Protective Effect of Genistein on
Lipopolysaccharide/D-galactosamine-induced Hepatic Failure in Mice

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Abstract

This study examined the effect of genistein from *Hydrocotyle sibthorpioides* on lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced acute hepatic failure. Compared to the model control, genistein treatment significantly protected against LPS/D-GalN-induced liver injury, as evidenced by the decrease in serum alanine and aspartate aminotransferases activities and the attenuation of histopathological changes. Furthermore, genistein alleviated the pro-inflammatory cytokines including TNF-α and NO/iNOS by inhibiting nuclear factor-κB (NF-κ B) activity. Genistein attenuated the elevated level of caspases-3, while augmented the expression of Bcl-2. In addition, LPS/D-GalN induced significant increase of HO, carbon monoxide and bilirubin levels and these alterations were augmented by genistein treatment. In conclusion, the protective effect of genistein on LPS/D-GalN-induced liver damage was mainly due to its ability to block NF-κ B signaling pathway for anti-inflammation response, attenuate hepatocellular apoptosis and increase HO level. These findings suggest that genistein can be considered as a potential agent for preventing acute hepatic failure.

**Keywords:** Genistein; *Hydrocotyle sibthorpioides*; Lipopolysaccharide/D-galactosamine; Acute hepatic failure
1. Introduction

Fulminant hepatic failure is a dramatic clinical syndrome that results from severe impairment of liver function. $\text{D}$-galactosamine ($\text{D}$-GalN) is selectively metabolized by hepatocytes. $\text{D}$-GalN can reduce the intracellular pool of uracil nucleotides in hepatocytes, thus inhibiting the synthesis of RNA and proteins.\textsuperscript{1)} Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is an endotoxin that is thought to contribute significantly to hepatic failure.\textsuperscript{2)} LPS in combination with $\text{D}$-GalN-induced liver injury is a well-known experimental model used to develop liver failure as a result of massive hepatocyte death.\textsuperscript{3)} It is generally accepted that the model mimics clinical liver dysfunction and is useful for evaluating the efficiency of treatment.

Due to thousands of years of experience, herbal medicines are considered as a rich source of new therapeutic agents. Many compounds with new structural features and mechanisms of actions have been isolated from herbal medicines. Natural products are potential sources of novel anti-hepatitis drugs that may be applicable to liver disease therapy. *Hydrocotyle sibthorpioides* (Apiaceae *Hydrocotyle sibthorpioides* Lam.) is a perennial herb widely distributed in parts of China and Taiwan. It has folkloric uses in treatment of illnesses such as fever, edema, detoxication, throat pain, psoriasis, and herpes zoster. In addition, it has been reported that *Hydrocotyle sibthorpioides* could functionally and structurally damage tumor cells, as well as help improve phagocytotic activity and enhance immune function.\textsuperscript{4)}

Previously, many traditional clinical treatments have confirmed that *Hydrocotyle sibthorpioides* has a curative effect in treatment of jaundice Hepatitis B, and this herb has been recommended in treatment of hepatitis B virus infections.\textsuperscript{5)} Recently, we have screened a number of hepatoprotective ingredients from *Hydrocotyle sibthorpioides*. Our study indicated that genistein, an active compound of *Hydrocotyle sibthorpioides*, exerted a preventative effect to ameliorate chronic liver injury and even liver fibrosis induced by chronic alcohol administration in rats.\textsuperscript{6)} Based on these reports, it would be of great interest to determine the effects of genistein on acute hepatic failure.

Therefore, this study examined the hepatoprotective effects of genistein isolated from *Hydrocotyle sibthorpioides* against fulminant hepatic failure induced by LPS/$\text{D}$-GalN, and
explored the mechanism of its protection.

2. Materials and methods

2.1. Chemicals

Genistein was isolated from *Hydrocotyle sibthorpioides* according to the protocol established in our previous study,\(^{6}\) its chemical structure was shown in Fig. 1. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and malondialdehyde (MDA) kits were obtained from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). Tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) kit was purchased from Wuhan Boster Bio-engineering Co. Ltd. (Wuhan, China).

2.2. Animals and treatments

Male C57BL/6 mice (20–22 g) were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China). The research was conducted according to protocols approved by the institutional ethical committee of Guangxi Medical University. After a period of one week, the mice were divided into six groups with 15 mice per group as follows:

- **Group I**, normal control, the mice received the same volume of saline;
- **Group II**, genistein control, the mice received genistein (2 mg/kg);
- **Group III**, model control, the mice received LPS (50 \(\mu\)g/kg) and \(D\)-GalN (800 mg/kg);
- **Group IV**–**VI**, genistein treatment, the mice received genistein (0.5, 1 or 2 mg/kg) plus LPS (50 \(\mu\)g/kg)/\(D\)-GalN (800 mg/kg);

The animals were administered intraperitoneally drugs once daily for 3 days prior to challenge experimentation. Mice in groups III–VI were then challenged intraperitoneally with LPS/\(D\)-GalN. Blood was collected from the mice eyes at 1.5 h after LPS/\(D\)-GalN administration. The mice were then euthanized by intraperitoneally injecting high dose of pentobarbitone sodium (150 mg/kg) at 6 h after LPS/\(D\)-GalN injection. Liver samples were dissected and washed immediately with ice-cold saline.

2.3. Histological analysis

Liver tissues were fixed in 4% phosphate buffered formalin, dehydrated in graded alcohols and embedded in paraffin blocks. Five micrometer thick paraffin sections were then
rehydrated and stained with hematoxylin and eosin (H&E). All histological examinations were performed by an experienced pathologist who was blinded to the experimental groups.

2.4. Estimating AST, ALT and total bilirubin activities

Determination of serum alanine aminotransferase (ALT) was carried out using a commercial kit (Nanjing Jiancheng, China). The principle of this test is to catalyze a reaction with pyruvate as one of the end products. Pyruvate reacts in the next reaction that involves oxidation of NADH to NAD. The rate of decrease of NADH is measured photometrically and is directly proportional to the rate of formation of pyruvate, which is indicative of ALT activity.

An aminotransferase kit by Nanjing Jiancheng (China) was used in determination of aspartate aminotransferase (AST) level. Briefly, AST is an enzyme that catalyzes conversion of 2-oxoglutarate into oxalacetate, which further reacts with NADH. The rate of decrease of NADH is measured photometrically and is directly proportional to the rate of formation of oxaloacetate, which in turn is indicative of AST activity.

Serum total bilirubin was measured using a BIL-Total kit (Nanjing Jiancheng, China). This method involves photometric measurement of azobilirubin dye that is produced when bilirubin reacts with sulfanilic acid.

2.5. Hepatic lipid peroxidation assays

To assess the anti-oxidant effect of genistein, lipid peroxidation in the liver was determined by measuring the level of malondialdehyde (MDA), an end product of lipid peroxidation, using a thiobarbituric acid method. In brief, the liver samples were hydrolyzed at pH 13 by incubation in 0.5 M NaOH solution at 60 °C for 30 min, and were then neutralized to pH 8.0 with concentrated HCl. The 2.5 ml of each sample was mixed in a screw capped tube with 2.5 ml TBA reagent. The tubes were heated in a boiling water bath for 30 min, cooled in water and centrifuged at 1500 g to assure a clear solution. The absorbance was measured at 532 nm.

2.6. Determination of serum TNF-α level

The serum TNF-α level was determined by enzyme-linked immunosorbent assay (ELISA) (Amersham Pharmacia Biotec, NJ, USA) according to the manufacturer’s protocol. Briefly,
polystyrene 96 well Nunc immunoplates were coated with 50 μl purified anti-mouse TNF-α antibody in 0.1 M bicarbonate buffer (pH 8.2). The plates were then washed in phosphate buffered saline with 0.05% Tween 20 (PBS/Tween) and blocked with PBS-25% PCS for 2 hours at room temperature. After 3 washes with PBS/Tween, 50 μl of diluted sera (1:4), or recombinant murine TNF-α standard in serial dilutions were added and incubated 4 hours at room temperature. After extensive washing, the plates were incubated for 1 hour with 100 μl of biotinylated anti-TNF-α monoclonal antibody (2 μg/ml). The washing procedure was repeated 4 times, and the plates were incubated at room temperature with avidin-peroxidase, (1:400). One hundred μl of OPD at a concentration of 0.4 mg/ml and hydrogen peroxide were added, and OD was read on a microplate reader at 450 nm.

2.7. Determination of NO production

Since the levels of nitrite (NO$_2^-$) and nitrate (NO$_3^-$) can be used to estimate NO production, we measured concentrations of these stable NO oxidative metabolites in liver homogenates. In brief, the NO$_2^-$ and NO$_3^-$ in the liver homogenates were separated by means of a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6 × 50 mm, Eicom), after which NO$_3^-$, was reduced to NO$_2^-$, in a reduction column packed with copper-plated cadmium filings (NO-RED, Eicom). The NO$_2^-$, was mixed with a Griese reagent to form a purple azo dye in a reaction coil. The separation and reduction columns and the reaction coil were placed in a column oven set at 35 °C. The absorbance of the colored product dye at 540 nm was measured by means of a flow-through spectrophotometer (NO-RED, Eicom). The mobile phase, which was delivered by a pump at a rate of 0.33 ml/min, was 10% methanol containing 0.15 M NaCl/NH$_4$Cl and 0.5 mg/ml 4Na-EDTA. The Griese reagent, which was 1.25% HCl containing 5 mg/ml sulfanilamide with 0.25 mg/ml N-naphthylethylenediamine, was delivered at a rate of 0.1 ml/min.

2.8. Analysis of TNF-α and iNOS mRNA expression

To examine the mRNA expression levels of TNF-α and iNOS in the liver, total RNA from snap-frozen tissues was extracted using a RNeasy mini kit (QIAGEN Inc.). First-strand cDNA was prepared by following the instructions in SuperScript™ First-Strand Synthesis System for RT-PCR kit (Life Technologies). Reverse transcription and amplification by PCR
were performed as described previously. The following primer sequences were used: TNF-α: 5′-ATG AGC ACA GAA AGC ATG ATC-3′ (sense) and 5′-TAC AGG CTT GTC ACT CGA ATT-3′ (antisense), 276 bp; iNOS: 5′-GTG AGG ATC AAA AAC TGG GG-3′ (sense) and 5′-ACC TGC AGG TTG GAC CAC-3′ (antisense), 306 bp; β-actin: 5′-GAT GGT GGG TAT GGG TCA GAA GGA-3′ (sense) and 5′-GCT CAT TGC CGA TAG TGA TGA CCT-3′ (antisense), 514 bp. The PCR products were subjected to electrophoresis and ethidium bromide staining. The gels were analyzed by densitometry. β-actin was used as the internal control.

2.9. Measurement of nuclear NF-κB p65 content

Nuclear was extracted from the liver by a Nuclear Extraction Kit (Active Motif, Carlsbad, USA). Nuclear NF-κB p65 content was measured using a NF-κB/p65 ActivELISA kit (Imgenex, San Diego, USA) according to the manufacturer’s protocol. This method combines the principle of the electrophoretic mobility shift assay with the 96-well-based enzyme linked-immunosorbent assay. During the assay, a double-stranded biotinylated oligonucleotide containing the flanked DNA binding consensus sequence for NF-κB (5′-GGGACTTTCC-3′) was mixed with cellular (nuclear) extract of liver tissue (50 μg/well) and transferred to the streptavidin-coated plate well. The bound NF-κB transcription factor subunit p65 was detected with a specific primary antibody (a Rabbit anti-NF-κB p65) followed by a highly sensitive HRP-conjugated secondary antibody, for which colorimetric signals were detected in a spectrophotometric plate reader.

2.10. Measurement of caspase-3 activity

The caspase-3 activity was measured using an in vitro fluorogenic peptide substrate, N-acetyl-Asp-Glu-Val-Asp-7-amino-trifluoromethyl-cumarine (DEVD-AFC; BioMol, Plymouth Meeting, PA, USA), according to the procedure reported by Kim et al. Briefly, a sample of liver tissue (1.0 g) was homogenized in 6 ml of buffer containing 25 mM Tris, 5 mM MgCl₂, 1 mM EGTA, and 50 μM protease inhibitor cocktail (Sigma). The homogenate was centrifuged for 15 min at 40,000g and the resulting supernatant was collected for determining the caspase-3 activity. Dithiothreitol (10 mM) was added to the samples immediately before freezing. The caspase-3 activity was assayed in a total volume of 100 μl. A 30-μg sample of cytosolic protein was incubated at room temperature for 15 min in buffer
containing 30 mM HEPES, 0.3 mM EDTA, 100 mM NaCl, 0.15% Triton X-100, and 10 mM dithiothreitol. The reaction was initiated by adding 200 μM DEVD-AFC and the samples were then incubated at 37 °C. The change in fluorescence (excitation at 400 nm and emission at 490 nm) was monitored after 120 min.

2.11. Western blot immunoassay for caspase-3 and Bcl-2

The cytosolic proteins of liver samples were prepared using cytoplasmic extraction reagents according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). Briefly, equivalent aliquots of protein extract were separated on 12% SDS-PAGE gel. After electrophoretic separation, proteins were transferred to polyvinylidene fluoride (PVDF) membranes, followed by incubation with primary antibodies of caspase-3, Bcl-2 or β-actin (Santa Cruz, CA). Then the membrane was treated with horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands intensity was determined by using a densitometer equipped with Image QuaNT software (Molecular Dynamics, Sunnyvale, CA). The signals were normalized to that of β-actin.

2.12. HO activity assay

HO enzymatic activity was measured by bilirubin generation as described previously. Briefly, frozen hepatic samples were homogenized in lysis buffer (250 mM Tris–HCl, pH7.4; 150 mM NaCl; 250 mM sucrose; 0.5 mM phenylmethylsulfonylfluoride (PMSF); 1 μg/μl leupeptin; 1 μg/μl aprotinin). Microsomal fraction was obtained by successive centrifugations and washed with 0.15 M KCl followed by centrifugation (95,000 × g for 35 min). The pellet was suspended in 0.1 M potassium phosphate by sonication and stored at −80 °C. The reaction was carried out in the mixture containing 2 mg/ml protein microsomal fraction, 1 mM glucose-6-phosphate, 0.2 unit/ml glucose-6-phosphate dehydrogenase, 0.8 mM NADPH, and 0.025 mg/ml hemin at 37 °C for 45 min. After chloroform extraction, the extracted bilirubin was calculated by the difference of absorbance at 464 and 530 nm (ε=40 mM⁻¹ cm⁻¹).

2.13. Measurement of total tissue carbon monoxide

For liver carbon monoxide (CO) determination, liver homogenates were incubated in CO-free septum-sealed vials containing 60% (w/v) sulphosalicylic acid on ice. The amount of
CO generated by the reaction and released into the vial headspace was quantitated by gas chromatography with a reduction gas analyzer (Trace Analytical, Menlo Park, CA, USA). Liver CO content was calculated as pmol/mg protein.

2.14. Statistical analysis

Statistical analysis was performed using SPSS 11.5 for Windows. Differences between the groups were assessed using a one-way analysis of variance (ANOVA) with a Tukey’s test for post hoc multiple comparisons. The data are presented as the means ± SE. A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Histopathological findings

The liver in both the normal control (Fig. 2 I) and genistein control (Fig. 2 II) groups revealed normal lobular architecture and cellular structure. However, livers exposed to LPS/D-GalN exhibited multiple and extensive areas of central inflammation and cellular necrosis and a moderate increase in inflammatory cell infiltration (Fig. 2 III). These pathological alterations were ameliorated by genistein treatment (Fig. 2 IV-VI).

3.2. Effects of genistein on AST, ALT and total bilirubin levels increased by LPS/D-GalN

Significant elevation of AST, ALT and total bilirubin levels in the serum which are released from the cytosolic part of the liver cells often suggest the pathological condition of severe liver injury. To evaluate the extent of liver injury in mice, we conducted an analysis of serum AST and ALT activities and total bilirubin level. As shown in Fig. 3, significant increases in serum aminotransferases AST (Fig. 3 A), ALT (Fig. 3 B) and total bilirubin (Fig. 3 C) levels were observed in LPS/D-GalN animals as compared to mice with saline treatment (negative control). Treatment with genistein significantly decreased the AST and ALT activities, while further increased the bilirubin level. Genistein had no effect on these basal serum biochemical indicators levels.

3.3. Effects of genistein on the contents of MDA, TNF-α and NO, as well as the mRNA expression levels of TNF-α and iNOS.

Free radicals generate the lipid peroxidation process in an organism. Malondialdehyde
(MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA. Its level is commonly known as a marker of oxidative stress.\(^\text{12}\) TNF-\(\alpha\), a pleiotropic pro-inflammatory cytokine, is rapidly produced by macrophages in response to tissue damage,\(^\text{13}\) and is essential for LPS/\(D\)-GalN-induced fulminant hepatic failure because it causes the production of reactive oxygen species, inflammatory responses and hepatic necrosis. In addition, NO has been shown to react with superoxide to form the highly reactive oxidant, peroxynitrite, \(\text{NO}_3^-\), which can cause tissue injury. It has been suggested that NO formation may be of critical importance in modulating the toxicity of superoxide or other oxidants.\(^\text{14}\) To assess the inhibitory effects of genistein on lipid peroxidation, pro-inflammatory cytokine and NO formation, the levels of hepatic MDA, serum TNF-\(\alpha\) and liver \(\text{NO}_2^-/\text{NO}_3^-\) were determined. As shown in Fig.4, the contents of hepatic MDA (Fig. 4A), serum TNF-\(\alpha\) (Fig. 4B) and liver \(\text{NO}_2^-/\text{NO}_3^-\) (Fig. 4C) were found to be significantly increased in LPS/\(D\)-GalN-treated mice, whereas genistein treatment significantly reduced the contents of these inflammatory indicators. Furthermore, administration of LPS/\(D\)-GalN markedly induced elevation of TNF-\(\alpha\) and iNOS mRNA expression, and treatment with genistein dose-dependently attenuated both the genes expression levels (Fig. 4D). In addition, genistein alone had no significant effect on the basal levels of these inflammatory cytokines.

3.4. Effect of genistein on nuclear NF-\(\kappa B\) p65 content

NF-\(\kappa B\) is a dimeric transcription factor that is involved in the regulation of a large number of genes that control various aspects of the inflammatory response.\(^\text{15}\) In this study, to explore the underlying mechanism of genistein on pro-inflammatory mediators, nuclear NF-\(\kappa B\) p65 content was measured. The results revealed that administration of LPS/\(D\)-GalN markedly induced increase of nuclear NF-\(\kappa B\) p65 content, however, treatment of mice with 1 or 2 mg/kg genistein significantly decreased nuclear NF-\(\kappa B\) p65 content. In addition, genistein alone had a negligible effect on this basal nuclear factor content (Fig. 5).

3.5 Effects of genistein on the activity of caspase-3, as well as the protein levels of caspase-3 and Bcl-2

Caspase-3 is essential for some typical hallmarks of apoptosis, and is indispensable for
apoptotic chromatin condensation and DNA fragmentation in all cell types examined.\textsuperscript{16} Bcl-2 is an integral membrane protein located mainly on the outer membrane of mitochondria. Overexpression of Bcl-2 prevents cells from undergoing apoptosis in response to a variety of stimuli.\textsuperscript{17} In this study, the activity of caspases 3 in the LPS/\textsubscript{D}-GalN-treated group was rapidly increased compared to the normal control group, and the elevated activity of caspases 3 was attenuated by treatment with 1 or 2 mg/kg genistein (Fig. 6A). Consistent with the result of caspases 3 activity, after injection of LPS/\textsubscript{D}-GalN, the level of caspase-3 protein increased remarkably compared with the normal control group, and administration of genistein reduced its expression (Fig. 6B). In addition, Bcl-2 protein showed little expression in the LPS/\textsubscript{D}-GalN group, treatment with genistein augmented it (Fig. 6B).

3.6. Effects of genistein on the HO activity and the CO content.

Heme oxygenase (HO) is the rate-limiting enzyme in the catabolism of heme to biliverdin, free iron and carbon monoxide (CO). Increases in HO activity have been implicated in tissue protection against oxidative stress.\textsuperscript{18} In the present study, LPS/\textsubscript{D}-GalN induced a moderate increase of HO level, and this response was additive to genistein in dose-dependent manner (Fig. 7A). Similarly, LPS/\textsubscript{D}-GalN administration induced a marked increase in hepatic CO content at 6 h, and treatment with genistein augmented the elevation of CO content (Fig. 7B).

4. Discussion

LPS/\textsubscript{D}-GalN-induced acute liver injury in mice has been widely used as an animal model for elucidating the mechanisms of clinical liver dysfunction and for evaluating the efficacy of hepatoprotective agents.\textsuperscript{19} In this study, mice exposed to LPS/\textsubscript{D}-GalN presented significant increase of ALT and AST activities, whereas, these increases were attenuated by treatment with genistein. Moreover, the histological analysis revealed that genistein administration significantly attenuated the structural degeneration observed at 6 h after LPS/\textsubscript{D}-GalN. These results suggest that genistein has hepatoprotective effect on this model of hepatic damage.

The underlying mechanisms by which genistein protects against LPS/\textsubscript{D}-GalN-induced liver damage remain to be defined, but may be associated with several postulated pathways such as: anti-oxidant, anti-inflammatory and anti-apoptotic functions. With this view in mind,
we first analyzed the anti-oxidant effect of genistein against acute liver damage. Determination of MDA in the liver of mice revealed that genistein significantly reduced hepatic MDA concentrations in LPS/D-GalN-treated mice, suggesting that genistein possessed the anti-oxidant effect that might partially contribute to its hepatoprotective effect. In addition, reduction of the production of inflammatory mediators might be another important mechanism responsible for genistein-mediated protection. Among the inflammatory mediators, TNF-α and NO in particular, play a pivotal role in inflammatory responses involving LPS/D-GalN-induced liver injury. TNF-α is a key pro-inflammatory cytokine that is distinctly associated with the initiation or progression of inflammatory response and induces massive apoptosis of hepatocytes. NO is a highly reactive oxidant, produced by parenchymal and nonparenchymal liver cells from L-arginine via the action of inducible nitric oxide synthase (iNOS). It is well-known that NO participates in diverse physiological and pathological processes, and may exert toxicological effects such as inhibition of mitochondrial respiration and DNA synthesis as well as formation of peroxynitrite, a more long-lived cytotoxic oxidant when over-generated under stress conditions. In our investigation, we found that genistein led to a significant decrease in the production of TNF-α level, and down-regulated the expression of hepatic iNOS protein and reduced NO content as well. These results suggest that genistein could produce anti-inflammatory effects that mediated protection from liver damage.

To explore the underlying mechanism of genistein on pro-inflammatory mediators, we further detected nuclear factor-kappa B (NF-κB) activity in the liver. Under basal conditions, NF-κB is present in the cytoplasm of hepatocytes in a latent form, bound to the NF-κB inhibitory protein, inhibitor kappa B (IκB). Upon exposure to pro-inflammatory stimuli, the IκB kinase (IKK) complex is activated and catalyses the phosphorylation of IκB. Phosphorylated IκB is then targeted for degradation by the 26S proteosome complex, thereby liberating NF-κB to migrate to the cell nucleus and direct transcription of target genes. Toady, it has become clear that NF-κB plays a key role in regulation of inflammatory cytokines. In the present study, administration of LPS/D-GalN significantly enhanced liver NF-κB activity, however, treatment of mice with genistein markedly decreased its activation. This result indicate that the suppression of NF-κB by genistein may lead to a decrease in a
number of NF-κB-target genes, especially inflammatory cytokines, expressed in hepatocytes, in other words, the inhibition of inflammatory cytokines by genistein is probably due to blocking of NF-κB signaling pathway.

Recently many studies reported that genistein could impede the proinflammatory cascade of chronic inflammation associated with the development of cancer, and was able to attenuate inflammatory responses via inhibition of NF-κB activation following adenosine monophosphate-activated protein kinase (AMPK) stimulation in LPS-treated RAW 264.7 macrophages. Similarly, our result revealed that genistein alleviated the pro-inflammatory cytokines such as TNF-α through inhibiting NF-κ B activity in mice, which was consistent with the previous studies. Taken together, these studies suggest that genistein is able to attenuate inflammatory responses in vitro and in vivo, i.e., the above data provide direct evidence for the potential application of genistein in the prevention and treatment of inflammatory diseases.

Subsequently, we examined whether genistein induction possessed the anti-apoptotic effect against LPS/D-GalN caused liver injury by analysis of capase-3 and Bcl-2 in the liver. Caspase-3 is thought to be related to both death receptor and mitochondrion-dependent apoptosis and its activation will lead to hepatocyte apoptosis and induce liver damage eventually. In addition, it is established that the Bcl-2 family, including pro-apoptotic and anti-apoptotic proteins, takes part in the control of apoptosis, and the overexpression of Bcl-2 is anti-apoptotic. In this study, treatment with genistein attenuated the elevated capase-3 activity and protein expression level, while augmented Bcl-2 protein expression. These results indicate that the protective effect of genistein against LPS/D-GalN induced hepatic injury might be mediated by its anti-apoptotic effect.

Actually, LPS-induced liver inflammatory process is tightly regulated by not only pro-inflammatory mediators that initiate and exacerbate inflammation but also anti-inflammatory mediators that switch off and resolute inflammation. Heme oxygenase (HO) is the rate-limiting enzyme in heme catabolism, which leads to the generation of biliverdin, free iron, and carbon monoxide. As an inducible heat shock protein, HO has been implicated in serving as a protective gene due to its abilities in antioxidant, anti-inflammatory and antiapoptotic actions. Studies have shown that HO and its product,
carbon monoxide, can reduce tissue edema, leukocyte adhesion and migration, and production of cytokines.\textsuperscript{30} Some researchers reported HO and its products biliverdin and carbon monoxide protected mice from LPS/D-GalN-induced liver injury, prolonged survival, and reduced cytokines expression, and treatment with HO enzyme inhibitor can abrogate these protective effects of HO in the inflammatory process.\textsuperscript{4} Thus, it was believed that HO may become a liver disease therapeutic target.

Previous studies have been shown that HO induction inhibits the production of pro-inflammatory cytokines including TNF-\(\alpha\) in response to LPS \textit{in vivo} and \textit{in vitro}.\textsuperscript{31} Hence, we hypothesized that HO mediated the protective effect of genistein on LPS/D-GalN induced acute liver injury by affecting TNF-\(\alpha\) production. To clarify the idea, we examined whether genistein affected HO activity. The results revealed that LPS/D-GalN induced significant increase of HO, carbon monoxide and bilirubin levels and these alterations were augmented by the genistein treatment, indicating that HO probably mediated the protective effect of genistein against LPS/D-GalN-induced liver injury, i.e., increased HO activity by genistein is construed as protective effect of genistein by induction of oxidative defense system. However, the mechanism involved in the activation of HO protein expression by genistein in mice remains to be further investigated.

In summary, our study provided evidence that genistein significantly prevented LPS/D-GalN-induced liver damage in mice \textit{via} the underlying mechanisms of blocking NF-kB signaling pathway for anti-inflammation response, attenuating hepatocellular apoptosis, and increasing HO level as well. These findings suggest that genistein can be considered as a potential agent in the prevention of acute hepatic failure.

\textbf{Conflict of interest statement}

The authors state that there is no conflict of interest.

\textbf{Acknowledgments}

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**Figure legends**

Fig.1. The chemical structure of genistein.

Fig.2. Histomorphological examination (H&E staining, 100×). I: normal group; II: genistein control group; III: LPS/D-GalN group (model control group); IV-VI: 0.5, 1 and 2 mg/kg genistein-treated groups.

Fig.3. The effects of genistein on the activities of AST, ALT and total bilirubin. The results are presented as the means ± SE (n=15). *P< 0.05 when compared with the model
control group.

**Fig.4. The effects of genistein on the contents of MDA, TNF-α and NO and the mRNA expression levels of TNF-α and iNOS.** The Hepatic MDA (A), serum TNF-α (B) and NO production (C) were determined using commercial kits, and the TNF-α and iNOS mRNA expression levels (D) were assayed using semi-quantitative PCR. I: normal group; II: genistein control group; III: LPS/D-GalN group (model control group); IV-VI: 0.5, 1 and 2 mg/kg genistein-treated groups. The results are presented as the means ± SE (n=15). *P< 0.05 when compared with the model control group.

**Fig. 5. The effect of genistein on the nuclear NF-κB p65 content.** The results are presented as the means ± SE (n=15). *P< 0.05 when compared with the model control group.

**Fig.6. The effects of genistein on the activity of caspase-3 and the protein levels of caspase-3 and Bcl-2.** The caspase 3 activity was measured using *in vitro* fluorogenic peptide substrates (A), and the expression levels of caspase-3 and Bcl-2 proteins were determined by Western blot immunoassay (B). I: normal group; II: genistein control group; III: LPS/D-GalN group (model control group); IV-VI: 0.5, 1 and 2 mg/kg genistein-treated groups. The results are presented as the means ± SE (n=15). *P< 0.05 when compared with the model control group.

**Fig.7. The effects of genistein on the HO activity and the CO content.** The activity of HO enzyme was measured by bilirubin generation (A), and the amount of CO generated by the reaction and released into the vial headspace was quantitated by gas chromatography with a reduction gas analyzer (B). The results are presented as the means ± SE (n=15). *P< 0.05 when compared with the model control group.
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.

A

B

C

D

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Fig. 5.

![Graph showing NF-κ B p65 (pg/mg protein) levels with various treatments.](image)

Legend:
- (-): Negative
- (+): Positive
- *: Statistically significant difference

Axes:
- Y-axis: NF-κ B p65 (pg/mg protein)
- X-axis: Treatments: Genistein (-), 2 mg/kg (-), 0.5 mg/kg (+), 1 mg/kg (+), 2 mg/kg (+), LPS/D-GalN (-), (-), (+), (+), (+, respectively.)
Fig. 6.

A

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B
Fig. 7.