Asia for many years. For the first time, our studies indicate that post-treatment with GL can attenuate the hepatic mitochondrial damage and inhibit the up-regulation of nNOS induced by APAP. Subsequently, the APAP induced hepatotoxicity is significantly decreased. Importantly, these effects are independent of inhibiting the metabolic activation of APAP. Glycyrrhizin as a commercial drug has a high potential to be used in treatment for APAP poisoning in clinical practices.

Abstract

Overdose of acetaminophen (APAP) is responsible for the most cases of acute liver failure worldwide. Hepatic mitochondrial damage mediated by neuronal nitric oxide synthase-(nNOS) induced liver protein tyrosine nitration plays a critical role in the pathophysiology of APAP hepatotoxicity. It has been reported that pre-treatment or co-treatment with glycyrrhizin can protect against hepatotoxicity through prevention of hepatocellular apoptosis. However, the majority of APAP-induced acute liver failure cases are people intentionally taking the drug to commit suicide. Any preventive treatment is of little value in practice. In addition, the hepatocellular damage induced by APAP is considered to be oncotic necrosis rather than apoptosis. In the present study, our aim is to investigate if glycyrrhizin can be used therapeutically and the underlying mechanisms of APAP hepatotoxicity protection. Hepatic damage was induced by 300 mg/kg APAP in balb/c mice, followed with administration of 40, 80, or 160 mg/kg glycyrrhizin 90 min later. Mice were euthanized and harvested at 6 h post-APAP. Compared with model controls, glycyrrhizin post-treatment attenuated hepatic mitochondrial and hepatocellular damages, as indicated by decreased serum glutamate dehydrogenase, alanine aminotransferase, and aspartate aminotransferase activities as well as ameliorated mitochondrial swollen, distortion, and hepatocellular necrosis. Notably, 80 mg/kg glycyrrhizin inhibited hepatic nNOS activity and its mRNA and protein expression levels by 16.9, 14.9, and 28.3%, respectively. These results were consistent with the decreased liver nitric oxide content and liver protein tyrosine nitration indicated by 3-nitrotyrosine staining. Moreover, glycyrrhizin did not affect the APAP metabolic activation, and the survival rate of ALF mice was increased by glycyrrhizin. The present study indicates that post-treatment with glycyrrhizin can dose-dependently attenuate hepatic mitochondrial damage and inhibit the up-regulation of hepatic nNOS induced by APAP. Glycyrrhizin shows promise as drug for the treatment of APAP hepatotoxicity.

Keywords: Glycyrrhizin, acetaminophen hepatotoxicity, mitochondrial damage, neuronal nitric oxide synthase, tyrosine nitration, protein adducts

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Introduction

Acetaminophen (APAP) is a most popular antipyretic and analgesic over-the-counter medicine worldwide. It is safe at recommended doses; however, overdose of APAP can cause severe liver damage,[1] which accounts for the major acute liver failure (ALF) cases in Europe and USA.[2–4]

Early mechanism studies in mice reveal that overdose of APAP leads to its toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) formed excessively. NAPQI depletes liver glutathione and binds to cellular proteins, which lead to the initiation of APAP hepatotoxicity.[5,6] This insight results in the rapid development of N-acetylcysteine (NAC), a precursor of glutathione to be an antidote to treat APAP poisoning.[7] However, NAC is the only available antidote approved by FDA since late 1970s,[8] despite its limited effectiveness and anaphylactic risk.[1,9] Therefore, novel therapeutic agents are urgently needed. There are substantial progresses in further elucidating the detailed mechanism of APAP hepatotoxicity.[10] Mitochondrial peroxynitrite formation is considered to be the later predominant cellular event in APAP hepatotoxicity.[11] Peroxynitrite is highly reactive and leads to protein tyrosine nitration.[12] The subsequent mitochondrial oxidant/nitrosative stress is further amplified by JNK,[13] and triggers permanently mitochondrial permeability transition pore opening.[14] Finally, the critical hepatic mitochondrial damage leads to the nuclear DNA damage, necrotic cell death, and sterile inflammation. [15,16] More recent studies have demonstrated that neuronal nitric oxide synthase (nNOS) identified in hepatocytes is mainly responsible for the peroxynitrite formation in APAP hepatotoxicity.[17,18] Hepatic nNOS is upregulated by APAP overdose.[19] APAP-induced primary hepatocytes death was significantly reduced by direct pharmacological inhibition of nNOS, or interference with calcium induction of nNOS.[19–21] Compared with wild-type mice, hepatic manganese superoxide dismutase nitration and liver damage significantly decreased at 6 and 8 h after administration of APAP 300 mg/kg in nNOS knockout mice.[22,23] This indicates that nNOS plays an crucial role in the mitochondrial damage induced by APAP.[19] Other researchers also found no effect of inducible nitric oxide synthase (iNOS) is involved in APAP hepatotoxicity.[13,24]

The molecule glycyrrhizin (GL), an active ingredient of liquorice, has been clinically used to treat various chronic liver diseases in Asia for many years.[25] For example, the GL formulation SNMC (Stronger Neo-Minophagen C) succeeds in treating viral hepatitis in China and Japan by inhibiting virus replication, anti-inflammatory, and immunomodulatory effects. A clinical randomized controlled trial showed that GL is effective in the treatment of severe acute exacerbation of chronic hepatitis B.[26] Animal experiments also showed that GL attenuates D-galactosamine/lipopolysaccharides induced acute liver failure in rat through anti-inflammatory effect.[27] In fact, acute liver failure caused by viral hepatitis only accounts for about 10% of all clinical acute liver failure cases, and drug-induced liver cell damage especially APAP-induced liver cell damage is the main cause of liver failure. Therefore, whether GL can play a protective role in non-inflammatory acute liver failure and whether there is any other protection mechanism are very attractive and should be investigated. More recently, it has been reported that preventive administration of GL protected against APAP hepatotoxicity via alleviating TNF-α mediated apoptosis.[28] However, it has been widely accepted that hepatocellular damage in mice and people induced by APAP overdose is oncotic necrosis rather than apoptosis.[29,30] In addition, if GL could protect hepatocyte from death through other mechanisms, the same results would be obtained with the consequence of less inflammation.[31] On top of that, the majority of patients with ALF induced by APAP are intentionally taking the drug to commit suicide.[32,33] Any preventive treatment is of little value in practice.[34] In the present study, our aim is to investigate the effects of post-treatment with GL on APAP-induced hepatic mitochondrial and hepatocellular damages in mice. Besides that, essential changes of hepatic nNOS activity and its mRNA levers and protein expressions were also investigated.

Materials and methods

Animals and treatments

Male Balb/c mice (six to eight weeks) were provided by the Experimental Animal Center of Fourth Military Medical University. The mice were fed adaptively for three days in environmentally conditions at $22 \pm 1^{\circ}C$, $55 \pm 5\%$ humidity, 12-h light/dark cycle, and unrestricted access to standard food and clean water.

After a 15 h overnight fast, 40 mice were randomly divided into five groups ($n = 8$ each). Mice in 2nd to 5th groups were treated intraperitoneally with 300 mg/kg APAP (1.5%, Sigma-Aldrich), which was dissolved and kept in warm saline in advance.[35] Ninety min post-APAP,[36] GL (0.2% for 40 mg/kg, 0.4% for 80 mg/kg and 0.8% for 160 mg/kg, Shanghai Aladdin Bio-Chem Technology Co., Ltd) was administered intraperitoneally to the mice in 3rd, 4th, and 5th groups, respectively. The mice in 1st group, as normal controls, and in 2nd group, as model controls, received equal injections of saline. All mice were euthanized under isoflurane anesthesia at 6 h post-APAP. Mice blood and liver samples were harvested right away. Portions from left lateral liver lobe were fixed properly for histology, transmission electron microscopy, and immunohistochemistry analysis. The residual liver tissues were quick-frozen and stored at $-80^{\circ}C$ for later analysis.[37] To investigate the protective effects of GL in APAP-induced ALF, mice were intraperitoneally injected with a lethal dose of APAP (750 mg/kg) and 80 mg/kg GL 90 min later, and survival was monitored every 24 h for seven days. Experimental procedures (approval no. 20161005) were approved by the Animal Ethics Committee of Fourth Military Medical University.

Biochemical assays

Serum was extracted by centrifugation of blood at 10,000 r/min for 5 min. Serum glutamate dehydrogenase (GDH) was detected using the kit (Beyotime Biotechnology). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using the kits (Mindray Bio-Medical Electronics Co., Ltd).

Histology and immunohistochemistry

The liver tissues were prepared and stained with hematoxylin-eosin (H&E) according to the conventional

method.[29] The degree of hepatocyte necrosis was evaluated using optical microscopy.

3-nitrotyrosine, the representative of APAP-induced protein tyrosine nitration was assessed by immunohistochemistry as described using mouse anti-3-nitrotyrosine antibody (1:100, Abcam).[24]

Transmission electron microscopy

In order to investigate whether APAP-induced mitochondrial damage was relieved by GL post-treatment, transmission electron microscopy was performed. Tissues from the liver were fixed in cold 2% glutaraldehyde–2% paraformaldehyde solution prepared in 0.1 M PBS for 8 h. Tissue samples were fixed in 2% osmium tetroxide for an additional 1 h. After that, tissue samples were embedded in a polymer resin. Ultrathin sections were prepared and stained with uranyl acetate and Reynold's lead citrate. Mitochondrial damage in the liver was evaluated using an electron microscope (JEM-1230; JEOL, Ltd).

LC-MS/MS analysis

In order to investigate whether GL protects APAP hepatotoxicity through interference with the APAP metabolism, LC-MS/MS analysis with a modified sample processing method was performed.[38] Liver tissue was homogenized and 4-aminobenzoic acid (PABA) (Kermel S.A.) was added into the homogenate as the internal standard. Proteins were precipitated using methanol and the supernatant was dried using nitrogen. The precipitate was re-dissolved and diluted to a concentration of 5%, and the expression levels of 3-(cystein-S-yl)-acetaminophen (APAP-cys), acetaminophen-glutathione (APAP-GSH), and 3-(N-Acetyl-L-cystein-S-yl)-acetaminophen (APAP-NAC) were determined and analyzed using an LC-MS/MS System (API 4000; Thermo Fisher Scientific, Inc.). The ratio of APAP-GSH and APAP-NAC standarded to PABA was calculated and normalized to the total protein to evaluate the effects of GL on APAP metabolites. The ratio of APAP-CYS from digested APAP-protein adducts represented the level of APAP-protein adduct formation.

Reverse transcription-quantitative PCR

Total RNA in the liver tissues was extracted and reverse transcribed to cDNA using the TRIzol® RNA extraction kit and cDNA synthesis kit, respectively (Invitrogen; Thermo Fisher Scientific, Inc.). After that, the changes of nNOS and iNOS at the gene expression levels were detected using a real-time PCR detection system and SYBR Green PCR Master Mix (Applied Biosystems). The primers used were: nNOS, 5′-CTACAAGGTCCGATTCAACAG-3′ (forwards) and 5′-CCCACACAGAAGACATCACAG-3′ (reverse); iNOS, 5′-TCGCTTTGCCACGGACGAGA-3′ (forwards) and 5′′-TGGCCAGCTGCTTTTGCAGG-3′ (reverse); β-actin, 5′-ACCACACCTTCTACAATGAG-3′ (forwards) and 5′-ACGACCAGAGGCATACAG-3′ (reverse).

Western blotting

The primary antibodies included Rabbit anti-nNOS polyclonal antibody, rabbit anti-iNOS, and mouse anti-β-actin (1:1000; Abcam). The secondary antibodies we used were horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (1:2000; Shenzhen Bioeasy Biotechnology Co., Ltd). Western blotting was performed as described.[13] Protein brands were detected using chemiluminescence (Shenzhen Bioeasy Biotechnology Co., Ltd) and gray levels were quantified by Image J system (National Institutes of Health).

Hepatic NO content analysis

In order to evaluate the effect of GL on the catalytic capacity of hepatic nNOS, hepatic nitric oxide (NO) content was detected. Mice livers were homogenized and treated with 0.5% Triton-X 100. The supernatants were prepared by centrifuging the mixtures at 10,000*g* for 10 min. The NO contents were detected according to the procedure of NO assay kit (Abcam, USA) using a multimode microplate reader (Infinite 200 PRO, Tecan, Switzerland).

Statistics

All data were presented as mean ± SE. Comparisons of groups were assessed using ANOVA followed by Tukey test through GraphPad Prism 8.02. $P < 0.05$ was considered significant.

Results

Effects of GL on hepatic mitochondrial damages and hepatotoxicity induced by APAP

In order to investigate the effects of GL post-treatment on APAP-induced hepatic mitochondrial and hepatocellular damages, the serum GDH, ALT, and AST activities were measured in the serum. The results revealed that serum GDH, ALT, and AST activities were markedly elevated after APAP overdose. Serum GDH, ALT, and AST were decreased by 24.7, 55.5, and 61.7% respectively, in the mice post-treatment with 80 mg/kg GL, and 30.4, 60.5, and 57.4% respectively, in the mice post-treatment with 160 mg/kg GL ($P < 0.01$, Figure 1(a) to (c)). The survival rate in GL-treated mice was much higher than that observed in APAP-treated mice throughout the observation period. At 24 h after APAP injection, 100% of mode mice were dead, whereas survival rate of GL-treated mice was 70%, and 40% at the end of the experiment ($P < 0.01$, Figure 1(d)) These results indicated that GL post-treatment could significantly attenuate hepatic mitochondrial damages and hepatotoxicity induced by APAP overdose in mice.

Effects of GL on hepatic histopathological lesion

To investigate the effects of GL post-treatment on hepatic histopathological lesions, the liver sections were evaluated with H&E staining. The results revealed that the liver injury was characterized by cell swelling and lysis, loss of

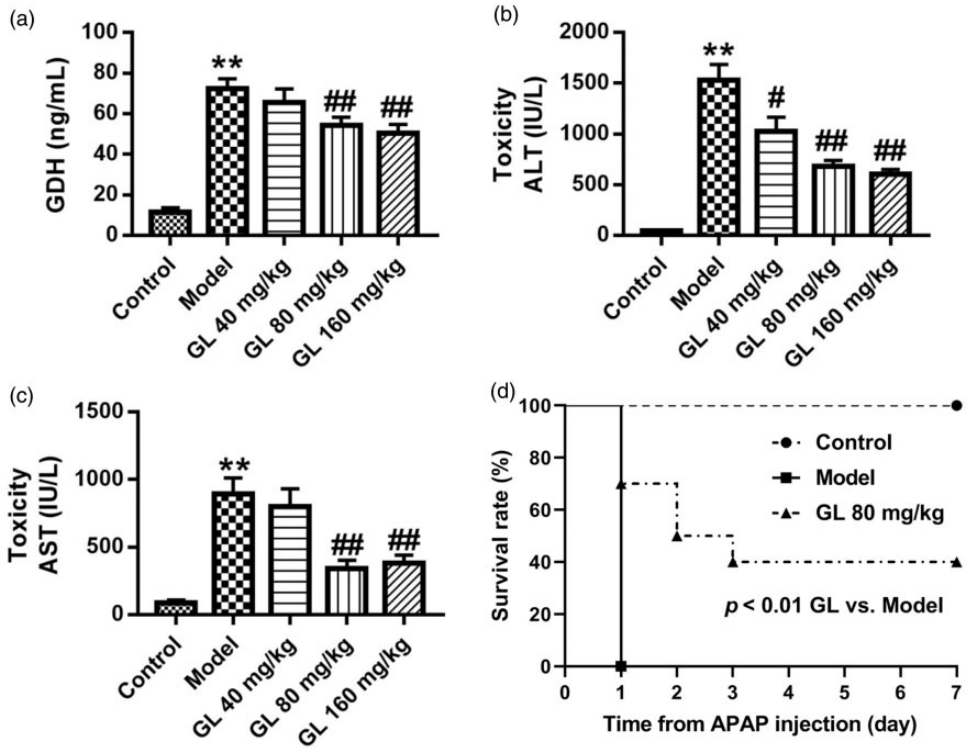

Figure 1. Glycyrrhizin (GL) attenuates hepatic mitochondrial and hepatocellular damages induced by acetaminophen as well as the survival rate of ALF. The activities of serum (a) glutamate dehydrogenase (GDH), (b) alanine aminotransferase (ALT), and (c) aspartate aminotransferase (AST) were significantly decreased by 80 or 160 mg/kg GL. $n = 8$ mice per group; $^{**}P < 0.01$ (versus normal control); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ (versus model control). (d) Mice were administered a lethal dose of APAP (750 mg/kg, i.p.), and survival rate was monitored at indicated time points ($n = 10$/group).

architecture, and inflammatory cell infiltration in the model controls, which represent oncotic necrosis,[29] rather than cell shrinkage or apoptotic bodies, which represent apoptosis. This lesion was markedly ameliorated in the mice administered GL (Figure 2(a) to (f)).

As the mitochondria are the organelles that are primarily damaged in APAP hepatotoxicity, electron microscopy detection was used to evaluate hepatic mitochondrial changes. The results demonstrated that the damaged mitochondria appeared swollen, distorted, and loss of double limiting membranes, which were visibly alleviated in the mice receiving GL (Figure 2(g) to (i)). This indicated that GL post-treatment can decrease the mitochondrial damages induced by APAP overdose.

GL did not affect APAP metabolic activation

In order to investigate whether GL interferes with the APAP metabolic activation, in the present study, APAP-protein adducts formation was detected with serious concern. Our results showed that APAP metabolites, including APAP-cys, APAP-GSH and APAP-NAC, were significantly generated after APAP overdose, but there were no differences of APAP metabolites formation between model controls and mice administered 80 mg/kg GL (Figure 3). These results indicated that post-treatment with GL did not affect APAP metabolism and APAP-protein adducts formation.

GL inhibited the up-regulation of hepatic nNOS

The levels of hepatic nNOS mRNA were investigated by RT-qPCR assay. The results showed that hepatic nNOS mRNA was significantly increased after APAP administration. Compared with the model controls, hepatic nNOS mRNA was decreased by 14.9% in the mice receiving 80 mg/kg GL (Figure 4(a)). Western blotting assay showed that the protein expression of hepatic nNOS was also significantly increased after APAP administration. The nNOS protein expression was decreased by 28.3% with administration of 80 mg/kg GL, compared with the model controls (Figure 4(b)). There is no significant difference in iNOS mRNA or protein levels among groups. These results indicated that GL post-treatment could inhibit the up-regulation of hepatic nNOS in APAP-induced hepatotoxicity mice.

GL inhibited the production of NO and protein tyrosine nitration

NO is the catalytic product of NOS. APAP-induced abnormal up-regulation of hepatic nNOS activity was assessed by detection of NO content in the liver homogenate. Hepatic NO content was increased in the model controls, but it was decreased by 16.9% in the mice receiving 80 mg/kg GL, compared with model controls (Figure 5(a)). These results indicated that GL post-treatment could inhibit the production of NO in APAP-induced liver injury mice.

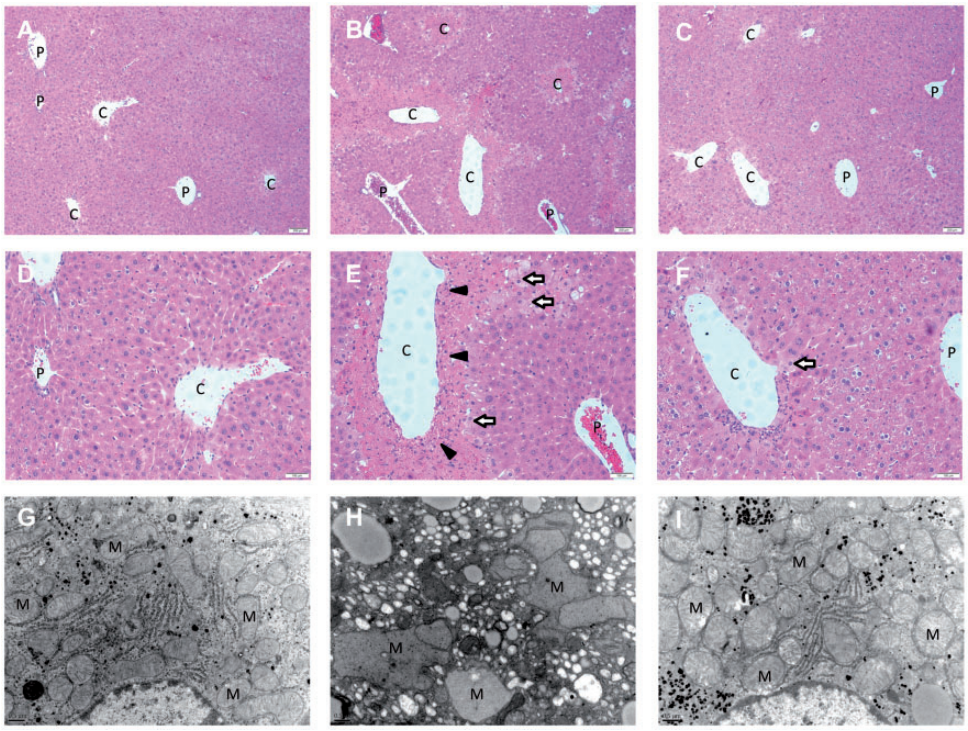

Figure 2. Glycyrrhizin (GL) reduces hepatic histopathological lesion induced by acetaminophen. Hematoxylin and eosin staining represented that hepatocytes swelling (arrow), lysis and loss of architecture and inflammatory infiltration (arrowhead) in the model control were alleviated by post-treatment with 80 mg/kg GL. Apoptotic hepatocytes were very rare. Electron microscopy represented that cellular edema and mitochondrial swollen in hepatocytes of models were partially recovered by post-treatment with 80 mg/kg GL. Normal controls (a,d,g), Model controls (b,e,h), 80 mg/kg GL post-treatment groups (c,f,i). C: central vein; P: portal area; M: mitochondria. Scale bars, 200 μm (a–c), 100 μm (d–f), 0.5 μm (g–i). $n = 8$ mice per group.

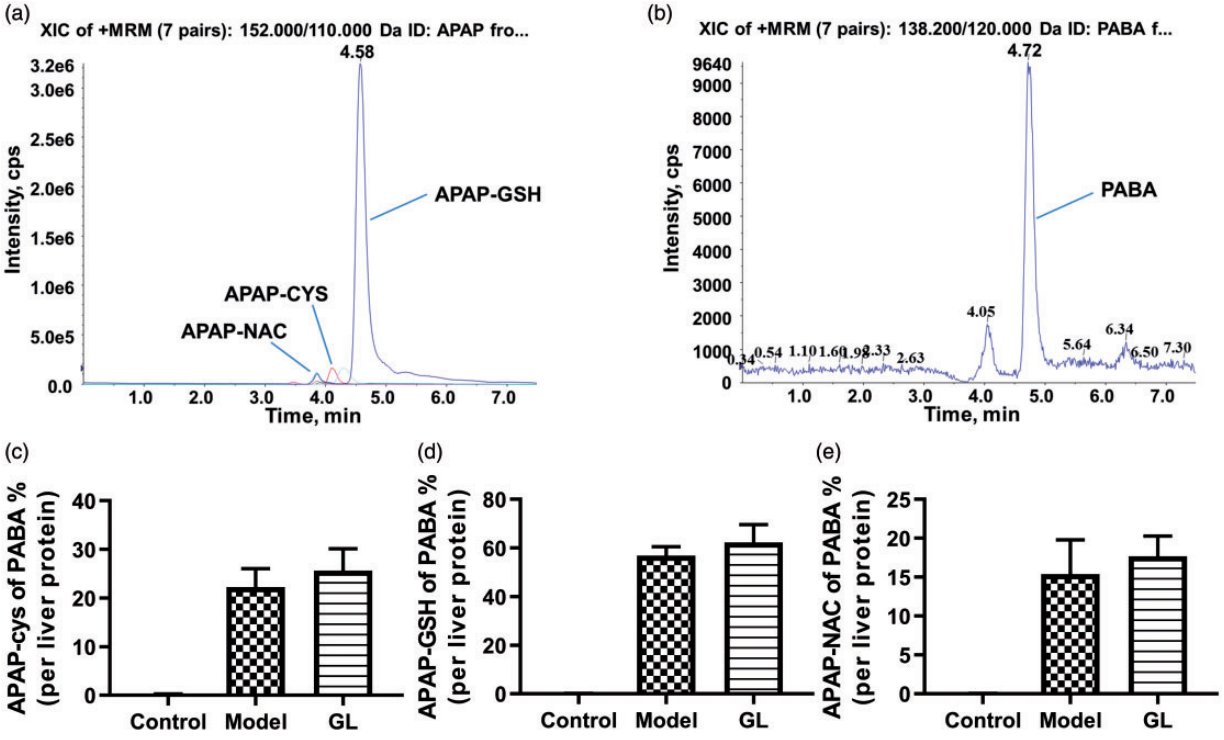

Figure 3. Glycyrrhizin (GL) has no effect on acetaminophen (APAP) metabolism and APAP-protein adducts formation. (a) HPLC-MS detected the metabolites of acetaminophen, which are marked by the various peaks. (b) HPLC-MS analysis of PABA in liver tissue homogenate, as an internal standard. The protein contents of (c) 3-(cystein-S-yl) acetaminophen (APAP-cys), (d) acetaminophen-glutathione (APAP-GSH), and (e) 3-(N-Acetyl-L-cystein-S-yl) acetaminophen (APAP-NAC) were not changed following GL treatment, as compared to the model control. $n = 8$ mice per group.

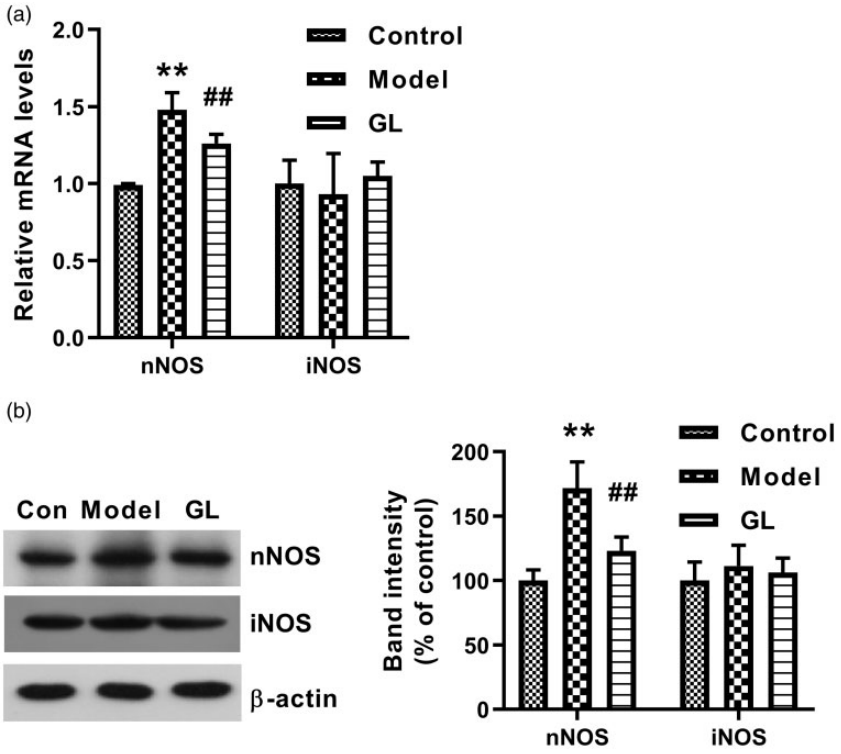

Figure 4. Glycyrrhizin (GL) inhibits the up-regulation of neuronal nitric oxide synthase (nNOS) in the liver tissue induced by acetaminophen overdose. (a) Reverse transcription-quantitative PCR showed that the elevated nNOS mRNA levels were decreased by post-treatment with 80 mg/kg GL, whereas inducible nitrogen oxide synthase (iNOS) mRNA levels were unchanged. $^{**}P < 0.01$ (versus normal control); $^{\#\#}P < 0.01$ (versus model control). (b) The protein expressions of nNOS, but not inducible nitrogen oxide synthase (iNOS), were increased in the liver tissue from the model control and were decreased following post-treatment with 80 mg/kg GL. $^{**}P < 0.01$ (versus normal control); $^{\#\#}P < 0.01$ (versus model control). $n = 8$ mice per group.

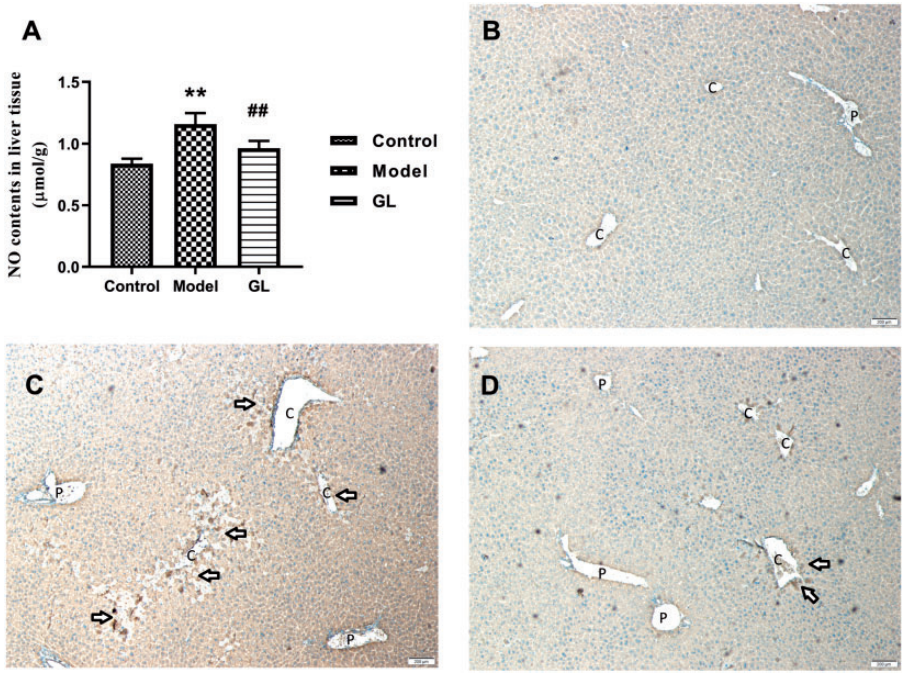

Figure 5. Glycyrrhizin (GL) inhibits the formation of nitric oxide (NO) and protein tyrosine nitration in liver tissue induced by acetaminophen overdose. (a) NO content in the liver tissue homogenate was analyzed using a spectrophotometer. Post-treatment with 80 mg/kg GL inhibited the production of NO following acetaminophen injury. $^{**}P < 0.01$ (versus normal control); $^{\#\#}P < 0.01$ (versus model control). (b–d) The 3-nitrotyrosine staining (arrow) of hepatocytes especially around the central veins was decreased by post-treatment with 80 mg/kg GL. Normal controls (b), model controls (c), 80 mg/kg GL post-treatment groups (d). C: central vein; P: portal area. $n = 8$ mice per group.

The 3-nitrotyrosine staining was performed in order to investigate the liver protein tyrosine nitration by peroxynitrite formation.[39] The results showed that the staining of 3-nitrotyrosine antibody in the liver was much higher in the model controls than in normal controls, which was more extensive around the hepatic vessels, especially around the central veins. Compared with the model controls, the staining of hepatic 3-nitrotyrosine was weakened in the mice administered 80 mg/kg GL. The results suggested that GL post-treatment could decrease liver protein tyrosine nitration (Figure 5(b) to (d)).

Discussion

Although it is quite urgent to develop novel antidotes against APAP hepatotoxicity,[40] due to the high cost of new drug development, re-purposing an existing drug may be the best choice for a new antidote development against APAP overdose.[41] The present study indicates that post-treatment with GL can dose-dependently attenuate acetaminophen hepatotoxicity in mice, which mimic human poisoning very well.[36] Because GL has been used clinically for many years, and it is very safe even in end-stage liver disease,[26,42] it has a high potential to be therapeutically used in treatment for APAP hepatotoxicity in clinical practices.

APAP is mainly metabolized into glucuronidated- and sulfated-APAP at recommended doses in liver. These metabolites are nontoxic and rapidly excreted into urine. However, a very small portion of APAP is oxidized to NAPQI, a toxic intermediate metabolite. Usually, NAPQI is highly reactive and rapidly detoxified by glutathione (GSH).[43] When APAP is overdose, a large portion of APAP is metabolized into NAPQI mainly by cytochrome P450 2E1. The excessive NAPQI depletes liver GSH and covalently binds with sulfhydryl groups in biomolecules, particularly mitochondrial proteins, which trigger the mitochondrial dysfunction.[44] Since the excessive NAPQI formation plays a key role for initiation of the APAP hepatotoxicity, it is not difficult to understand why an inhibitor of P450 2E1 is effective for APAP hepatotoxicity, only when administered as pre-treatment or co-administered with APAP. In fact, so many natural products are only effective for APAP-induced acute liver damage as pre-treatment.[31,45] In our study, GL post-treatment did not affect the metabolism of APAP, because there was no difference of APAP metabolites formation between the mice receiving GL and model controls, as demonstrated by the LC-MS/MS assay.

The present study revealed that post-treatment of GL (80 mg/kg) could markedly attenuate hepatic mitochondrial and hepatotoxicity induced by APAP, as shown by blood biochemical indexes, survival rate test, transmission electron microscopy, and histopathological assays. Notably, the up-regulation of hepatic nNOS, at early stages of APAP poisoning, was also inhibited by GL post-treatment. Compared with model controls, hepatic nNOS mRNA levels, nNOS protein expression, and NO contents significantly decreased in the mice receiving GL. Meanwhile, we found that the protein tyrosine nitration in liver tissue decreased markedly as detected by 3-nitrotyrosine immunohistochemistry assay in GL treated mice. Importantly, all of these results are consistent with the findings that peroxynitrite, which lead to protein tyrosine nitration is formed by the reaction of superoxide with nNOS originated NO in APAP hepatotoxicity.[12,46] We can conclude that GL can alleviate APAP-induced mitochondrial damage, and this effect is related to the inhibition of nNOS. However, the exact underlying mechanisms of GL post-treatment in attenuating the hepatic mitochondrial damage induced by APAP remain unknown, except inhibiting the up-regulation of hepatic nNOS and it is possible that there are other protective effects. Due to the different protective mechanisms between GL and NAC, their synergistic effects also need to be further investigated.

In conclusion, GL post-treatment can dose-dependently attenuate the hepatic mitochondrial damage and inhibit the up-regulation of nNOS induced by APAP. It is necessary to further investigate the clinical application value of GL and the detailed molecular mechanisms for GL protection against APAP hepatotoxicity in mice.

AUTHORS' CONTRIBUTIONS

ADW and PY designed the present study, PY, LS, and PH designed the methodology, XLD, PY, LFY, JC, ZBJ, and LFL performed the investigation, PH performed the data curation, XLD, LFY, and PY drafted the manuscript, XLD worked with the software, ADW and PY reviewed and edited the manuscript, ADW and PY provided supervision and ADW provided project administration.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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