

Basic Study

Protective effect of *Salvia miltiorrhiza* and *Carthamus tinctorius* extract against lipopolysaccharide-induced liver injury

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Author contributions: Gao LN and Yan K performed the majority of experiments; Gao LN and Cui YL performed the literature research; Gao LN, Fan GW, Wang YF and Yan K performed the data acquisition and data analysis; Gao LN drafted the manuscript and made critical revisions related to important intellectual content of the manuscript; Cui YL designed the whole study and reviewed the manuscript; all authors read and approved the final manuscript.

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Animal care and use statement: The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (24 °C ± 1 °C, 12 h/12 h light/dark, 55% ± 5% humidity, ad libitum water) for

two weeks prior to experimentation. Intraperitoneal injection administration was carried out with conscious animals, using syringes of 1 mL. All animals were given ether anesthesia for tissue and blood collection.

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Abstract

AIM: To investigate the hepatoprotective effects and mechanisms of an extract of *Salvia miltiorrhiza* and *Carthamus tinctorius* *in vivo*.

METHODS: C57BL/6J mice were randomly assigned to five groups and intraperitoneally administered 0.9% saline, *Salvia miltiorrhiza* and *Carthamus tinctorius* extract [Danhong injection (DHI), 0.75 and 3 g/kg mixed extract] or reduced glutathione for injection (RGI, 300 mg/kg) for 30 min before exposure to lipopolysaccharide (LPS, 16 mg/kg). After intraperitoneal LPS stimulation for 90 min or 6 h, the mice were sacrificed by ether anaesthesia, and serum and liver samples were collected. Histological analysis (H&E) and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) staining were performed. Alanine transferase (ALT), aspartate transaminase (AST), total bilirubin (TBil), glutathione-S-transferase (GST), malondialdehyde (MDA), tumour necrosis factor (TNF)- α , interleukin (IL)-6, and caspase-3 levels were measured. Bax, Bcl-2, P-I κ B α , I κ B α , P-NF- κ B p65, and NF- κ B p65 protein levels were determined by Western blot. TNF- α , IL-6, caspase-3, Bax and Bcl-2 mRNA expression was measured by real-time reverse transcription-polymerase chain reaction (RT-PCR).

RESULTS: Hematoxylin-eosin staining and TUNEL results suggested that DHI (3 g/kg) treatment alleviated inflammatory and apoptotic ($P < 0.01$) injury in the liver of mice. DHI treatment dose-dependently blunted the abnormal changes in biochemical parameters such as ALT (72.53 ± 2.83 for 3 g/kg, $P < 0.01$), AST (76.97 ± 5.00 for 3 g/kg, $P < 0.01$), TBil (1.17 ± 0.10 for 3 g/kg, $P < 0.01$), MDA (0.81 ± 0.36 for 3 g/kg, $P < 0.01$), and GST (358.86 ± 12.09 for 3 g/kg, $P < 0.01$). Moreover, DHI (3 g/kg) remarkably decreased LPS-induced protein expression of TNF- α (340.55 ± 10.18 for 3 g/kg, $P < 0.01$), IL-6 (261.34 ± 10.18 for 3 g/kg, $P < 0.01$), and enzyme activity of caspase-3 (0.93 ± 0.029 for 3 g/kg, $P < 0.01$). The LPS-induced mRNA expression of TNF- α , IL-6 and caspase-3 was also decreased by DHI. Western blot analysis revealed that DHI antagonised LPS-stimulated decrease of Bcl-2 and increase of Bax protein expression. Furthermore, DHI inhibited LPS-induced I κ B α and NF- κ B p65 phosphorylation.

CONCLUSION: DHI may be a multi-function protectant against acute hepatic injury in mice through its anti-inflammatory, anti-oxidative and anti-apoptotic activities.

Key words: *Salvia miltiorrhiza*; *Carthamus tinctorius*; Apoptosis; Anti-inflammatory; Antioxidant; Acute liver injury

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Core tip: *Salvia miltiorrhiza* and *Carthamus tinctorius* extract [Danhong injection (DHI)] effectively protected against hepatic injury. DHI intervention significantly reduced alanine transferase, aspartate transaminase, total bilirubin, malondialdehyde, glutathione-S-transferase, tumour necrosis factor- α , interleukin-6, and caspase-3 levels in an lipopolysaccharide (LPS)-induced mouse model of acute liver injury. Moreover, DHI antagonised LPS-induced Bcl-2 and Bax expression and inhibited I κ B α and nuclear factor- κ B p65 phosphorylation. These findings suggest that *Salvia miltiorrhiza* and *Carthamus tinctorius* extract (such as DHI) acts as a multi-function protectant against acute hepatic injury in mice through its anti-inflammatory, anti-oxidative and anti-apoptotic activities.

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INTRODUCTION

Substantial evidence suggests that *Salvia miltiorrhiza* Bge. (Lamiaceae) is hepatoprotective against hepatic toxicity and ischemia/reperfusion injury *via* anti-oxidative effects and improved microcirculation^[1]. Similarly, Carthamus Red from *Carthamus tinctorius* L. (Composite) has hepatoprotective effects against CCl₄-induced liver injury^[2]. The combination of *Salvia miltiorrhiza* and *Carthamus tinctorius* extracts has been traditionally used for synergistic therapeutic effects on activating blood circulation and resolving stasis and to treat ischemic encephalopathy and coronary heart disease in the clinic^[3-5]. As a classic prescription of the *Salvia miltiorrhiza* and *Carthamus tinctorius* herbal pair, Danhong injection (DHI) is composed of a 30% ethanol extract of the *Salvia miltiorrhiza* root and rhizome and an aqueous extract of the *Carthamus tinctorius* flower according to standard extraction processes. High-performance liquid chromatography (HPLC) fingerprinting of DHI has been widely performed. The primary active components of DHI include tanshinone IIA, danshensu, protocatechuic aldehyde, rosmarinic acid, hydroxysafflor yellow A, and salvianolic acid B^[6-9]. Based on the literature, we proposed that the *Salvia miltiorrhiza* and *Carthamus tinctorius* extract (namely DHI in this study) may be beneficial for hepatic protection.

Hepatic diseases are a global human health problem, with high morbidity and mortality^[10]. There is no effective treatment for fatal liver disease, such as hepatic failure. Although hepatic transplantation is

associated with a high survival rate (50%-70%)^[11], insufficient organ donation and high expenses limit its application. Therefore, the hepatoprotective effects of various drugs have been evaluated in animal models.

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria, and the core-lipid A region is the toxic moiety of LPS. LPS impairs the liver by acting as a hepatotoxin^[12,13]. The liver provides the first line of defence against bacteria and their products^[14]. In animal models, various reagents, such as carbon tetrachloride^[15], D-galactosamine/LPS^[16] and concanavalin A^[17], have been used to induce acute liver injury. In this study, we induced acute liver injury in mice by LPS alone; a 16 mg/kg dose of LPS is sufficient to cause lung, liver and kidney injury^[18]. Simultaneously, we employed reduced glutathione for injection (RGI) as a positive control drug against LPS-induced acute liver injury. We investigated the hepatoprotective effects of DHI and explored the underlying mechanisms.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (18-20 g) were purchased from Beijing HuaFukang Bio-technology Co., Ltd and housed under standard laboratory conditions with controlled light (on at 7:00 am and off at 7:00 pm), comfortable temperature (24 °C ± 1 °C) and standard humidity (55% ± 5%). All mice in this study were used in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Ethics Committee of Tianjin University of Traditional Chinese Medicine (TCM-2009-037-E10).

Reagents

DHI was purchased from Shandong Buchang Pharmaceutical Co., Ltd (Jinan, China) (Drug approval number: Z20026866). LPS from *Escherichia coli* 0111: B4, anti-mouse IgG peroxidase conjugate and β -actin monoclonal antibody were obtained from Sigma-Aldrich Co. (St. Louis, United States). Interleukin (IL)-6 and tumour necrosis factor (TNF)- α mouse ELISA kits and anti-mouse Bax and Bcl-2 monoclonal antibodies were obtained from eBioscience (San Diego, United States). Mammalian Cell Lysis Kits, Bradford Protein Assay Kits and UNIQ-10 column Trizol total RNA extraction kits were obtained from Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). FastStart Universal SYBR Green Master (ROX) kits were purchased from Roche (Mannheim, Germany). Detection kits for alanine transferase (ALT), aspartate transaminase (AST), total bilirubin (TBil) and glutathione-S-transferase (GST) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The assay kits for malondialdehyde (MDA), terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL), cell apoptosis detection,

and Nuclear and Cytoplasmic Protein Extraction were purchased from Beyotime Institute of Biotechnology (Haimen, China). RGI was obtained from LvYe Pharmaceutical Co., Ltd (Yantai, China). The BCA Protein Assay Kit was purchased from Thermo Pierce (Rockford, United States). The caspase-3 colorimetric assay kit was obtained from Enzo Life Science (Farmingdale, United States). Antibodies against phospho-I κ B- α , I κ B α , phospho-NF- κ B p65, NF- κ B p65 and the peroxidase-conjugated secondary antibody were purchased from Cell Signaling Technology (Beverly, United States). Goat anti-rabbit IgG peroxidase conjugate was purchased from Calbiochem (Darmstadt, Germany).

DHI extraction, preparation and quality control standard

According to the standard extraction process drafted by Shandong BuChang Pharmaceutical Co. Ltd and approved by the State Food and Drug Administration (SFDA) of China, *Salvia miltiorrhiza* and *Carthamus tinctorius* extracts were prepared as DHI. First, *Salvia miltiorrhiza* (750 g) was immersed in 7.5 L of 30% ethanol, extracted twice for 1 h at 50 °C, and filtered. *Carthamus tinctorius* (250 g) was mixed with the material remainder by filtration of the *Salvia miltiorrhiza* extract and immersed twice in 2.5 L water for 1 h at 35 °C. Second, the aqueous solution was mixed with the alcoholic extract and vacuum evaporated to a relative density of 1.0-1.2 (65 °C). Third, isotonic sodium chloride injection was added to adjust the pH level to 6-7. DHI was then prepared after filtration, potting and sterilisation.

The DHI quality control standard stipulated by SFDA of China is that the total amount of Danshensu (molecular formula: C₉H₁₀O₅) and protocatechuic aldehyde (molecular formula: C₇H₆O₃) should not be less than 0.5 mg in a 1-mL injection analysed by HPLC. In addition, the total flavonoids determined by visible spectrophotometry should not be less than 5.0 mg/mL as determined using rutin as a reference (molecular formula: C₂₇H₃₀O₁₆)^[3,19].

Experimental design

In the present study, we performed two parallel animal experiments. In each experiment, mice were randomly assigned to 5 groups (8 animals each): blank control; LPS (dissolved in sterile pyrogen-free saline solution, 16 mg/kg body weight); LPS + DHI (0.75 and 3 g/kg, relative to human clinical dosage); positive control, LPS + RGI (300 mg/kg). The DHI dosage representation was in g/kg (crude drugs weight/body weight). The quantities of the crude drugs were calculated such that the extract contained 750 g of *Salvia miltiorrhiza* and 250 g of *Carthamus tinctorius* per litre. Therefore, the g/kg dosage representation is equivalent to the mL/kg^[3] found in many other references. Mice were intraperitoneally injected with 0.9% saline (for the control group), DHI and RGI 30 min prior to a single LPS injection (0.9% saline for the blank control group).

Table 1 Oligonucleotide primers for real-time reverse transcription polymerase chain reaction

Gene	Primer	Sequence (5'-3')	PCR product (bp)
<i>β-actin</i> (NM_007393.3)	Forward	TGTTACCAACTGGGACGACA	165
	Reverse	GGGGTGTGAAGGCTCTCAA	
<i>TNF-α</i> (NM_013693.2)	Forward	TAGCCAGGAGGAGAACAGA	127
	Reverse	TTTTCTGGAGGAGATGTGG	
<i>IL-6</i> (NM_031168.1)	Forward	TCCAGTTGCCTTCTTGGGAC	140
	Reverse	GTGTAATTAAGCCTCCGACITG	
<i>Bax</i> (NM_007527.3)	Forward	CTGCAGAGGATGATTGCTGA	174
	Reverse	GATCAGCTCGGGCACTTTAG	
<i>Bcl-2</i> (NM_177410.2)	Forward	GGACTTGAAGTGCCATTGGT	127
	Reverse	AGCCCCCTCTGTGACAGCTTA	
<i>Caspase-3</i> (NM_009810.2)	Forward	ATGGGAGCAAGTCAGTGGAC	137
	Reverse	CGTACCAGAGCGAGATGACA	

PCR: Polymerase chain reaction.

Different volumes of DHI and RGI were dissolved in 0.9% saline to the same total volume of the maximum administration dosage. After LPS exposure for 90 min or 6 h, the mice were anaesthetised with ether, and blood was collected. Subsequent cardiac perfusion was performed prior to liver tissue collection to avoid influencing the liver tissue results. Serum was separated by centrifugation at 3000 rpm for 15 min at 4 °C. Both serum and liver tissues were frozen at -80 °C until use.

Haematoxylin and eosin staining and TUNEL assay

For histological analysis, liver specimens obtained after 90-min LPS exposure were fixed in 10% neutral-buffered formalin, routinely processed, and sectioned (5 μm thick). After haematoxylin and eosin (H&E) staining, live specimens were photographed under a light microscope (Olympus, Tokyo, Japan) at magnification × 200. For hepatocyte apoptosis detection, liver tissues were collected after 6-h LPS stimulation. The TUNEL assay was performed to label the 3'-end of fragmented DNA in liver sections according to the manufacturer's instructions (Beyotime, China). Finally, slides were examined by fluorescence microscopy (Nikon, Japan), and quantitative statistical analysis was performed using NIH Image J software.

ALT, AST and TBil, IL-6, TNF-α, MDA, GST and caspase-3 measurements

For ALT, AST and TBil detection, serum samples were collected after 6-h LPS stimulation. For IL-6 and TNF-α analysis, liver tissues were harvested from mice stimulated with LPS for 90 min. In addition, liver tissues collected for measuring MDA production and GST activity were stimulated with LPS for 6 h. Liver tissues were homogenised in phosphate-buffered saline (PBS) and centrifuged at 8000 rpm for 15 min at 4 °C. Supernatant aliquots were stored at -80 °C until use. All ALT, AST, TBil, IL-6, TNF-α, MDA, GST

and caspase-3 measurements were performed using commercially available kits. Caspase-3 activity was determined using a modified protocol in which a standard curve was prepared with pNA diluted in buffer (assay buffer and lysis buffer were mixed at a ratio of 9:1). One unit was the amount of enzyme that cleaved 10⁻⁹ M Ac-DEVD-pNA colorimetric substrate per hour at 37 °C under saturated substrate concentrations.

Real-time RT-PCR analysis of TNF-α, IL-6, caspase-3, Bcl-2 and Bax mRNA expression in mouse liver

Liver tissues were stimulated with LPS for 90 min for TNF-α and IL-6 expression analysis and for 6 h for the detection of caspase-3, Bcl-2 and Bax. Real-time RT-PCR was performed as described previously^[12], and oligonucleotide primers used for mouse TNF-α, IL-6, caspase-3, Bcl-2, Bax and β-actin (an internal control) are listed in Table 1.

Western blot analysis for Bcl-2, Bax and NF-κB proteins in mouse liver

For Bcl-2 and Bax analysis, livers were obtained after LPS treatment for 6 h. For NF-κB family detection, livers were collected after LPS treatment for 90 min. Livers were lysed using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's protocol. The protein concentration in the supernatant was determined using the BCA method. For Western blot analysis, equal quantities (20 μg) of protein were boiled for 5 min and subjected to SDS-PAGE, followed by electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with TTBS (0.5% Tween 20, 10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl) containing 5% non-fat milk for 1 h at room temperature, followed by incubation with antibodies against phospho-IκB-α, IκBα, phospho-NF-κB p65, NF-κB p65 (1:1000, Cell Signaling Technology, United States), Bax, Bcl-2 (1:1000, eBioscience, United States) or β-actin (1:5000, Sigma-Aldrich Co., United States) overnight at 4 °C. The membranes were washed and incubated with HRP-conjugated secondary antibodies against mouse (1:1000 for IκBα, Bax and Bcl-2, and 1:10000 for β-actin, Sigma-Aldrich Co., United States) or rabbit (1:1000 for phospho-IκB-α, phospho-NF-κB p65 and NF-κB p65, Calbiochem, Germany) for 1 h at room temperature. After washing, protein bands were detected using an ECL detection kit (Millipore, United States) and exposed to Kodak BioMax Light films. Films were quantified using NIH Image J software.

Statistical analysis

Statistical analyses were performed with Origin 7.5 software (MicroCal, United States). Data are expressed as the mean ± SE. Differences between the values of various experimental groups were assessed by one-way analysis of variance (ANOVA), and *P*-values less

than 0.05 were considered statistically significant.

The statistical methods of this study were reviewed by Jing-Bo Zhai from Tianjin University of Traditional Chinese Medicine.

RESULTS

Effects of DHI on hepatic histopathology and apoptosis

H&E staining analysis of liver sections (Figure 1A) revealed intact lobular architecture and normal hepatocyte structure in mice that did not receive LPS (a). However, upon LPS exposure (b), histological changes primarily characterised by fat degeneration occurred, which are indicated by arrows in Figure 1A. Moreover, hepatic cells became loosely and irregularly arranged. By contrast, DHI administration prior to LPS exposure (c, d) ameliorated these changes, as only slight fat degeneration or few inflammatory granulocyte and monocyte infiltrates were visible. RGI (e) also alleviated inflammatory injury, although not to the same extent as 3 g/kg DHI (d).

To confirm that DHI prevented hepatocyte apoptosis, we performed TUNEL assays on liver sections (Figure 1B). Following LPS treatment, mouse livers (g) displayed increased hepatocyte damage and abundant TUNEL-positive hepatocytes, whereas we did not observe any TUNEL-positive cells in the blank control group (f). When we administered DHI or RGI (h-j) prior to LPS, DHI (3 g/kg) and RGI both significantly alleviated LPS-induced apoptotic injury ($P < 0.01$, Figure 1C). Taken together, these results indicate that DHI attenuated massive LPS-induced hepatocyte apoptosis in a dose-dependent manner.

Effects of DHI on LPS-induced increase in ALT and AST activities and TBil production in serum

To examine hepatic function, we examined ALT and AST activities and TBil production. LPS alone increased ALT (A) and AST activity (B) and TBil production (C) by 363.3%, 265.7% and 170.1%, respectively, in mouse serum (Figure 2). However, DHI or RGI pre-treatment significantly and dose-dependently decreased these activities ($P < 0.05$ or $P < 0.01$).

Effects of DHI on LPS-induced increase in GST activities and MDA production in liver homogenates

To assess the degree of oxidative stress, we measured MDA and GST levels. As shown in Figure 3, liver levels of MDA (A) and GST (B) increased by 2.68-fold and 1.44-fold, respectively, in mice stimulated with LPS or saline as a control. However, DHI or RGI administration prior to LPS markedly ameliorated oxidative injury, as evidenced by reductions in MDA (0.81 ± 0.36 for 3 g/kg, $P < 0.01$) and GST (358.86 ± 12.09 for 3 g/kg, $P < 0.01$) levels.

Effects of DHI on liver TNF- α and IL-6 expression

Compared to the LPS-alone group, DHI (3 g/kg)

significantly reduced the LPS-induced increase in TNF- α protein (Figure 4A, $P < 0.01$) and mRNA expression (Figure 4B, $P < 0.01$). Similarly, LPS alone increased IL-6 protein and mRNA expression by 317.0% and 363.1%, respectively, compared to saline treatment. After DHI administration, IL-6 protein levels significantly decreased in a dose-dependent manner (Figure 4C, $P < 0.01$). However, only the high dose of DHI (3 g/kg) exhibited a marked inhibitory effect ($P < 0.01$) on IL-6 mRNA expression (Figure 4D, 3.06 ± 0.043 , $P < 0.01$) and caspase-3 activity (0.93 ± 0.029 , $P < 0.01$).

Effects of DHI on caspase-3 activity in the liver

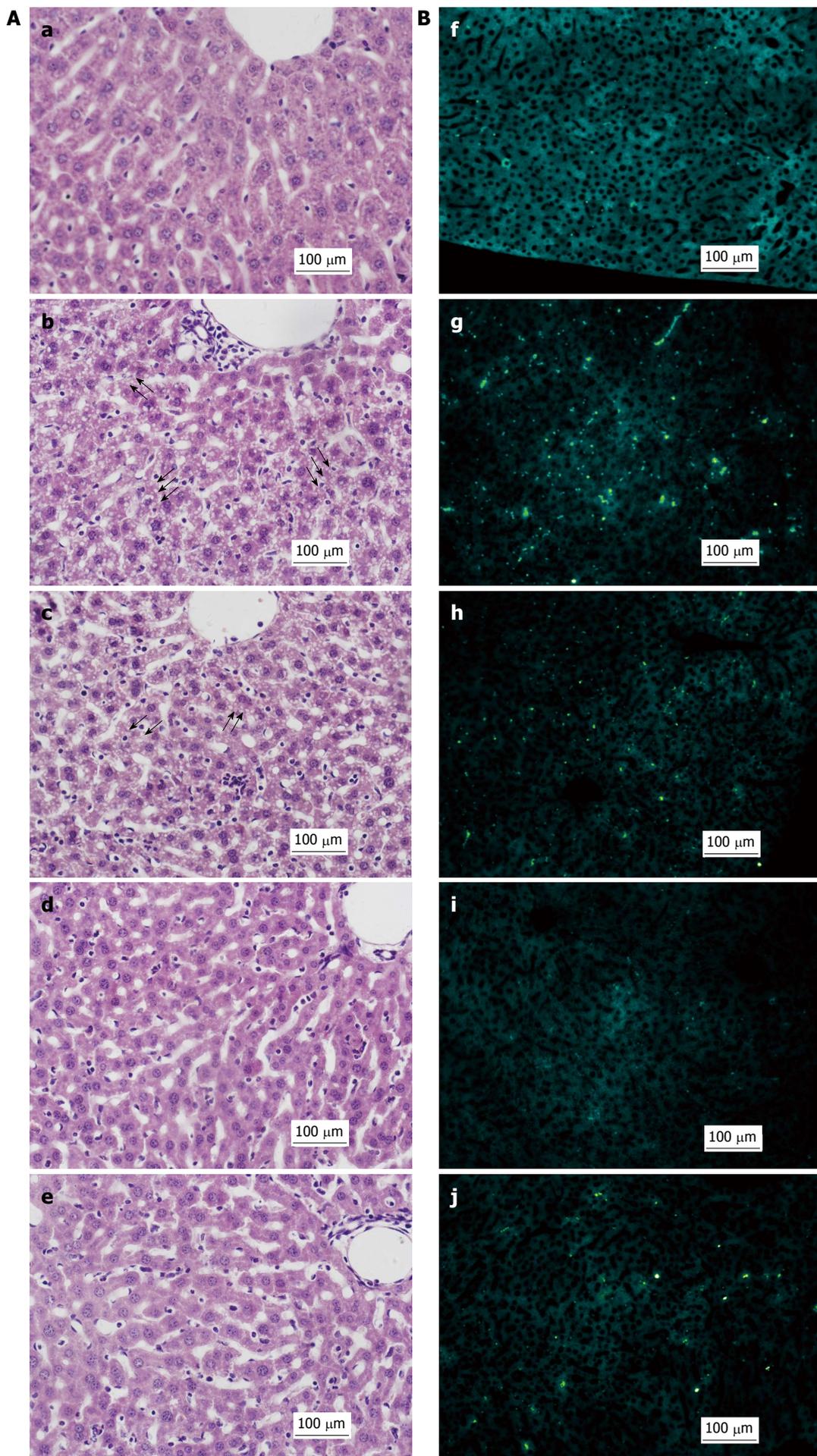
To further investigate the anti-apoptotic and hepatoprotective effects of DHI, we measured caspase-3 activity (Figure 5) by examining enzyme activity and mRNA expression. Compared to the blank control group, LPS significantly increased caspase-3 activity by 217.6%, whereas DHI markedly blunted caspase-3 activity (0.93 ± 0.029 for 3 g/kg, $P < 0.01$). DHI (3 g/kg) also significantly decreased caspase-3 mRNA expression compared to LPS treatment alone (0.63 ± 0.044 , $P < 0.01$), but RGI treatment had no effect on caspase-3 mRNA expression compared to LPS treatment alone.

Effects of DHI on liver Bcl-2 and Bax expression

Real-time RT-PCR (Figure 6A and B) revealed that LPS treatment led to an increase in Bax mRNA expression (242.2%, $P < 0.01$) and a decrease in the mRNA expression of Bcl-2 (44.2%, $P < 0.01$), an integral membrane anti-apoptotic protein. However, the aberrant changes in Bax and Bcl-2 expression were alleviated by DHI administration ($P < 0.05$ or $P < 0.01$). Moreover, we analysed Bcl-2 and Bax protein expression by Western blot (Figure 6E). The relative optical density (Figure 6C and D) showed that LPS up-regulated Bax protein expression ($P < 0.01$). However, DHI antagonised this LPS-induced protein expression ($P < 0.01$). Moreover, DHI significantly increased Bcl-2 protein expression compared to LPS treatment alone ($P < 0.01$). In summary, DHI protected hepatocytes from apoptotic injury by balancing Bax and Bcl-2 expression.

Effects of DHI on liver NF- κ B protein expression

The NF- κ B family plays a key role in pro-inflammatory and anti-apoptosis processes. Generally, NF- κ B/Rel proteins enter the cytoplasm as homodimers or heterodimers in an inactive state in complexes with inhibitory I κ B proteins. Upon LPS treatment (Figure 7), we observed I κ B α degradation and phosphorylation, which revealed that DHI alleviated the LPS-induced phospho-I κ B α up-regulation ($P < 0.05$). DHI (3 g/kg) significantly antagonised the LPS-induced I κ B α increase ($P < 0.05$). To determine if NF- κ B p65 translocation and phosphorylation occurred during acute



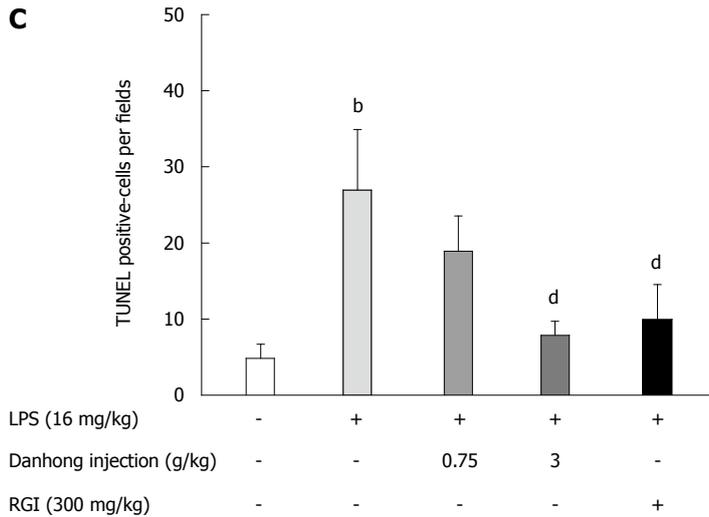


Figure 1 Effects of Danhong injection on hepatic histopathology and apoptosis. A: Hematoxylin-eosin staining (magnification $\times 200$); B: TUNEL assay (magnification $\times 200$), (a/f) Blank control group, (b/g) Lipopolysaccharide (LPS)-stimulated group, (c/h) Danhong injection (DHI) (0.75 g/kg) + LPS-stimulated group, (d/i) DHI (3 g/kg) + LPS-stimulated group, and (e/j) RGI (300 mg/kg) + LPS-stimulated group; C: Analysis of apoptotic cell numbers. The number of apoptotic cells per field (1 mm^2) was determined using NIH Image J software ($n = 6$). ^b $P < 0.01$ vs blank control group, ^d $P < 0.01$ vs LPS-treated group. Arrows indicate fat degeneration.

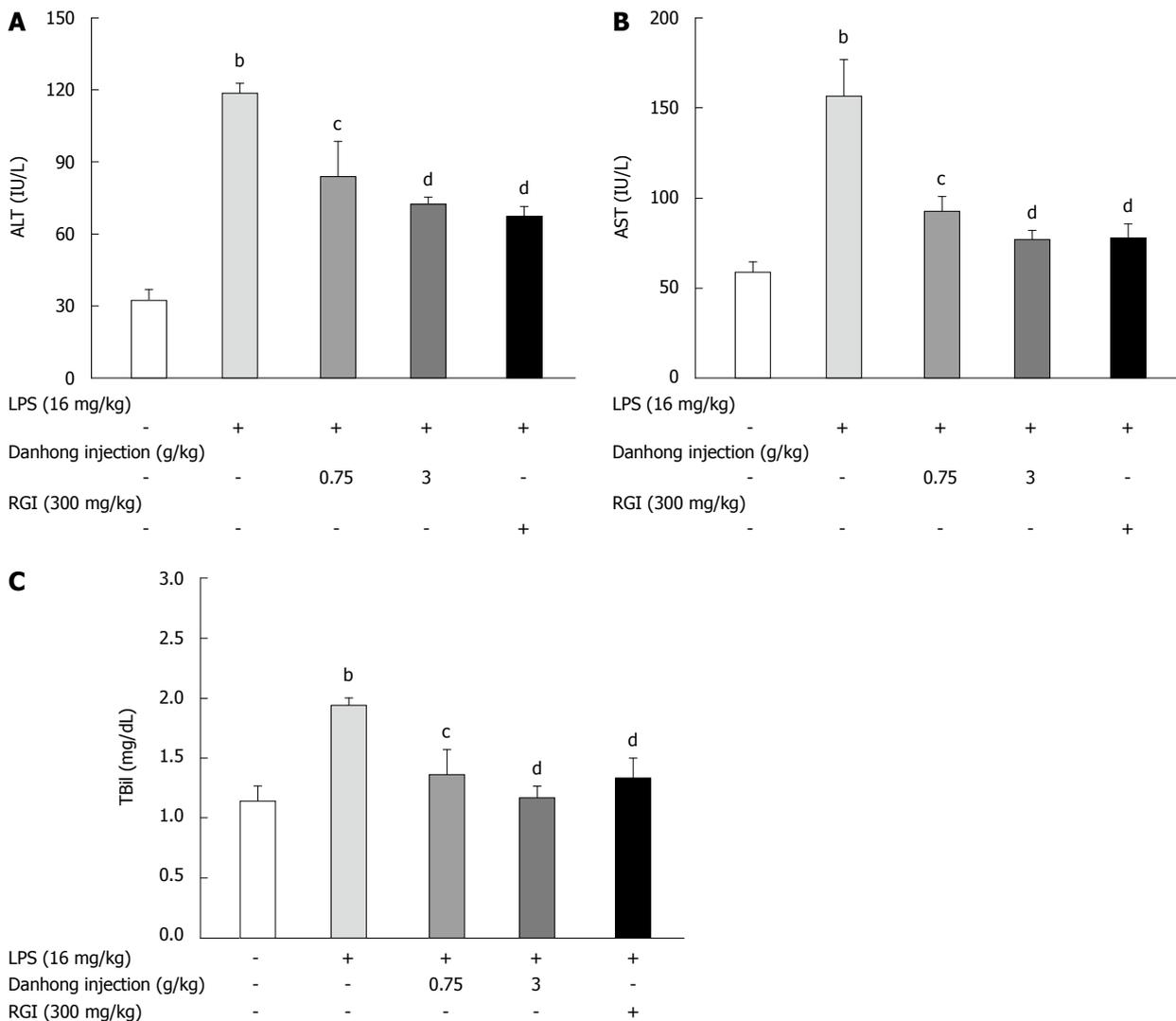


Figure 2 Effects of Danhong injection on serum alanine transferase and aspartate transaminase activities and total bilirubin production in lipopolysaccharide-treated mice. Different groups of mice were pre-treated with glutathione for injection (RGI) or 0.9% saline for 30 min before lipopolysaccharide (LPS) or 0.9% saline injection for 6 h. Values are mean \pm SE ($n = 8$). ^b $P < 0.01$ vs blank control group, ^c $P < 0.05$, ^d $P < 0.01$ vs LPS-treated group.

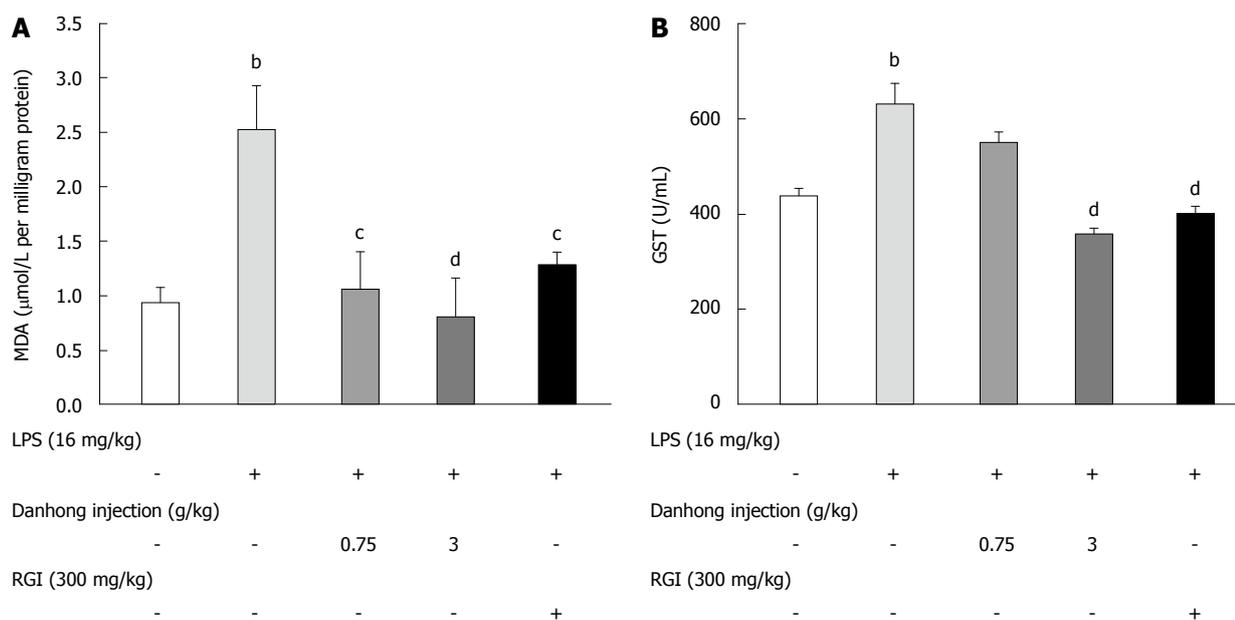


Figure 3 Effects of Danhong injection on malondialdehyde production and glutathione-S-transferase activity in liver homogenates of lipopolysaccharide-induced mice. Mice were pre-treated with Danhong injection (DHI), glutathione for injection (RGI) or 0.9% saline for 30 min before lipopolysaccharide (LPS) or 0.9% saline injection for 6 h. Values are mean \pm SE ($n = 8$). ^b $P < 0.01$ vs blank control group, ^c $P < 0.05$, ^d $P < 0.01$ vs LPS-treated group.

liver injury, we examined the effects of DHI treatment on LPS-induced NF- κ B p65 and phospho-NF- κ B p65 by Western blot, which revealed that DHI significantly reduced the up-regulation of NF- κ B p65 ($P < 0.01$) and phospho-NF- κ B p65 ($P < 0.05$ or $P < 0.01$).

DISCUSSION

Studies have established that *Salvia miltiorrhiza* plays a vital role in vasorelaxation^[20], scavenging oxygen free radicals, preventing lipid peroxidation, and combatting inflammation^[21]. The primary pharmacological action of *Carthamus tinctorius* is to improve microcirculation, antagonise coagulation and thrombosis, and inhibit the inflammatory reaction^[22-25]. Modern pharmacological studies have determined that *Salvia miltiorrhiza* and *Carthamus tinctorius* extracts in the form of a compound preparation (such as DHI, a classic preparation) are a potential anti-oxidative^[26,27] agent and an anti-inflammatory drug^[11]. The anti-inflammatory activity of DHI is primarily due to hydroxysafflor yellow A, and its anti-oxidative capacity relies on salvianolic acid B. Danshensu has the strongest anti-apoptotic effect^[6]. To evaluate the safety of DHI, Wang *et al.*^[28] investigated its acute and chronic toxicity by intravenous injection, determining an LD₅₀ of 39.5 mL/kg, more than 10-fold higher than the high dosage used here.

We performed novel pharmacological explorations of the effects of DHI on liver injury. H&E staining indicated that DHI largely ameliorated fat degeneration, indicating its hepatic protective capability. Second, DHI regulated ALT, AST, TBil and MDA levels and GST enzyme activities, suggesting that DHI repairs hepatic

function and alleviates oxidative stress.

The regulation of liver function is dependent on communication between cells (hepatocytes, endothelial cells, Kupffer cells and others) and cytokines. TNF- α is required for the proliferation of normal hepatocytes and exerts an anti-apoptotic effect by inducing NF- κ B^[26,29]. However, excessive TNF- α may mediate hepatotoxicity by inducing apoptosis^[30,31]. Furthermore, TNF-induced hepatocyte injury provides a vital signal for polymorphonuclear (PMN) transmigration from sinusoids into the parenchymal tissues, which leads to cellular necrosis^[32]. IL-6 is secreted from activated macrophages and is a potent inducer of the hepatic acute phase response^[33]. For mice with LPS-induced acute liver injury, DHI notably inhibited the increase in IL-6 and TNF- α protein and mRNA expression. These results confirm that DHI can improve hepatic function and protect the liver from oxidative stress and inflammatory damage.

The transcription factor NF- κ B is involved in the regulation of many important cellular and physiological processes, such as growth, apoptosis, and the immune and inflammatory responses^[33-35]. Further exploring the mechanisms of DHI in acute mouse liver injury, we observed that LPS induced the NF- κ B signalling pathway and TNF- α secretion *via* I κ B α phosphorylation triggered NF- κ B p65 translocation to the nucleus stimulated the inflammatory cascade reaction. The released inflammatory mediators, such as TNF- α and IL-6, could then multiply the toxic effects of LPS on multiple organs and lead to multiple organ dysfunctions. However, DHI treatment inactivated the NF- κ B pathway and protected against hepatic injury-induced deterioration.

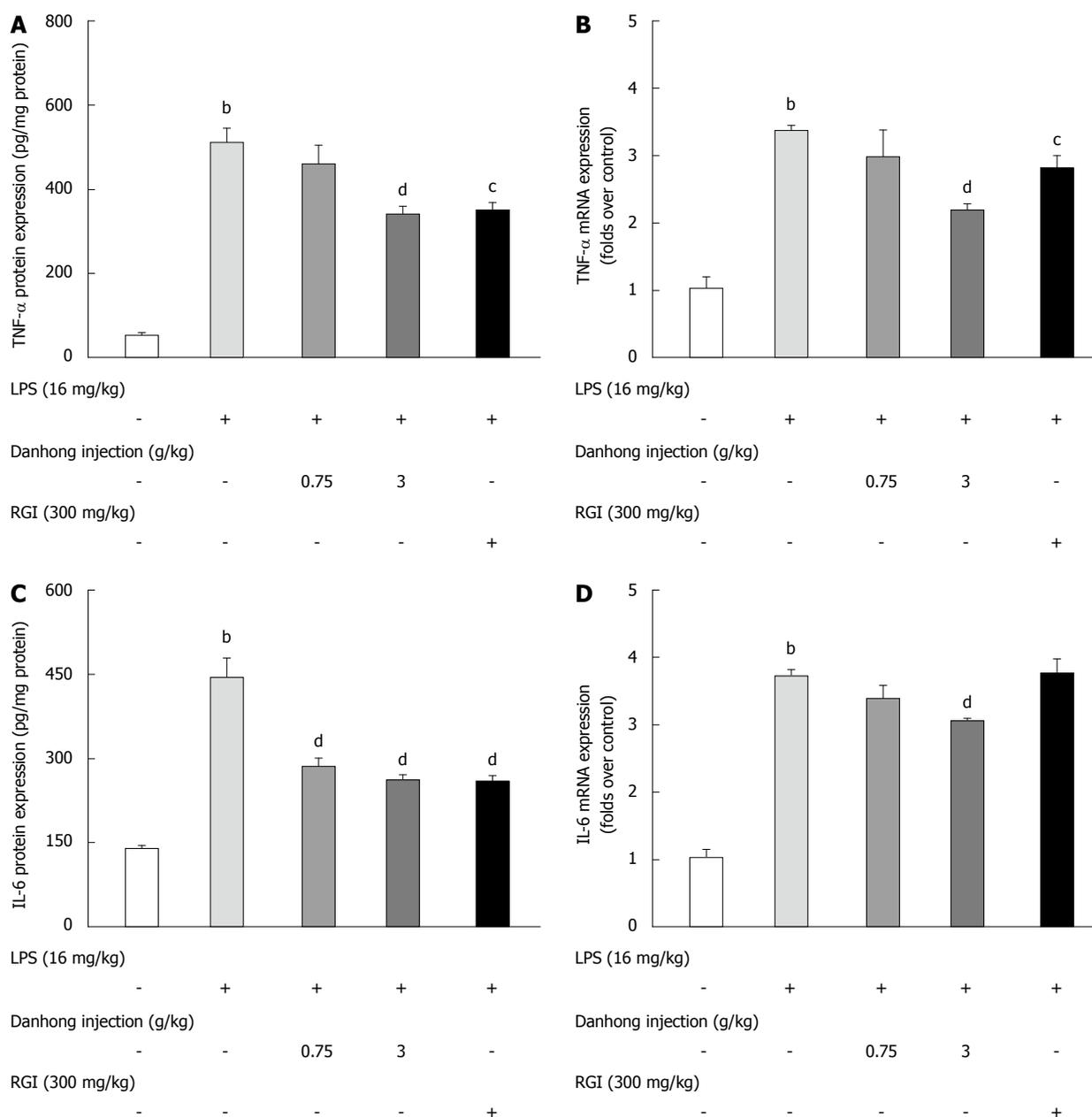


Figure 4 Effects of Danhong injection on tumour necrosis factor- α and interleukin-6 expression in mouse liver. The protein expression levels of tumour necrosis factor (TNF)- α (A) and interleukin (IL)-6 (C) were measured by ELISA. Values are mean \pm SE ($n = 8$). TNF- α (B) and IL-6 (D) mRNA expression was detected by real-time RT-PCR. Values are mean \pm SE ($n = 3$) of three independent experiments. ^b $P < 0.01$ vs blank control group, ^c $P < 0.05$, ^d $P < 0.01$ vs lipopolysaccharide (LPS)-treated group.

Acute inflammation can also induce apoptotic injury^[36]. Excess TNF- α is an important inducer of endotoxemia-induced liver injury and accelerates hepatocyte apoptosis^[37]. To explore whether apoptosis is involved in LPS-induced liver injury, we performed TUNEL assays. Our results revealed that LPS administration led to striking hepatocyte apoptosis with focal concentration. However, DHI blunted the apoptotic response. Caspase-3 is indispensable for some typical characteristics of apoptosis, such as chromatin condensation and DNA fragmentation^[38]. The detection of caspase-3 revealed that DHI treatment alleviated LPS-induced caspase-3 activity

at both the enzymatic activity and mRNA expression level. We also propose that the reduced caspase-3 activity was due to a decrease in mRNA expression or the dual role of mRNA expression and enzyme activity. We did not observe a significant change in caspase-8 activity. The key anti-apoptotic protein Bcl-2 can inactivate caspases^[35,39]. Further investigating the possible anti-apoptotic mechanism of DHI, we observed that DHI reduced Bax expression and increased Bcl-2 expression. As mentioned above, we propose that DHI inhibits hepatic apoptosis by balancing Bcl-2 and Bax levels.

In conclusion, this study focused on the effect of

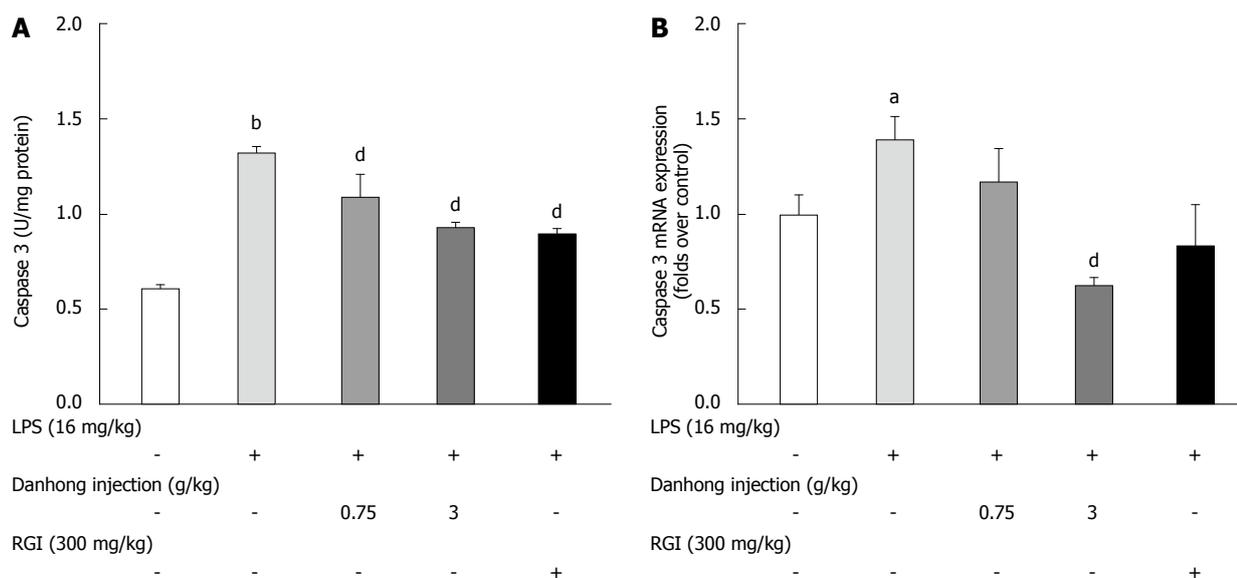
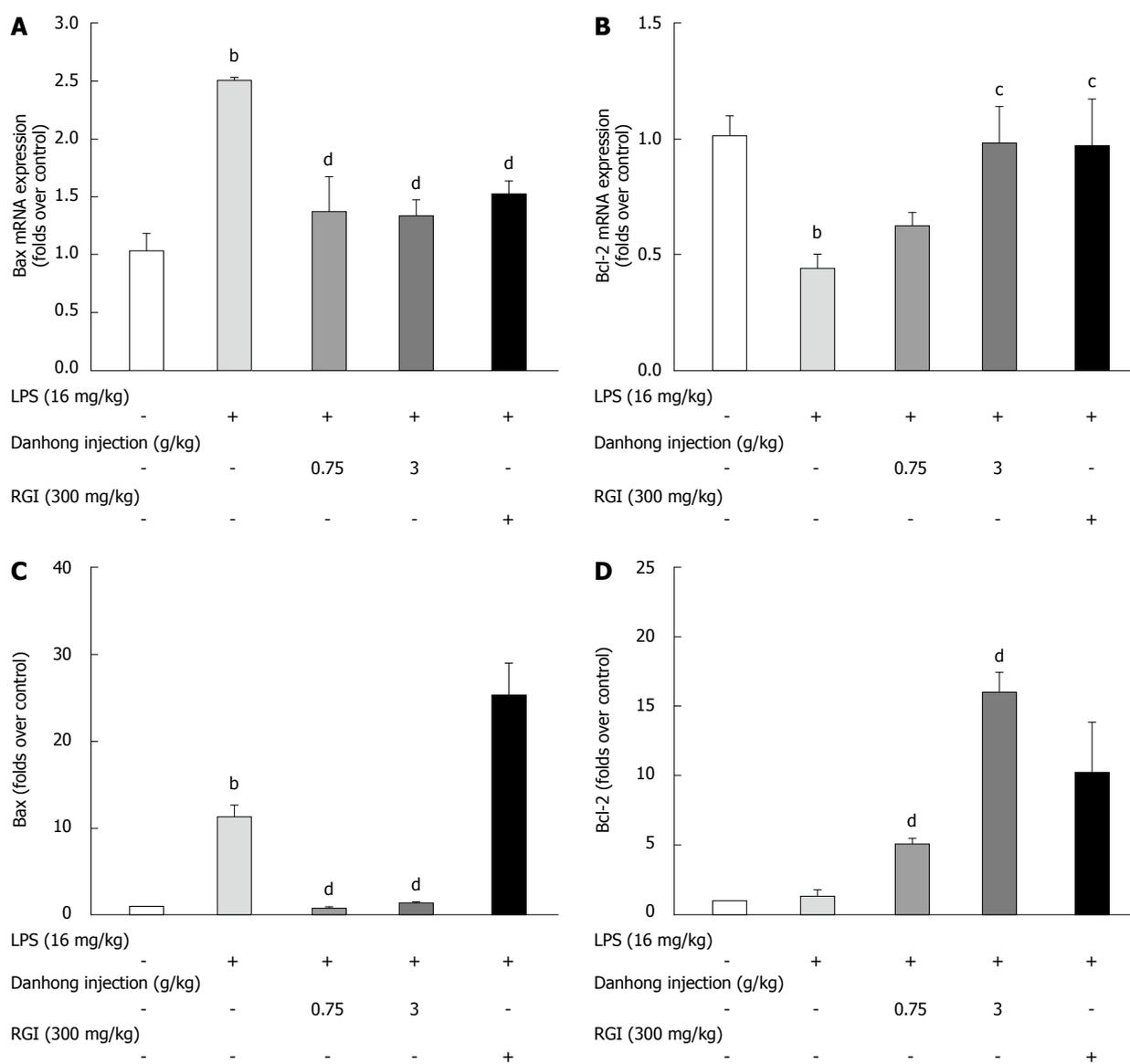


Figure 5 Effects of Danhong injection on liver caspase-3 activity. A: Caspase-3 enzymatic activity was measured using a modified commercial kit. Values are mean \pm SE ($n = 8$); B: Caspase-3 mRNA expression was detected by real-time reverse transcription-polymerase chain reaction. Values are mean \pm SE ($n = 3$) of three independent experiments. ^b $P < 0.05$, ^d $P < 0.01$ vs blank control group, ^a $P < 0.01$ vs lipopolysaccharide (LPS)-treated group.



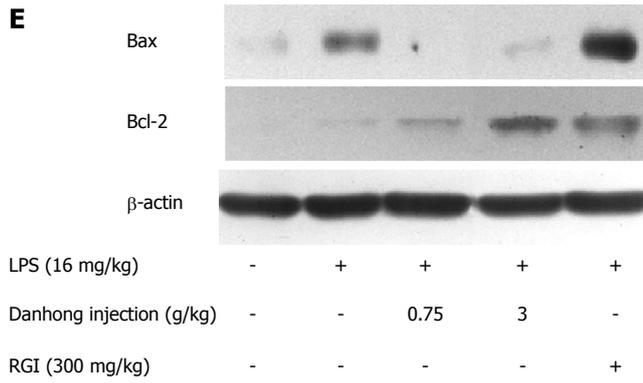
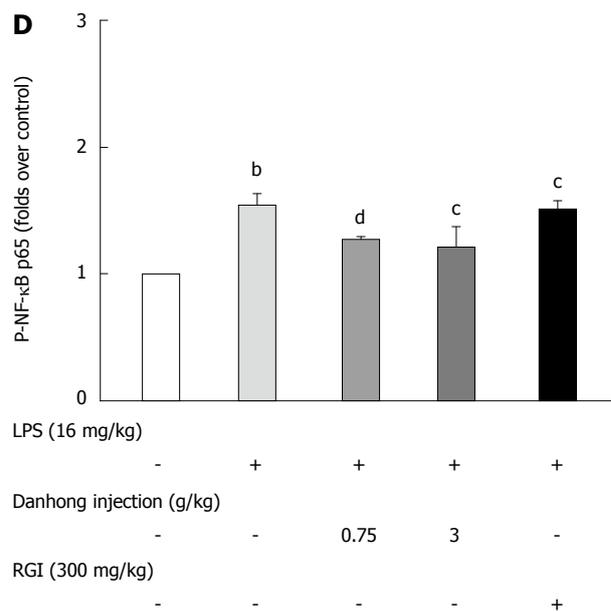
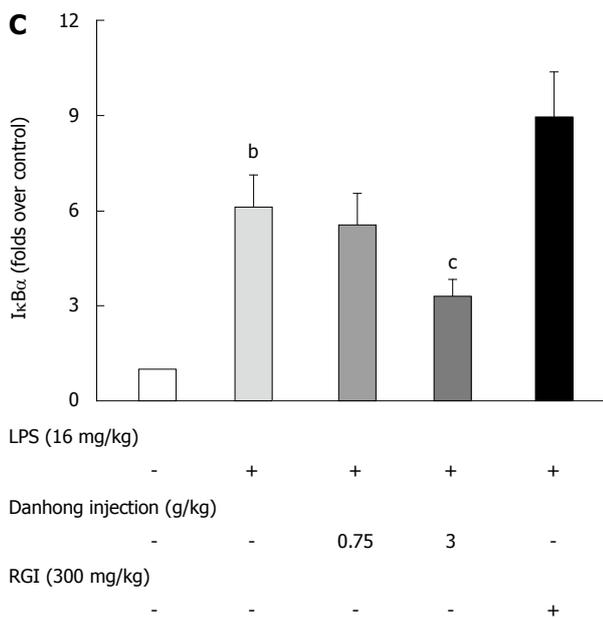
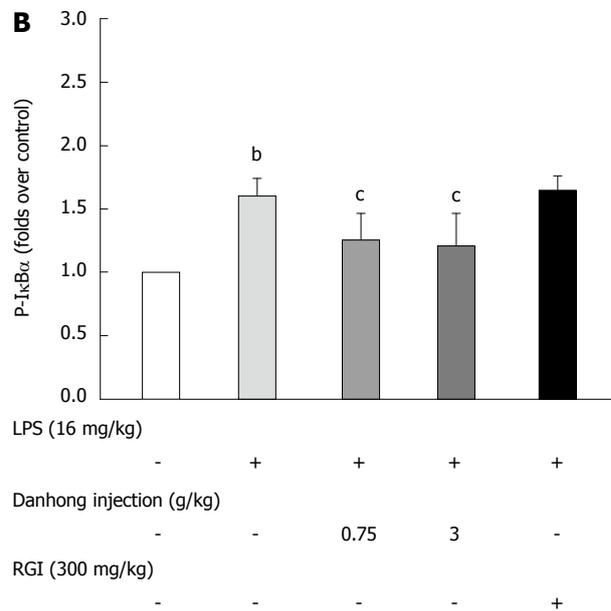
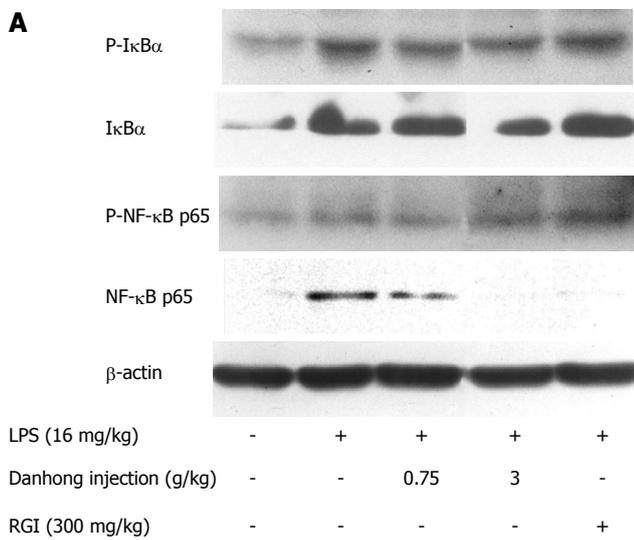


Figure 6 Effects of Danhong injection on liver Bcl-2 and Bax expression. The mRNA expression of Bax (A) and Bcl-2 (B) was detected by real-time RT-PCR. Values are mean \pm SE ($n = 3$) of three independent experiments. Bcl-2 and Bax protein expression was detected by Western blot (E). The relative optical density is shown (C and D). ^b $P < 0.01$ vs blank control group, ^c $P < 0.05$, ^d $P < 0.01$ vs lipopolysaccharide (LPS)-treated group. β -actin was used as an internal control.



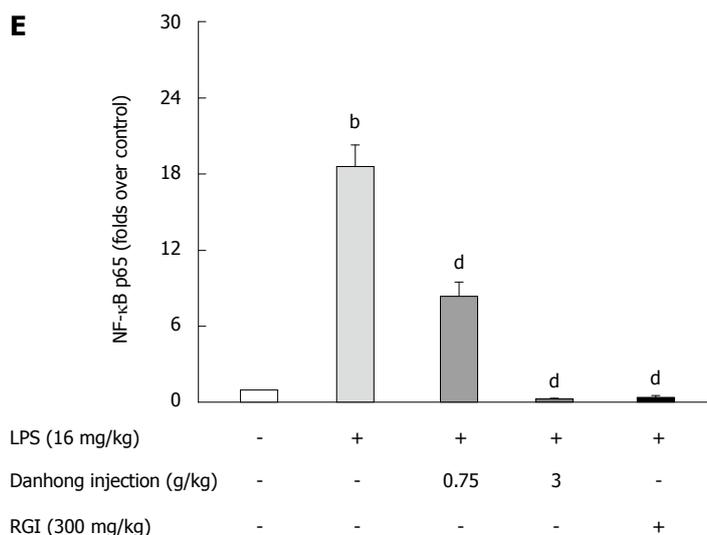


Figure 7 Effects of Danhong injection on liver nuclear factor- κ B family protein expression. Phospho-I κ B- α , I κ B α , phospho-NF- κ B p65 and NF- κ B p65 protein expression in the mouse liver was examined by Western blot (A). The relative optical density is shown (B-E). ^b*P* < 0.01 vs blank control group, ^c*P* < 0.05, ^d*P* < 0.01 vs lipopolysaccharide (LPS)-treated group. β -actin was used as an internal control.

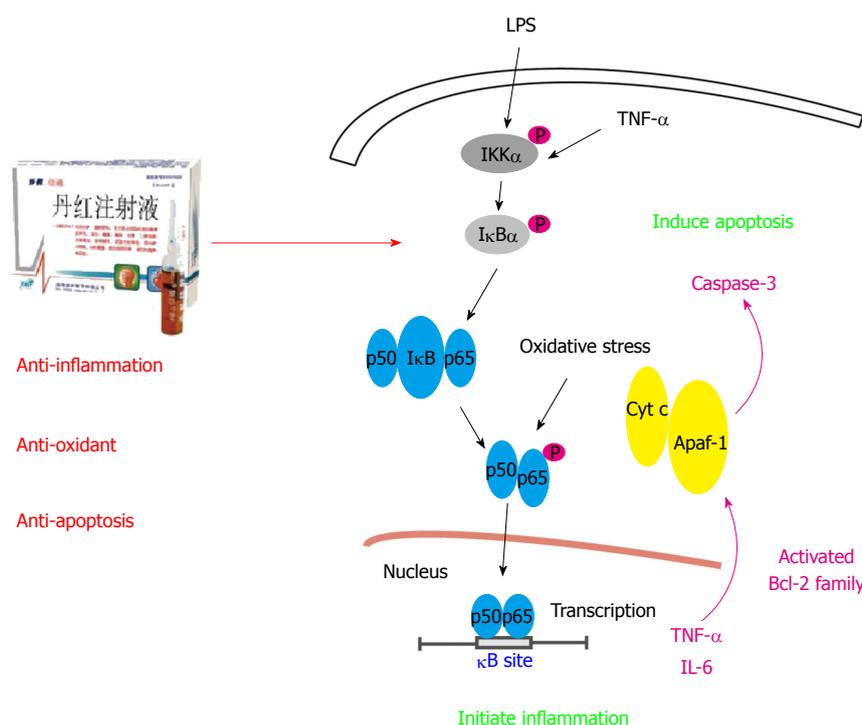


Figure 8 Pharmacological mechanism of Danhong injection on lipopolysaccharide-induced acute liver injury. Danhong injection (DHI) protected against lipopolysaccharide (LPS)-induced acute liver injury by regulating I κ B α , and nuclear factor (NF)- κ B p65 phosphorylation as well as NF- κ B p65 translocation from the cytoplasm to the nucleus. Subsequently, TNF- α and IL-6 secretion, Bax and Bcl-2 expression, and caspase-3 activity were modified and balanced.

Salvia miltiorrhiza and *Carthamus tinctorius* extract on hepatic protection. DHI is a popular Chinese medicine consisting of *Salvia miltiorrhiza* and *Carthamus tinctorius*. LPS injection activated the NF- κ B signalling pathway, resulting in the subsequent release of cytokines, including TNF- α and IL-6. TNF- α secretion induces oxidative stress injury, which deteriorates the NF- κ B-related signalling cascade. In addition, excessive

TNF- α production stimulates Bcl-2 family activation to release cytochrome C, leading to caspase-3 activation and hepatocyte damage. DHI administration prior to LPS alleviated acute liver injury through its anti-oxidative capacity and anti-inflammatory effects by inhibiting NF- κ B pathway activation and through its anti-apoptotic effect by balancing Bcl-2 and Bax levels (Figure 8).

COMMENTS

Background

Substantial evidence suggests that *Salvia miltiorrhiza* Bge. (Lamiaceae) is hepatoprotective against hepatic toxicity and ischemia/reperfusion injury via anti-oxidative effects and improved microcirculation. Similarly, flavonoids from *Carthamus tinctorius* L. (Compositae) have hepatoprotective effects. The combination of *Salvia miltiorrhiza* and *Carthamus tinctorius* extracts (Danhong injection, DHI) has been traditionally used for synergistic therapeutic effects on activating blood circulation and resolving stasis and to treat ischemic encephalopathy and coronary heart disease in the clinic. The present study aimed to investigate the hepatoprotective effects of DHI and explore the underlying mechanisms.

Research frontiers

Hepatic injury always accompanies with inflammatory infiltration, oxidative stress and apoptotic injury. Lipopolysaccharide (LPS) injection activates the nuclear factor (NF)- κ B signalling pathway, resulting in the subsequent release of cytokines, including tumor necrosis factor (TNF)- α and interleukin (IL)-6. TNF- α secretion induces oxidative stress injury, which deteriorates the NF- κ B-related signalling cascade. In addition, excessive TNF- α production stimulates Bcl-2 family activation to release cytochrome C, leading to caspase-3 activation and hepatocyte damage.

Innovations and breakthroughs

The present study first reported the protective effect of DHI on LPS-induced liver injury in mice. DHI may be a multi-function protectant against acute hepatic injury in mice through its anti-inflammatory, anti-oxidative and anti-apoptotic activities.

Applications

The results of the present study highlighted the protective effect of DHI on LPS-induced liver injury. It may be a potent drug for developing new therapeutic treatments for liver diseases.

Terminology

LPS is the major component of the outer membrane of Gram-negative bacteria, and the core-lipid A region is the toxic moiety of LPS. LPS impairs the liver by acting as a hepatotoxin. The liver provides the first line of defence against bacteria and their products.

Peer-review

This is an interesting study in which authors indicated that *Salvia miltiorrhiza* and *Carthamus tinctorius* extract (Danhong injection, DHI) protected from LPS-induced hepatic injury depending upon anti-inflammation, anti-oxidation and anti-apoptosis in mice. It may be valuable for new therapeutic treatments for liver diseases.

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