

Protective effect of Xuebijing injection on D-galactosamine- and lipopolysaccharide-induced acute liver injury in rats through the regulation of p38 MAPK, MMP-9 and HO-1 expression by increasing TIPE2 expression

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Abstract. Xuebijing injection (XBJ) has long been used to treat infectious diseases in China. The therapeutic effect of XBJ is probably associated with anti-inflammatory effects. However, the precise mechanisms responsible for the effects of XBJ remain unknown. The present study was conducted in order to evaluate the protective effects of XBJ in a rat model of D-galactosamine (D-Gal)- and lipopolysaccharide (LPS)-induced acute liver injury. In the present study, the rats were injected with D-Gal and LPS intraperitoneally to induce acute liver injury. Two hours prior to D-Gal and LPS administration, the treatment group was administered XBJ by intravenous infusion. The effects of XBJ on D-Gal- and LPS-induced expression of tumor necrosis factor (TNF)-alpha-induced protein 8-like 2 (TIPE2), nuclear factor- κ B (NF- κ B), matrix metalloproteinase-9 (MMP-9) and heme oxygenase-1 (HO-1) as well as mitogen-activated protein kinase (MAPK) signaling

was examined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blot analysis, immunofluorescence, as well as by analysing the serum levels of pro-inflammatory cytokines and the transaminases, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Myeloperoxidase (MPO), malondialdehyde (MDA) and superoxide dismutase (SOD) levels in the rat liver tissues were also measured. For histological analysis, hematoxylin and eosin (H&E)-stained liver samples were evaluated. The results showed that XBJ upregulated TIPE2 and HO-1 expression, reduced the expression of NF- κ B65 and MMP-9, inhibited the LPS-induced gene expression of c-jun N-terminal kinase (JNK) and p38 MAPK, decreased the generation of pro-inflammatory cytokines [interleukin (IL)-6, IL-13 and TNF- α], inhibited ALT and AST activity, and ameliorated D-Gal- and LPS-induced liver injury. The histological results also demonstrated that XBJ attenuated D-Gal- and LPS-induced liver inflammation. It was found that XBJ may prevent LPS-induced pro-inflammatory gene expression through inhibiting the NF- κ B and MAPK signaling pathways by upregulating TIPE2 expression, thereby attenuating LPS-induced liver injury in rats. The marked protective effects of XBJ suggest that it has the potential to be used in the treatment of LPS-induced liver injury.

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Abbreviations: TIPE2, tumor necrosis factor-alpha-induced protein 8-like 2; MMP-9, matrix metalloproteinase-9; HO-1, heme oxygenase-1; XBJ, Xuebijing injection; ALT, alanine aminotransferase; AST, aspartate aminotransferase; MAPK, mitogen-activated protein kinase; TNF- α , tumor necrosis factor- α ; MDA, malondialdehyde; LPS, lipopolysaccharide; MPO, myeloperoxidase; SOD, superoxide dismutase

Key words: liver injury, tumor necrosis factor-alpha-induced protein 8-like 2, Xuebijing injection, lipopolysaccharide, mitogen-activated protein kinase

Introduction

In the event of infection, severe trauma or burns, the transfer of endotoxins from Gram-negative, enteric bacteria in the intestinal tract to the blood and tissues may cause endotoxemia and a systemic inflammatory response, which can damage body tissues, and even develop into multiple organ dysfunction syndrome (MODS), potentially resulting in death (1-3). The main pathogenic factor of endotoxemia is lipopolysaccharide (LPS), found in bacterial cell walls. Following the entry of the endotoxin into the blood circulation, direct and indirect effects are exerted on the liver. Direct effects include interference with energy metabolism and the development of a liver microcirculation disorder. A large amount of LPS may cause Kupffer cell hyperactivity and low phagocytosis which may induce the synthesis

and release of a large number of inflammatory mediators and cytokines, thus intensifying the degree of liver injury caused by endotoxemia (4,6). The indirect effect mainly involves the induction of inflammatory cytokines in the cells after LPS binding with receptors on the cell membrane, activating internal and external mononuclear phagocyte systems through mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)- κ B (7). A variety of inflammatory mediators, such as tumor necrosis factor (TNF)- α and interleukin (IL)-13, increase the generation of oxygen free radicals, whereas reductions in the levels of anti-inflammatory cytokines such as IL-10, cause an imbalance in the inflammatory response and eventually lead to liver damage.

The gene TNF- α -induced protein 8-like 2 (TIPE2 or TNFAIP8L2), belongs to the TNFAIP8 family, and was detected and identified during a study by Sun *et al* into experimental allergic encephalomyelitis (EAE) (8), which found that TIPE2 is mainly expressed in the lymphatic tissue and sites of inflammation. Gene knockout and transgenic experiments have shown that TIPE2 principally participates in T cell activation mediated by the negative regulation of T cell receptors (TCRs), and in macrophage activation mediated by Toll-like receptors (TLRs) (9,10). This indicates that TIPE2 is an important negative regulatory factor of inflammation, and plays an important role in minimizing tissue damage by sustaining autoimmune stability and preventing excessive inflammation.

The traditional Chinese medicine Xuebijing injection (XBJ), which promotes blood circulation and removing blood stasis, is mainly composed of *Paeonia lactiflora* (Radix Paeoniae Rubra; Chishao), *Ligusticum chuanxiong* (Radix Ligustici Chuanxiong; Chuanxiong), *Salvia miltiorrhiza* (Radix Salviae Miltiorrhizae; Danshen), *Carthamus tinctorius* (Flos Carthami; Honghua) and *Angelica sinensis* (Radix Angelicae Sinensis; Dangui). These constituents have been found to exert effects that combat bacterial infection and toxins, reduce endotoxin levels, adjust immune and inflammatory mediators, improve microcirculation, protect vascular endothelial cells and enhance the abnormal blood coagulation mechanism (11,12). Clinically, XBJ injection is used for the treatment of sepsis and MODS, and has achieved significant curative effects (13). However, the specific mechanisms through which XBJ achieves therapeutic effects remain unclear and warrant further investigation.

In this experiment, we investigated whether XBJ injection inhibits the p38 mitogen-activated protein kinase (MAPK) and heme oxygenase 1 (HO-1) pathways, and whether it is capable of reducing inflammation and oxidative stress through enhancing TIPE2 expression in rats with D-galactosamine (D-Gal)- and LPS-induced hepatic injury. We also explored the protective effect of XBJ injection on hepatic injury induced by endotoxin. Finally, we examined the mechanism responsible for these effects. We present evidence for a novel biphasic role of XBJ injection on the expression of TIPE2, and p38 MAPK and HO-1 pathways in a rat model of D-Gal and LPS-induced hepatic injury, and provide novel insights into the role of XBJ injection in acute liver injury.

Materials and methods

Source of XBJ injection and components. XBJ injection was obtained from Tianjin Chase Sun Pharmaceutical Co., Ltd.

(Tianjin, China) (no. Z20040033), and was comprised of Chuanxiong, Chishao, Danshen, Honghua and Dangui. Chuanxiong, Chishao, Danshen, Honghua and Dangui were provided by Professor Li Shixia of Central South University (Changsha, China) and deposited in the pharmacy centre.

Preparation of XBJ from Chuanxiong, Chishao, Danshen and Honghua. Referring to the methods in literature (12), the detailed method is described below. The appropriate amounts of dried Chuanxiong, Chishao, Danshen and Honghua were weighed and backflow extractions were performed twice with chloroform (Tianjin Jinqiang Chemical Co., Ltd., Tianjin, China):methanol (Qingdao Yuyin Chemical Co., Ltd., Qingdao, China) (2:1) in a water bath at 50°C, for 2 h each time. The liquid was discarded and ultrasonic extraction was performed with the residue and 80% ethanol (Hebei Kejin Rio Di Chemical Co., Ltd., Hebei, China) three times, for 20 min each time. It was then filtered and the filtrate was discarded prior to performing ultrasonic extractions with the residue and water three times, for 20 min each time, and then filtered. The temperature of the combined filtrate was lowered, 95% ethanol was added (Hebei Kejin Rio Di Chemical Co., Ltd.), and then left to stand at a low temperature for 24 h. The extracted and filtered solids were washed with absolute ethanol and acetone, and then vacuum dried to obtain XBJ. Every 10-ml XBJ injection contained 1 g crude drug.

Animals. Adult male Sprague-Dawley (SD) rats, weighing 200–225 g, were purchased from the Laboratory Animal Center of Kunming Medical University (Kunming, China) for use in this study. The animals were housed under conditions of constant temperature (22°C) and humidity (50 \pm 5%) with a 12-h light/dark cycle and had access to chow and water *ad libitum* throughout the study. All experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee of the Kunming Medical University.

Reagents. LPS from *Escherichia coli* serotype O111:B4, and D-Gal were purchased from Sigma (St. Louis, MO, USA). Antiactivin A antibody was obtained from Sigma. TRIzol reagent was provided by Invitrogen (Carlsbad, CA, USA). SYBR-Green RT-PCR kit was purchased from Takara Bio, Inc. (Otsu, Japan). IL-1 β , IL-6 and TNF- α were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). All other chemicals were of the highest grade commercially available.

Animal treatment. In the first experiment, the rats were weighed and randomly divided into 5 groups (five rats/group) to assess the protective effect of XBJ on acute liver injury. To induce acute injury, SD rats were injected intraperitoneally (i.p.) with LPS from *E. coli* (10 μ g/kg) plus D-Gal (500 mg/kg), or saline as previously described (3). Group 1 was untreated (injected with saline) and served as the control group; group 2 received LPS plus D-Gal for the induction of liver injury and served as the model group. In groups 3, 4 and 5, 2 h prior to D-Gal and LPS administration to induce liver injury the rats were also treated with an intravenous infusion of XBJ at doses of 5, 10 and 15 ml/kg body weight (BW), respectively. The animals in each group were anesthetized with ether at 24 h, and the right

Table I. Primer sequences for the genes used to validate the microarray analysis by RT-qPCR.

Gene	Primer 5'→3'	Product (bp)
TIPE2	F: GGGAACATCCAAGGCAAG R: AGCTCATCTAGCACCTCACT	259
NF-κB	F: GCAACAGCCTGTCTGTCTGCA R: TAGAGGTGTCGTCCCATCGTAG	368
p38 MAPK	F: CACTGCTGCTTCCTCACTCCA R: AGGGTTCAGGTGCTCTGTTCG	297
JNK	F: GCCCGATGAAACCTCGCAGAT R: ACGCAGGCAATCCTACTGGA	352
β-actin	F: CCACACCCGCCACCACTTCG R: CTTGCTCTGGGCCTCGTCGC	349

TIPE2, tumor necrosis factor-α-induced protein 8-like 2; F, forward; R, reverse.

internal carotid artery was isolated. Blood was extracted (5 ml), centrifuged to collect the supernatant, dispensed into two sterile tubes, sealed with sealing glue, and placed in a freezer at -20°C until use. Extracted peripheral venous blood (2 ml) was placed in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation to detect the gene and protein expression of TIPE2. The rats were anesthetized by an overdose of barbiturate (intravenous injection, 150 mg/kg pentobarbital sodium), and tissue blood samples were collected. Parts of the hepatic tissue samples were stored at -80°C for western blot analysis, immunohistochemical staining and PCR analyses. Other parts of the hepatic tissue samples were placed in formaldehyde (10%) for histological evaluation. Blood samples were collected from the vena cavae of the rats using a Bioclean injector (5 ml) and centrifuged at 3,000 x g for 10 min to obtain the serum, which was stored at -80°C until further use.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from liver samples and PBMC homogenate using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The isolated RNA was treated with RNase-free DNase (Ambion, Austin, TX, USA) to remove traces of genomic DNA contamination. One microgram of total RNA was reverse transcribed to cDNA using SuperScript II (Invitrogen). The target gene expression was quantified with Power SYBR-Green PCR Master Mix using an ABI HT7900 Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA). Each amplified sample in all wells was analyzed for homogeneity using dissociation curve analysis. After denaturation at 95°C for 2 min, 40 cycles were performed at 95°C for 10 sec and at 60°C for 30 sec. Relative quantification was calculated using the comparative CT method (2-ΔΔCt method: ΔΔCt = ΔCt sample - ΔCt reference). Lower ΔCT values and lower ΔΔCT reflect a relatively higher amount of gene transcript. Statistical analyses were performed for at least 6-15 replicate experimental samples in each set. The primers sequences are listed in Table I.

Western blot analysis. TIPE2 protein expression in PBMCs, as well as phosphorylated (p-)IκB-α, p-IκB, NF-κB65, p-p38 MAPK, matrix metalloproteinase 9 (MMP-9) and HO-1 protein expression in the livers of D-Gal plus LPS-exposed rats was determined using western blot analysis. Fifty micrograms of protein from each sample was applied to a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and the proteins were transferred to polyvinylidene fluoride membranes electrophoretically. The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.5% Tween-20 and then incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal TIPE2 (1:1,000; Cat.no.15940-1-AP) and rabbit polyclonal p-p38 MAPK (1:1,000; Cat. no. SAB4301534) (both from Sigma-Aldrich, St. Louis, MO, USA), goat polyclonal MMP-9 (1:1,000; Cat. no. sc-8839; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit p-IκB-α (1:1,000; Cat. no. 2859), rabbit NF-κB65 (1:1,000; Cat. no. 9936) and mouse HO-1 (1:1,000; Cat. no. 70081) (all from Cell Signaling Technology, Inc., Danvers, MA, USA), mouse monoclonal β-actin (1:2,000; Cat. no. sc-47778 B) and goat polyclonal p-JNK (1:1,000; Cat. no. sc-46006) (both from Santa Cruz Biotechnology, Inc.). The membranes were then incubated with horseradish-peroxidase conjugated anti-mouse (1:2,000; Cat. no. 7076) and anti-rabbit IgG (1:2,000; Cat. no. 7074) (both from Cell Signaling Technology, Inc.), or donkey anti-goat IgG (1:5,000; Cat. no. sc-2749; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The protein bands were developed using (electrochemiluminescence) ECL detection reagent (Perkin-Elmer Life Sciences, Boston, MA, USA) and quantified using the GeneSnap program (SynGene; Synoptics Ltd., Cambridge, UK). The protein expression of TIPE2, p-IκB-α, NF-κB65, p-p38 MAPK, MMP-9 and HO-1 was normalized to β-actin levels.

Immunohistochemical analysis. Immunostaining was performed on the liver sections following antigen retrieval using Retrieval A (Zymed Laboratories, Inc., San Francisco, CA, USA) at 100°C for 20 min, and endogenous peroxidases were quenched with 3% H₂O₂ (Tianjin Jinqiang Chemical Co., Ltd.). The sections were blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) followed by staining with primary anti-cleaved caspase-3 (BD Pharmingen, San Jose, CA, USA) at room temperature for 1 h. The sections were washed and following the application of the secondary antibody (R&D Systems, Inc.), the tissues were developed using Vectastain ABC and 3,3'-diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA, USA). Following staining, five high-power fields (magnification, x200) were randomly selected in each slide, and the average proportion of positive expression in each field was counted using the true color multi-functional cell image analysis management system (Image-Pro Plus; Media Cybernetics, Inc., Rockville, MD, USA), and expressed as positive unit (pu).

Immunoassay for cytokines. Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc.) were used to quantify the levels of transforming growth factor (TGF)-β1, TNF-α, IL-13, IL-10 and IL-6 in the rat serum. The absorbance was read at 450 nm by a microplate reader (model 680; Bio-Rad Laboratories, Mississauga, ON, Canada), with the wavelength correction set at 550 nm. To

calculate the concentrations of TGF- β 1, TNF- α , IL-13, IL-10 and IL-6, a standard curve was constructed using serial dilutions of cytokine standards provided with the kit.

Determination of superoxide dismutase (SOD) activity. SOD is a key enzyme that catalyses the dismutation of superoxide radicals resulting from cellular oxidative metabolism into hydrogen peroxide, and prevents LPS-induced penetration. The stored samples were homogenized in 100 mmol/l Tris-HCl buffer and centrifuged at 10,000 \times g for 20 min, and then SOD activity was determined using assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and expressed as units per microgram of total protein (u/mg). The total protein content in samples was analyzed using a bicinchoninic acid protein assay kit (R&D Systems, Inc.).

Measurement of malondialdehyde (MDA). MDA was quantified as thiobarbituric acid reactive substances (TBARS) according to previously published methods (11) as a measure of lipid peroxidation. Briefly, the weighed samples were homogenized in 1 ml 5% trichloroacetic acid. The samples were centrifuged (10,000 \times g) and 250 μ l of the supernatant was reacted with the same volume of 20 mM thiobarbituric acid for 35 min at 95°C, followed by 10 min at 4°C. Sample fluorescence was read using a spectrophotometric plate reader (Victor3 1420-050; Perkin Elmer, Waltham, MA, USA) with an excitation wavelength of 515 nm and an emission wavelength of 553 nm.

Liver myeloperoxidase (MPO) assay. The liver tissue was homogenized (50 mg/ml) in 0.5% hexadecyltrimethylammonium bromide (Shanghai Hungsun Chemical Co., Ltd., Shanghai, China) in 10 mM 3-(N-morpholino)propanesulfonic acid (Shanghai Huayi Bio-technology Co., Ltd., Shanghai, China) and centrifuged at 15,000 \times g for 40 min. The suspension was then sonicated three times for 30 sec at 1-min intervals. An aliquot of supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine (Beijing Huaxin Rui Technology Co., Ltd., Beijing, China) and 1 mM H₂O₂ (Tianjin Jinqiang Chemical Co., Ltd.). The activity was measured spectrophotometrically as the change in absorbance at 37°C with a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The results are expressed as milliunits of MPO activity per milligram of protein, as determined by the Bradford assay.

Measurement of hepatic injury. The markers of hepatic damage, serum alanine aminotransferase (ALT) and aspartate transaminase (AST) levels were measured using an automated biochemistry clinical analyzer (Hitachi, Tokyo, Japan) according to an automated procedure.

Histological analysis. The liver tissue was fixed in 10% formalin for 24 h followed by dehydration. The liver tissue was embedded in paraffin wax, sectioned into 5- μ m slices and stained with Mayer's hematoxylin and eosin (H&E) (Merck Millipore, Darmstadt, Germany). The damage scores were estimated by counting morphological alterations in 10 randomly selected microscopic fields from 6 samples of each group and from at least 3 independent experiments. The morphological liver integrity was graded on a scale of 1 (excellent) to 5 (poor). Grading was adapted from t'Hart *et al* (14)

and described as: 1, normal rectangular structure; 2, rounded hepatocytes with an increase in the sinusoidal spaces; 3, vacuolization; 4, nuclear picnosis; and 5, necrosis. Histological evaluations of the damage scores were performed in a blinded fashion by three different observers.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay. Apoptosis was assessed by the TUNEL assay (Roche Molecular Biochemicals, Mannheim, Germany). The TUNEL-positive nuclei were counted in 10 random high power fields (640 objective) (15). The apoptotic index was calculated as a ratio of the apoptotic cell number to the total cell number in each field and expressed as a percentage. The ratio of the apoptotic cell number were performed in a blinded fashion by three different observers.

Survival analysis. Another 75 rats were divided into the following 5 groups (15 rats/group): i) control group, ii) model group (LPS plus D-Gal group), iii) treatment group [XBJ 5 ml/kg; LPS plus D-Gal + XBJ (5 ml/kg) group], iv) treatment group [XBJ 10 ml/kg; LPS plus D-Gal + XBJ (10 ml/kg) group], and v) treatment group [XBJ 15 ml/kg; LPS plus D-Gal + XBJ (15 ml/kg) group] in order to observe survival. The treatments were the same as mentioned above. The observation began at the time of XBJ treatment and the endpoint was set at 120 h after XBJ treatment.

Statistical analysis. Data are expressed as the means \pm SEM. All data were analyzed using SigmaStat version 3.5 (Systat Software, Inc., San Jose, CA, USA). The Student's t-test was used to compare data between two groups. For multiple group comparisons, one-way or two-way analysis of variance was performed followed by Student-Newman-Keuls post hoc analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

XBJ decreases serum ALT and AST levels. The effect of XBJ on liver injury remains unknown. For this purpose, we examined the effect of XBJ, a traditional Chinese herbal medicine on liver injury. The levels of serum ALT and AST, two markers of liver injury, were assessed after 24 h of XBJ and LPS plus D-Gal exposure *in vivo*. As shown in Fig. 1, LPS (10 μ g/kg) plus D-Gal (500 mg/kg) injection *in vivo* induces significantly higher serum ALT and AST levels as compared with the saline-injected controls after 24 h. XBJ administered at 5, 10 and 15 ml/kg BW produced significant reductions in serum ALT and AST levels at all three doses. The most significant effect was observed in the group of XBJ-treated rats that received XBJ (10 and 15 ml/kg BW) as the serum ALT and AST concentrations of these rats were restored to the levels of the saline-injected control group. Importantly, these enzyme levels were reduced in the LPS plus D-Gal-exposed rats pre-treated with XBJ, in a dose-dependent manner (5, 10 and 15 mg/kg, *i.p.*).

XBJ improves survival in LPS plus D-Gal-exposed rats. To determine the effect of XBJ on the survival of rats with LPS plus D-Gal-induced liver injury, the rats were treated with different concentrations of XBJ (5, 10 and 15 mg/kg BW) for 24 h in the

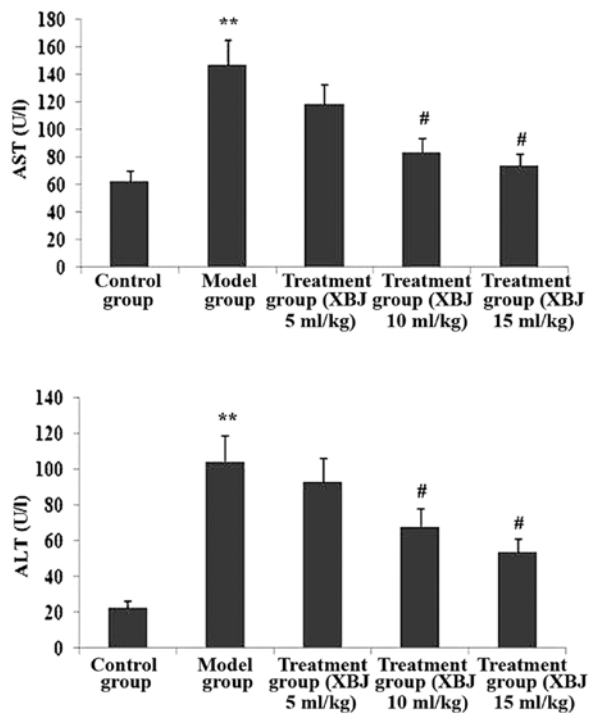


Figure 1. Effect of Xuebijing injection (XBJ) on markers of hepatic injury in lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. Rats were treated with various concentrations of XBJ (5, 10 and 15 ml/kg body weight) for 24 h and exposed to LPS, 10 μ g/kg plus D-Gal, 500 mg/kg. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured. Data are expressed as the means \pm SE values from three independent experiments in each group. ** P <0.01 vs. the control group and # P <0.05 vs. the model group.

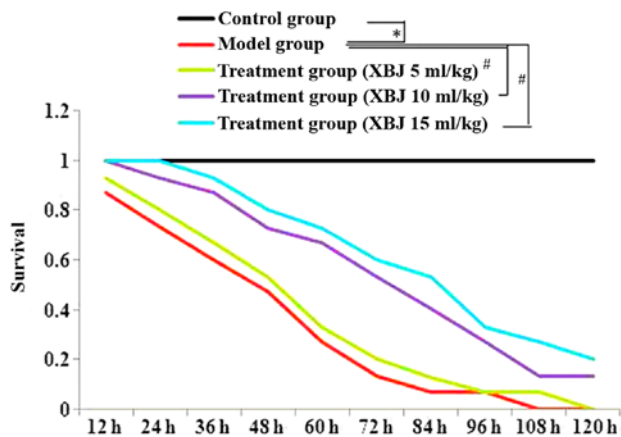


Figure 2. Administration of Xuebijing injection (XBJ) ameliorates survival in lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. Groups of rats were challenged with LPS, 10 μ g/kg plus D-Gal, 500 mg/kg and treated with XBJ (5, 10 and 15 ml/kg body weight) 24 h later. The survival of LPS plus D-Gal exposed rats was determined. Data were analyzed using log-rank, * P <0.05 vs. the control group and # P <0.05 vs. the model group.

presence of LPS (10 μ g/kg) plus D-Gal (500 mg/kg), as shown in Fig. 2. Following LPS plus D-Gal challenge, the survival of rats decreased significantly at different time points. However, XBJ increased survival in the LPS plus D-Gal-exposed rats in a concentration-dependent manner. A concentration as low as 10 ml/kg XBJ was effective at improving survival in LPS plus D-Gal-exposed rats.

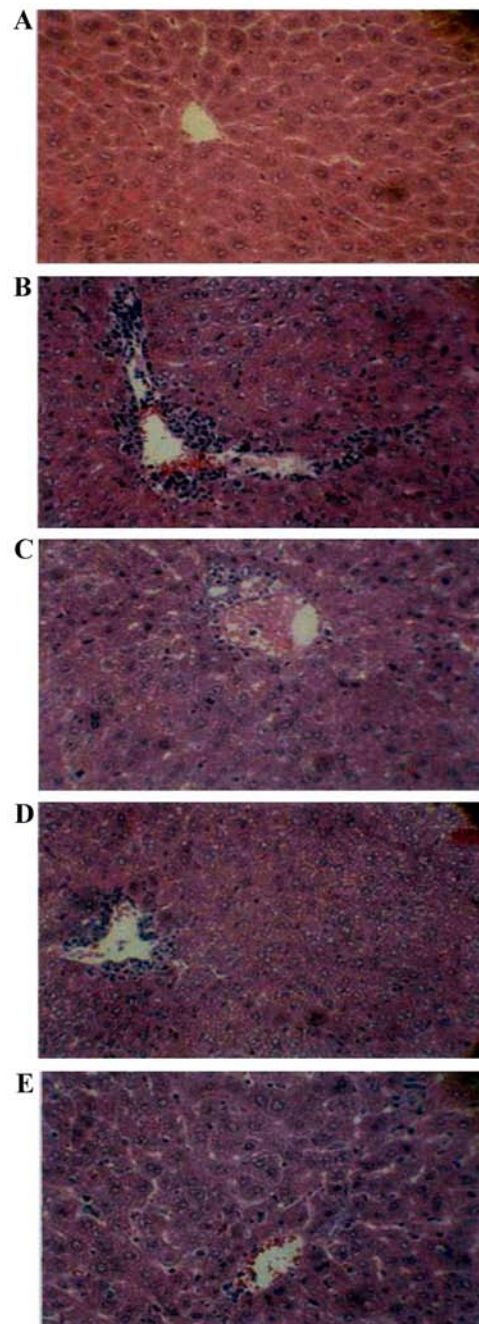


Figure 3. Administration of Xuebijing injection (XBJ) ameliorates the histopathological changes in the liver tissues of lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. Groups of rats were challenged with LPS, 10 μ g/kg plus D-Gal, 500 mg/kg and treated with XBJ (5, 10 and 15 ml/kg body weight) 24 h later. Representative images of H&E-stained liver sections from the five experimental groups (magnification, x400): (A) control group, (B) model group and (C-E) treatment groups (XBJ, at doses of 5, 10 and 15 ml/kg, respectively).

XBJ ameliorates histological liver damage in LPS plus D-Gal exposed rats. To determine the effect of XBJ on histological liver damage, histopathological analysis was performed on H&E-stained liver sections. Histological analysis of liver samples following LPS exposure revealed, as shown in Figs. 3 and 4, that normal histological structures of hepatic lobules were observed in livers from rats in the control group. The model group, treated with LPS + D-Gal exhibited complete damage to hepatocytes with hepatocellular vacuolization and focal hepatic necrosis,

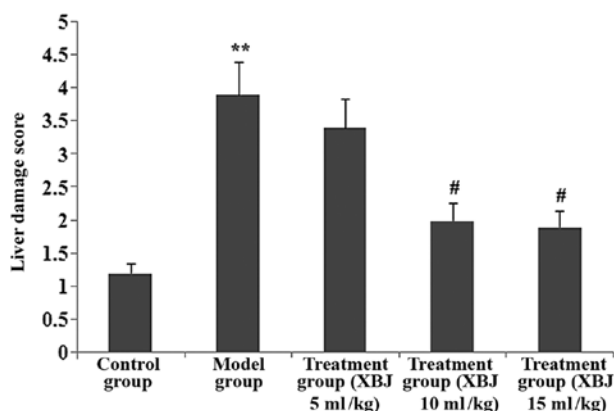


Figure 4. Administration of Xuebijing injection (XBJ) decreases liver damage scores in lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. Groups of rats were challenged with LPS, 10 μ g/kg plus D-Gal, 500 mg/kg and treated with XBJ (5, 10 and 15 ml/kg body weight) 24 h later. A histological evaluation of the therapeutic potential of XBJ on LPS+D-Gal-induced liver injury in rats was analyzed and the liver damage score was determined. Data are presented as the means \pm SD of one experiment consisting of three replicates. ** $P < 0.01$ vs. the normal control group; # $P < 0.05$ vs. model group.

and the liver damage score was significantly increased ($P < 0.01$). The administration of XBJ (at doses of 5, 10 and 15 ml/kg BW) ameliorated damage to the hepatocytes as compared with that observed in the LPS + DGal-treated animals. When comparing the liver cell morphology of the control group, LPS plus D-Gal, and LPS plus D-Gal pre-treated with XBJ (5, 10 and 15 ml/kg) groups, only the control and XBJ (10 and 15 ml/kg)-pretreated groups exhibited normal liver cell structures, with a well-defined cytoplasm and nucleus in the cells and ribbon-like hepatocyte arrangements, and the liver damage score was significantly decreased ($P < 0.05$).

XBJ inhibits the activation of cleaved caspase-3 in LPS plus D-Gal-exposed rat livers. To examine the effect of XBJ on the activation of cleaved caspase-3 in LPS plus D-Gal exposed rats, immunohistochemical staining was performed on the liver sections. As shown in Figs. 5 and 6, the activation of cleaved caspase-3 was very weak at 24 h in the livers of rats in the control group. However, cleaved caspase-3 expression was markedly increased in the LPS + D-Gal-treated group and the XBJ + LPS plus D-Gal groups ($P < 0.05$). At 24 h after XBJ (10 and 15 ml/kg) administration, the activation of cleaved caspase-3 was markedly inhibited in the LPS plus D-Gal-exposed rat livers ($P < 0.05$) compared with the model group. However, compared with the LPS plus D-Gal group, the XBJ (5 ml/kg) + LPS plus D-Gal group showed no significant differences ($P > 0.05$).

XBJ reduces the apoptotic rate of hepatocytes in LPS plus DGal-exposed rats. To determine the effect of XBJ on the apoptosis of hepatocytes from LPS plus D-Gal-exposed rats, TUNEL assays were performed. As shown in Figs. 7 and 8, after the groups of rats were exposed to LPS plus D-Gal, the apoptotic rate of hepatocytes was markedly enhanced. Notably, compared with the LPS plus D-Gal group, the groups pre-treated with XBJ (10 and 15 ml/kg) exhibited a markedly decreased apoptotic rate of hepatocytes ($P < 0.05$), and the XBJ (5 ml/kg) + LPS plus DGal group showed no significant differences ($P > 0.05$).

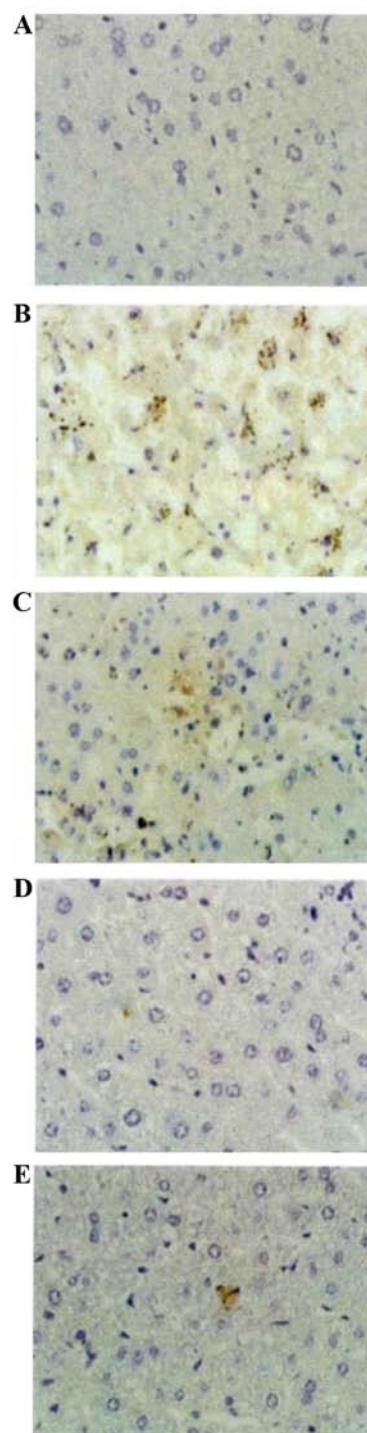


Figure 5. Effect of the administration of Xuebijing injection (XBJ) on the positive expression of cleaved caspase-3 in the liver tissue of lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. The groups of rats were challenged with LPS, 10 μ g/kg plus D-Gal, 500 mg/kg and treated with XBJ 24 h later. Immunostaining was performed on the liver sections following antigen retrieval using Retrieval A. Representative images of immunostaining show the positive expression levels of cleaved caspase-3 in the five groups of rats (immunofluorescence staining; magnification, $\times 200$): (A) control group, (B) model group and (C-E) treatment groups (XBJ, at doses of 5, 10 and 15 ml/kg, respectively).

Effect of XBJ on the mRNA expression of TIPE2, p38 MAPK, JNK and NF- κ B in LPS + D-Gal-exposed rats. RT-qPCR was used to evaluate the effect of XBJ on the mRNA expression of TIPE2, p38 MAPK, JNK and NF- κ B in the livers of rats treated

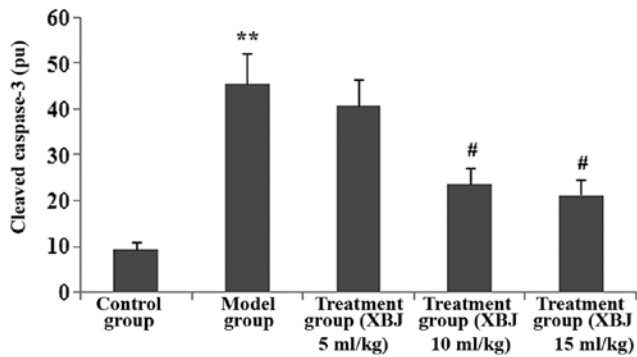


Figure 6. Administration of Xuebijing injection (XBJ) decreases the positive expression of cleaved caspase-3 in the liver tissues of lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. The groups of rats were challenged with LPS, 10 μ g/kg plus D-Gal, 500 mg/kg and treated with XBJ 24 h later. Immunostaining was performed on the liver sections following antigen retrieval using Retrieval A. Using Image-Pro Plus image analysis software, the cleaved caspase-3 positive expression levels in the liver tissue were calculated. Data are presented as the means \pm SD of one experiment consisting of three replicates. The experiments were performed in triplicate. * $P < 0.01$ vs. the normal control group; # $P < 0.05$ vs. model group.

with LPS + D-Gal and their control group in comparison with XBJ (5, 10 and 15 mg/kg BW) pre-treated groups. As shown in Figs. 9 and 10, LPS + D-Gal exposure caused a marginal decrease in TIPE2 expression, and an increase in p38 MAPK, JNK and NF- κ B levels in the rat livers when compared with the normal control livers. XBJ (5, 10 and 15 ml/kg BW) treatment resulted in an increase in the mRNA level of TIPE2, and a decrease in p38 MAPK, JNK and NF- κ B mRNA levels back to normal in a dose-dependent manner. The strongest inhibitory effect occurred after pre-treatment with 15 ml/kg XBJ. Therefore, XBJ upregulated the mRNA expression of TIPE2, and reduced the mRNA expression of p38 MAPK, JNK and NF- κ B in LPS + D-Gal-induced liver injury.

Effect of XBJ on the protein expression of TIPE2, p-p38 MAPK, p-I κ B- α , p-JNK and NF- κ B65 in LPS + D-Gal-exposed rats. Western blot analysis was performed in order to evaluate the effect of XBJ on the protein expression of TIPE2, p-p38 MAPK, p-I κ B- α , p-JNK and NF- κ B65 in the livers of rats treated with LPS + D-Gal and the control group in comparison with XBJ (5, 10 and 15 ml/kg BW) pre-treated groups. As shown in Figs. 11 and 12, LPS + D-Gal exposure caused a marginal decrease in TIPE2 protein levels, and an increase in the protein levels of p-p38 MAPK, p-JNK, p-I κ B- α and NF- κ B65 in the livers when compared with the normal control livers. XBJ treatment (5, 10 and 15 ml/kg BW) resulted in an increase in the protein levels of TIPE2, and a decrease in p-p38 MAPK, p-JNK, p-I κ B- α and NF- κ B65 back to normal in a dose-dependent manner. The strongest inhibition occurred following pre-treatment with 10 and 15 ml/kg XBJ. However, there was no significant difference between the 10 and 15 ml/kg groups. Thus, XBJ upregulated the protein expression of TIPE2, and inhibited the protein expression of p-p38 MAPK, p-JNK, p-I κ B- α and NF- κ B65 in LPS + D-Gal-exposed livers.

XBJ decreases serum levels of IL-13, IL-6, and TNF- α . As LPS + D-Gal-induced liver injury is largely mediated by pro-inflammatory cytokines, we further assessed the

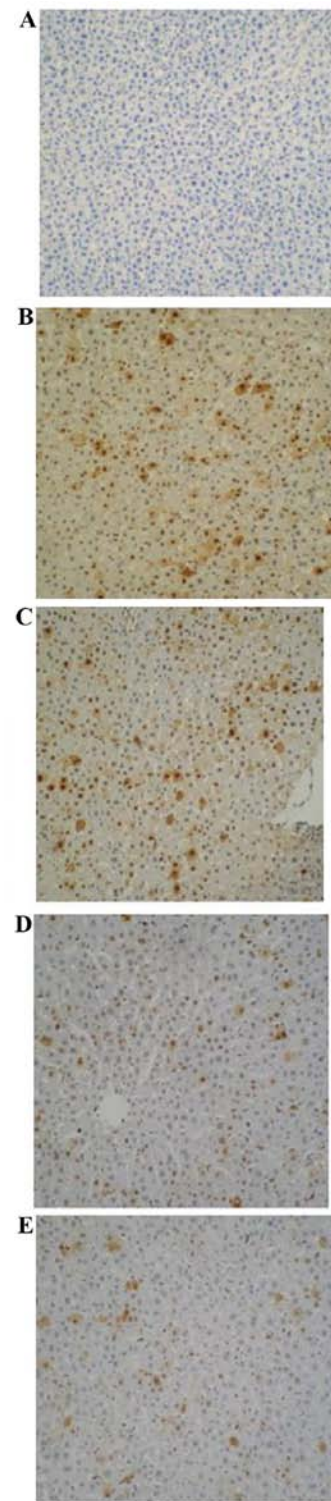


Figure 7. Effect of administration of Xuebijing injection (XBJ) on the apoptosis of hepatocytes in rats. The groups of rats were challenged with lipopolysaccharide (LPS), 10 μ g/kg plus D-galactosamine (D-Gal), 500 mg/kg and treated with XBJ 24 h later, followed by TUNEL analysis. Representative TUNEL images show the apoptosis of hepatocytes in rats at 24 h after XBJ administration. (A) Control group, (B) model group and (C-E) treatment groups (XBJ, at doses of 5, 10 and 15 ml/kg, respectively); magnification, $\times 200$.

anti-inflammatory effect of XBJ by detecting the levels of pro-inflammatory cytokines and anti-inflammatory cytokines in the plasma using ELISA. As shown in Fig. 13, the plasma levels of the pro-inflammatory cytokines, TNF- α , IL-13 and IL-6,

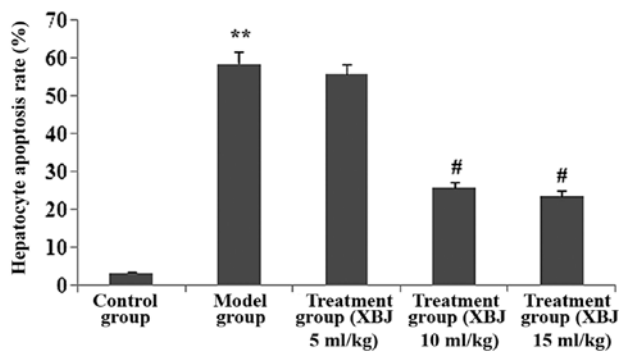


Figure 8. Administration of Xuebijing injection (XBJ) inhibits the apoptosis of hepatocytes in rats according to TUNEL analysis. The percentage of TUNEL-stained hepatocytes in rats exposed to LPS, 10 μ g/kg plus D-galactosamine (D-Gal), 500 mg/kg and treated with XBJ 24 h later was determined by statistical evaluation of the histochemical images. Values are expressed as the means \pm SD of three experiments. ** P <0.01 vs. the normal control; # P <0.05 vs. model group.

were all significantly elevated in response to LPS challenge. By contrast, the administration of XBJ (5, 10 and 15 ml/kg BW) effectively decreased the levels of pro-inflammatory cytokines and chemokines in a dose-dependent manner. LPS challenge

also increased the serum concentration of the anti-inflammatory cytokine, IL-10. The IL-10 concentration was not altered by the intravenous administration of XBJ. In particular, the highest dose of XBJ (15 ml/kg) resulted in the most powerful inhibition of TNF- α , IL-13 and IL-6. These results demonstrated that LPS + D-Gal administration increased the production of pro-inflammatory cytokines, including TNF- α , IL-13 and IL-6, and these increases were inhibited by pre-treatment with XBJ.

XBJ decreases MPO activity in liver. To ascertain the effect of XBJ injection on neutrophil infiltration into the liver tissues, we examined the MPO activity in the liver tissues of LPS + D-Gal-exposed rats. As shown in Fig. 14, MPO activity in the liver tissues was increased in the model group. However, XBJ treatment resulted in a significant reduction of MPO activity in a dose-dependent manner in the LPS + D-Gal-exposed liver tissues of rats.

Effect of XBJ on the levels of HO-1, MDA and SOD in liver tissue. To elucidate the possible mechanism responsible for the protective effect of XBJ on LPS-induced oxidative liver injury, we detected the levels of HO-1, MDA and SOD in liver tissue from rats exposed to LPS + D-Gal with or without XBJ. As shown in Fig. 15, liver HO-1 expression decreased markedly

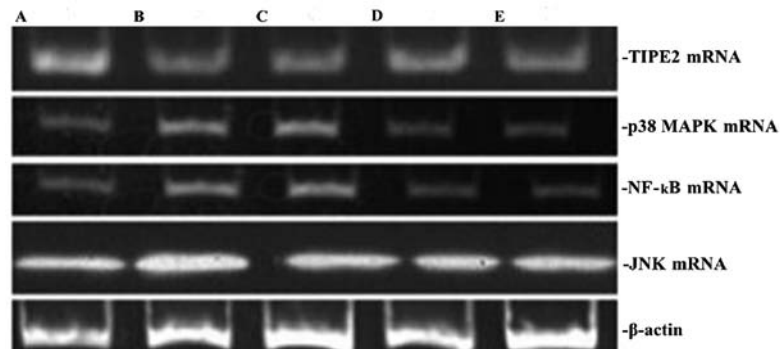


Figure 9. Effect of Xuebijing injection (XBJ) on the mRNA expression of TIPE2, p38 MAPK, JNK and NF- κ B in lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. The effect of XBJ on the mRNA expression of TIPE2, p38 MAPK, JNK and NF- κ B in LPS+DGal-exposed rats, was evaluated using RT-qPCR. Representative RT-qPCR results show the mRNA expression of TIPE2, p38 MAPK, JNK and NF- κ B in the five rat groups: (A) control group, (B) model group and (C-E) treatment groups (XBJ at doses of 5, 10 and 15 ml/kg, respectively).

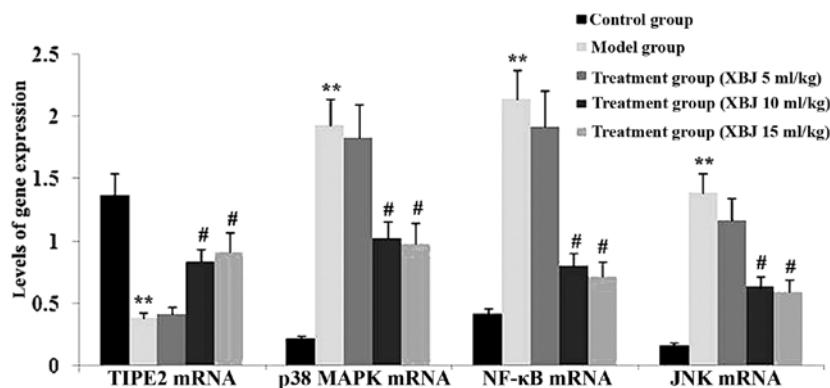


Figure 10. Effect of Xuebijing injection (XBJ) on the mRNA expression of TIPE2, p38 MAPK, JNK and NF- κ B in lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. The effect of XBJ on the mRNA expression of TIPE2, p38 MAPK, JNK and NF- κ B in LPS+DGal-exposed rats, was evaluated using RT-qPCR. Statistical summary of the densitometric analysis of TIPE2, p38 MAPK, JNK and NF- κ B mRNA expression in the five rat groups. ** P <0.01 versus the control group, # P <0.05 vs. the model group.

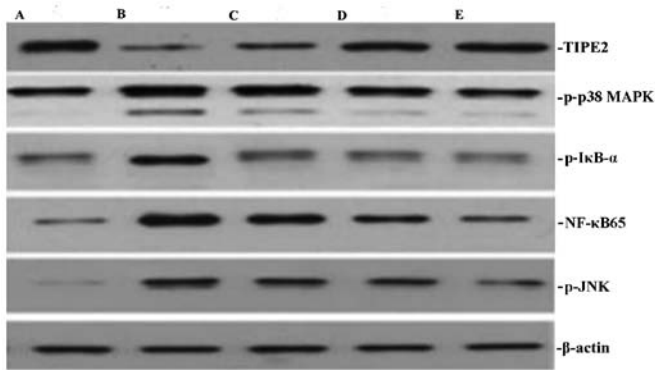


Figure 11. Effect of Xuebijing injection (XBJ) on the protein expression of TIPE2, phosphorylated (p-)p38 MAPK, p-IκB-α, p-JNK and NF-κB65 in lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. Rats were treated with various concentrations of XBJ (5, 10 and 15 ml/kg body weight) for 24 h and then exposed to LPS, 10 μg/kg plus D-Gal, 500 mg/kg. The protein expression of TIPE2, p-p38 MAPK, p-IκB-α, p-JNK and NF-κB65 in liver tissues was measured by western blot analysis and representative western blots show the protein expression of TIPE2, p-p38 MAPK, p-IκB-α, p-JNK and NF-κB65 in the five groups of rats: (A) control group, (B) model group and (C-E) treatment groups (XBJ, at doses of 5, 10 and 15 ml/kg, respectively).

in the model group compared with the control group, whereas the decrease was significantly upregulated in the LPS plus D-Gal + XBJ groups. As shown in Figs. 15 and 16, in the LPS + D-Gal-exposed (model) group, the liver levels of HO-1 and SOD was significantly lower at 24 h, and the MDA level was significantly higher. However, after the administration of XBJ, the HO-1 and the SOD levels in the liver tissues were markedly elevated, and the MDA levels were significantly attenuated in a dose-dependent manner.

XBJ inhibits MMP-9 protein expression in liver tissue, and decreases TGF-β1 serum levels in rats with LPS + D-Gal-induced liver injury. We next determined MMP-9 expression in the liver tissue and TGF-β1 expression levels in serum to evaluate the formation of early liver fibrosis as a marker for liver injury. As shown in Fig. 17, we found

that MMP-9 and TGF-β1 expression levels in the rats with LPS + D-Gal-induced liver injury were markedly increased. Following XBJ treatment, the TGF-β1 and the MMP-9 levels decreased significantly in a dose-dependent manner. Thus, XBJ inhibited the formation of early liver fibrosis by lowering TGF-β1 and MMP-9 expression.

Discussion

LPS is a glycolipid complex that is a major component of the outer membrane of Gram-negative bacterial cell walls, and is a principal factor causing septicopyemia and systemic inflammatory response syndrome (1). The liver is one of the most vulnerable organs in sepsis. LPS not only causes disturbances to the microcirculation leading to direct damage to the liver cells, but also stimulates mononuclear phagocyte systems in the liver to generate a great amount of inflammatory factors such as TNF-α, IL-1 and IL-6 (3). LPS also activates cascade reactions that lead to extensive liver damage. It has been demonstrated that following D-Gal sensitization, the sensitivity to LPS and the liver organ damage specificity of the animal is enhanced (5). In the present experiment, an animal model of acute hepatic injury, induced by an i.p. injection of D-Gal combined with LPS, was established and significant pathological changes was found to occur in the liver tissue 24 h after administering XBJ. Multifocal and small patchy necrosis accompanied by inflammatory cell infiltration were visible in the hepatic lobules, which is in line with acute liver injury.

Currently, there are many therapies for liver injury, which are associated with certain advantages and disadvantages (16). Liver transplantation therapy may be of limited value, as it is dependent on various factors including the age of the patient and the availability of sufficient donors of human leukocyte antigen, as well as being associated with multiple complications such as infection, great difficulties in treating graft vs. host disease and a high fatality rate (17). In the present study, we showed that XBJ reduces the mortality rate of rats with acute liver injury induced by D-Gal combined with LPS.

The level of serum aminotransferases is an important marker for judging the severity of acute hepatic injury (16).

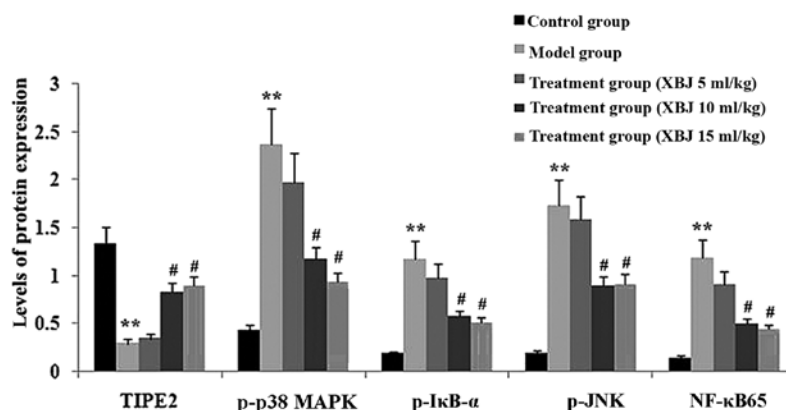


Figure 12. Xuebijing injection (XBJ) treatment upregulates TIPE2 protein expression and attenuates phosphorylated (p-p38) MAPK, p-IκB-α, p-JNK and NF-κB65 protein expression. Groups of rats were challenged with LPS, 10 μg/kg plus D-galactosamine (D-Gal), 500 mg/kg and treated with XBJ (5, 10 and 15 ml/kg body weight) 24 h later. TIPE2, p-p38 MAPK, p-IκB-α, p-JNK and NF-κB65 protein expression were measured by western blot analysis and a statistical summary of the densitometric analysis of TIPE2, p-p38MAPK, p-IκB-α, p-JNK and NF-κB65 protein expression levels in the five groups of rats is shown. Data are presented as the means ± SD of one experiment consisting of three replicates. The experiments were performed in triplicate. **P<0.01 vs. the control group, #P<0.05 vs. the model group.

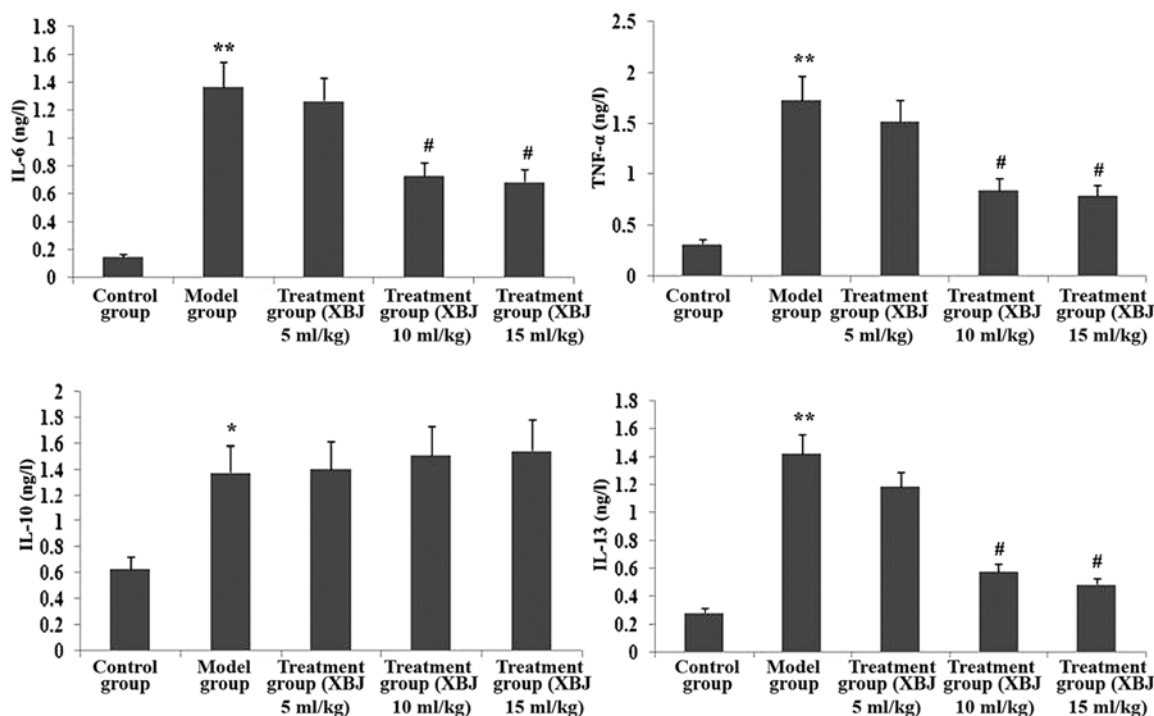


Figure 13. Effects of Xuebijing injection (XBJ) on the production of cytokines in lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. Rats were treated with various concentrations of XBJ (5, 10 and 15 ml/kg body weight) for 24 h and exposed to LPS, 10 μ g/kg plus D-Gal, 500 mg/kg. We measured the serum levels of TNF- α , IL-6, IL-13, and IL-10 using ELISA. Data are expressed as the means \pm SE values from three independent experiments in each group. * P <0.05, ** P <0.01 vs. the control group. # P <0.05 vs. the model group.

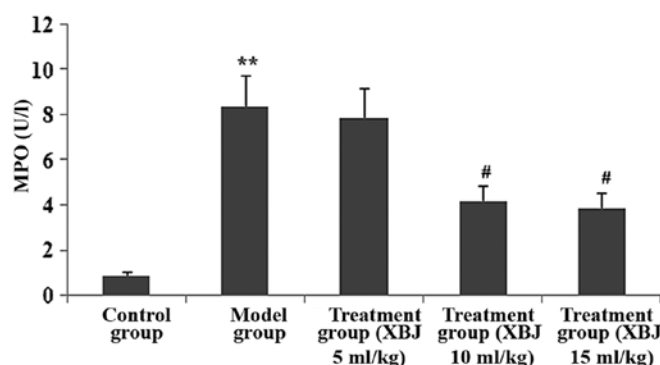


Figure 14. Xuebijing injection (XBJ) reduces myeloperoxidase (MPO) activity in lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. Rats were treated with various concentrations of XBJ (5, 10 and 15 ml/kg body weight) for 24 h and exposed to LPS, 10 μ g/kg plus D-Gal, 500 mg/kg. We measured MPO activity in liver tissue. Data are expressed as the means \pm SE values from three independent experiments in each group. ** P <0.01 vs. the control group, # P <0.05 vs. the model group.

This study showed that XBJ reduced the generation of ALT and AST in a dose-dependent manner, and significantly ameliorated pathological changes in the livers of rats with acute liver injury induced by D-Gal combined with LPS.

The active constituents of XBJ injection include carthamin yellow A, ligustrazine, tanshinol, ferulic acid, paeoniflorin and protocatechualdehyde (20). Among these, carthamin yellow A increases tissue hypoxia tolerance and reduces capillary permeability; ferulic acid removes oxygen radicals, regulates immune function and prevents pulmonary fibrosis (20); and ligustrazine removes oxygen

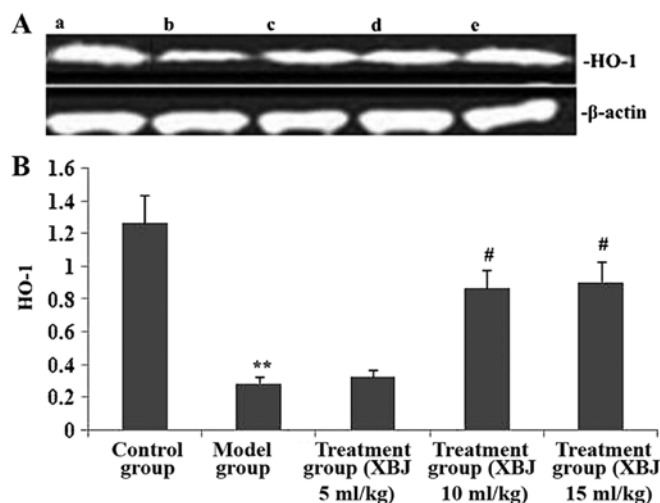


Figure 15. Xuebijing injection (XBJ) upregulates (heme oxygenase-1) HO-1 protein expression. Rats were treated with various concentrations of XBJ (5, 10 and 15 ml/kg body weight) for 24 h and exposed to LPS, 10 μ g/kg plus D-Gal, 500 mg/kg. We measured HO-1 protein expression in rat liver tissues. (A) Representative RT-qPCR shows the level of HO-1 protein expression in the five rat groups: (a) control group, (b) model group, and (c-e) treatment groups (XBJ, at doses of 5, 10 and 15 ml/kg). (B) Statistical summary of the densitometric analysis of the mRNA expression of HO-1 in the five rat groups. Data are expressed as the means \pm SE values from three independent experiments in each group. ** P <0.01 vs. the control group, # P <0.05 vs. the model group.

radicals and reduces vasopermeability to prevent and treat acute respiratory distress syndrome (ARDS) and acute lung injury (19,20). Tanshinol ameliorates ischemia/reperfusion injury, removes oxygen free radicals, regulates immunity,

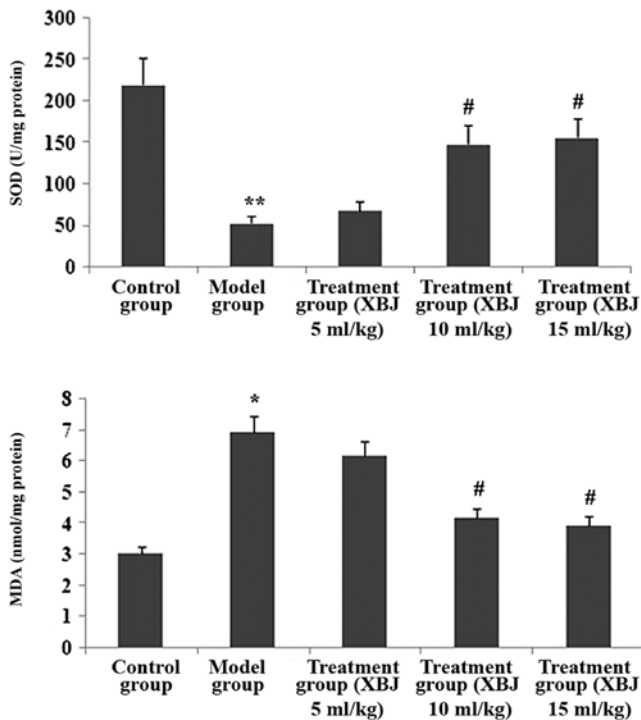


Figure 16. Xuebijing injection (XBJ) inhibits malondialdehyde (MDA) activation and increases superoxide dismutase (SOD) activation in lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. Rats were treated with various concentrations of XBJ (5, 10 and 15 ml/kg body weight) for 24 h and exposed to LPS, 10 μ g/kg plus D-Gal, 500 mg/kg. We measured MDA and SOD activation in liver tissue. Data are expressed as the means \pm SE values from three independent experiments in each group. * $P < 0.05$, ** $P < 0.01$ vs. the control group, # $P < 0.05$ vs. the model group.

antagonizes endotoxin and hypoxic pulmonary microvascular contraction to prevent ARDS, and paeoniflorin has effects on ARDS balance and prevention or regulation of thromboxane A₂/prostacyclin I₂ (TXA₂/PGI₂) (21). XBJ exerts powerful anti-endotoxin effects and causes the uncontrollable release of endogenous inflammation medium induced by LPS and generated by monocyte-macrophages (22). It has been demonstrated to inhibit the mRNA expression of procollagen protein, reduce the content of lipid peroxide, improve the activity of SOD so as to eliminate oxygen free radicals, protect vascular endothelial cells, reduce capillary permeability, relieve acute lung injury, reduce fibroblasts, inhibit the deposition of extracellular matrix (ECM) thereby attenuating the degree of pulmonary fibrosis and inhibiting the development of pulmonary fibrosis (23,24). From the experimental results, we concluded that XBJ injection increases the expression of TIPE2 in rats with acute liver injury induced by exposure to D-Gal in combination with LPS, inhibits the expression of the pro-inflammatory genes of acute hepatic injury, reduces oxidative stress and inflammation, ameliorates pathological changes in the livers of rats with acute liver injury, and reduces the mortality rate of acute liver injury induced by exposure to D-Gal in combination with LPS.

Existing research has shown that the severity of acute liver injury is closely associated with levels of prognostic cytokines. TNF- α is the main cytokine involved in hepatic injury, and IL-6 may inhibit liver cell regeneration (18). As a proteinase highly expressed in neutrophils, MPO is a major indicator of

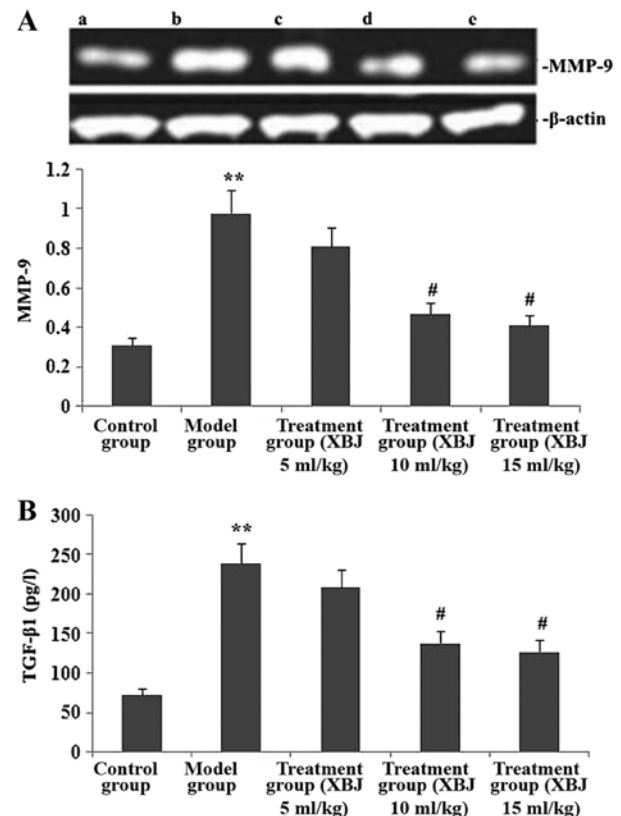


Figure 17. Xuebijing injection (XBJ) suppresses transforming growth factor β 1 (TGF- β 1) and matrix metalloproteinase-9 (MMP-9) expression in lipopolysaccharide (LPS) plus D-galactosamine (D-Gal)-exposed rats. Rats were treated with various concentrations of XBJ (5, 10 and 15 ml/kg body weight) for 24 h and exposed to LPS, 10 μ g/kg plus D-Gal, 500 mg/kg. (A) MMP-9 protein expression in the liver tissue was determined by western blot analysis [(a) control group, (b) model group and (c-e) treatment groups (XBJ at doses of 5, 10 and 15 ml/kg, respectively)]. (B) TGF- β 1 levels in serum were assayed by ELISA. Data are expressed as the means \pm SE values from three independent experiments in each group. ** $P < 0.01$ vs. the control group, # $P < 0.05$ vs. the model group.

neutrophil infiltration (25). We observed the increased secretion of pro-inflammatory mediators in rat livers exposed to D-Gal in combination with LPS, such as TNF- α , IL-13 and IL-6. XBJ reduced the secretion of TNF- α , IL-13 and IL-6 in a dose-dependent manner, whereas IL-10 levels remained unchanged. Simultaneously, the study also found that XBJ injection inhibits the activity of MPO, reduces intrahepatic gathering of inflammatory cells and further reduces inflammatory liver injury induced by D-Gal in combination with LPS.

It has been shown that by influencing gene transcription for a variety of cytokines, NF- κ B exerts complex effects on inflammation and the immune regulatory network (26-28). It has been suggested that activated NF- κ B enhances the transcription of cytokines, such as TNF- α and IL-1 β , which increases the duration of synthesis of inflammatory mediators (27). I κ B- α is the inhibiting factor of NF- κ B, which combines with NF- κ B under quiescent conditions (26). Following stimulation by LPS and cytokines, I κ B- α is phosphorylated enabling NF- κ B to transfer freely from the cytoplasm to the nucleus, where NF- κ B initiates gene transcription and the translation of various cytokines, thereby inducing the activation of inflammatory cells. The experimental results showed that D-Gal in combination with LPS stimulated the expression of NF- κ B65, promoted

I κ B- α phosphorylation, enhanced the inflammatory response and aggravated hepatic injury. After administering the XBJ injection, the expression of NF- κ B65 was reduced, I κ B- α phosphorylation was reduced, the inflammatory response was attenuated and the extent of hepatic injury was alleviated.

The MAPKs are important mediators for signal transduction from the cell surface to the inside of the cell, and play important roles in the regulation of the inflammatory response (29). The continuous activation of MAPKs may cause the constant generation of inflammatory cytokines and lead to cascade 'waterfall' effects. It is believed that p38 MAPK is mostly associated with the activation of NF- κ B in the MAPK signaling pathway (28,29). It has been shown in a rat model of sodium taurocholate-induced acute pancreatitis that the activity of macrophage p38 MAPK is enhanced, along with activation of NF- κ B, so as to inhibit the expression of p38 MAPK, and inhibit NF- κ B activity (30,31). In the present study, we found that XBJ injection promotes TIPE2 expression, inhibits the activity of p38 MAPK, reduces the activation of NF- κ B and reduces the degree of hepatic injury induced by D-Gal in combination with LPS. The JNK pathway is the principal way of inducing inflammation and cell apoptosis. It has been demonstrated that JNK signaling is stimulated by various external factors such as stress (ultraviolet radiation, high pressure infiltration and ischemia/reperfusion injury), cytokines (TNF- α and IL-1) and growth factors (30). p38 and JNK are activated by damage, such as liver injury, and the regulatory effects are the result of a collaborative relationship (31). The findings of this study also indicated that XBJ inhibits the p38 MAPK pathway, inhibits the phosphorylation of JNK and attenuates the degree of hepatic injury.

TIPE2 is a member of the TNFAIP8 family (9), which is expressed preferentially in the lymphatic tissue and myeloid-derived suppressor cells, and is constructed in a characteristic way (8). Namely, the large hydrophobic cavity located at the center of TIPE2 may play an important role in maintaining immunological homeostasis. In the case of accepting immunostimulation, other accessory factors are capable of competitively occupying the cavity, releasing immune activation factors and causing activation of the immune response. Previous findings have confirmed that TIPE2 gene knockout rats gradually exhibit weight loss, splenomegaly, leukocytosis and multiple organ spontaneous inflammatory responses. After LPS attack, an excessive inflammatory response may occur, which may cause sepsis or even premature death (8). The current data show that TIPE2 participates in the regulation of multiple signal transduction pathways. It has been suggested that TIPE2 inhibits JNK and activates p38 MAPK, and down-regulates activator protein-1 (AP-1) transcription factor so as to generate negative regulation signaling of JNK and p38 MAPK transduction pathways (32,33). However, TIPE2 does not play a role in the extracellular signal-related kinase (ERK) signaling pathway. TIPE2-deficient cells are hyper-responsive to the signal activation of TLRs and TCRs (8). TIPE2 expression is upregulated in a number of acute and chronic diseases, including sepsis, and it plays a key role in both the adaptive and innate immune systems, manifesting a negative regulatory effect in the maintenance of immune homeostasis (8,32,33).

HO-1 is a stress response protein. It is associated with the inhibition of cell apoptosis, anti-inflammatory effects and

enhanced resistance to oxidative stress, and the degradation products bilirubin, biliverdin and carbon monoxide (CO) play an important role in these functions. Bilirubin and biliverdin alleviate the adhesion between leukocyte and vascular endothelial cells, inhibit the activation of NADPH-oxidizing enzymes and inhibit the chemotaxis of monocytes. Bilirubin has an anti-complement effect to protect cells from inflammation and oxidative damage mediated by complement activation (34). MDA is the main product of lipid peroxidation, and the majority of tests define the degree of oxidative damage in the body by determining the amount of MDA (35). SOD is a key enzyme involved in the dismutation of superoxide radicals resulting from cellular oxidative metabolism into hydrogen peroxide, and prevents LPS-induced penetration (35). In the present study, we determined that following XBJ treatment, the expression of TIPE2 was upregulated, the expression of HO-1 was enhanced, the activity of MDA was inhibited, the activity of SOD was enhanced, and the degree of hepatic injury induced by D-Gal in combination with LPS was attenuated.

MMP-9 is mainly found in inflammatory cells and epithelial cells, and is capable of degrading many ECM ingredients, particularly type IV collagen, and it plays an important role in the occurrence and development of tissue fibrosis (36,37). A previous study has indicated that JNK1/2 was involved in *Moraxella catarrhalis* lipooligosaccharide (LOS)-induced MMP-9 expression (38). p38 MAPK and NF- κ B have also been shown to mediate MMP-9 expression (39). TGF- β 1 is known to enhance the fibrotic process by enhancing fibroblast growth and collagen production, as well as by promoting the differentiation of fibroblasts into myofibroblasts, which secrete collagen and other ECM components (40). In addition, it has been found that TGF- β 1 affects several signal transduction pathways in a Smad-independent manner, such as MAPK, including ERK, p38 MAPK and JNK (41). In the present study, we have demonstrated that XBJ injection reduced the expression of MMP-9 and TGF- β 1 by inhibiting the p38 MAPK and JNK pathways, which contributed to a reduction in the development of liver fibrosis induced by D-Gal in combination with LPS.

As expected, following LPS/D-Gal exposure, Kupffer cells secrete various proinflammatory cytokines including TNF- α , IL-1 β , IL-6, as well as the anti-inflammatory cytokine, IL-10. TNF- α induces the necrosis and apoptosis of hepatocytes which have been identified as the most important mechanisms of LPS/D-Gal-induced liver injury (42). LPS-induced TNF- α , IL-6 and IL-1 β mainly depend on the activation of two distinct signaling pathways, the MAPK and NF- κ B pathways (43). Caspase-3 is known as a downstream "effector" caspase which is activated by initiator caspases and has substrate specificity for the DEXD motif. In most cases, caspase-3 activation is essential for complete cell apoptosis (44). In the present study, our results indicated that XBJ inhibited the MAPK and NF- κ B pathways, inhibited caspase-3 activation and attenuated the apoptosis of hepatocytes in LPS plus D-Gal-exposed rats.

In conclusion, XBJ injection exerts a protective effect against hepatic injury induced by D-Gal in combination with LPS. XBJ exerts a therapeutic effect through the upregulation of TIPE2 expression, the inhibition of p38 MAPK and NF- κ B pathways, the enhanced activity of HO-1, reduced inflammation and oxidative stress, the attenuation of hepatic injury and apoptosis of hepatocytes, the inhibition of MMP-9 and

TGF- β 1 expression as well as the inhibition of the occurrence and development of hepatic fibrosis. This has provided an experimental basis for the clinical application of XBJ injection in the treatment of critical diseases, such as sepsis and MODS.

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References

- Nolan JP: The role of intestinal endotoxin in liver injury: a long and evolving history. *Hepatology* 52: 1829-1835, 2010.
- Kudo H, Takahara T, Yata Y, Kawai K, Zhang W and Sugiyama T: Lipopolysaccharide triggered TNF- α -induced hepatocyte apoptosis in a murine non-alcoholic steatohepatitis model. *J Hepatol* 51: 168-175, 2009.
- Yang SQ, Lin HZ, Lane MD, Clemens M and Diehl AM: Obesity increases sensitivity to endotoxin liver injury: implications for the pathogenesis of steatohepatitis. *Proc Natl Acad Sci USA* 94: 2557-2562, 1997.
- Peng X, Nie Y, Wu J, Huang Q and Cheng Y: Juglone prevents metabolic endotoxemia-induced hepatitis and neuroinflammation via suppressing TLR4/NF- κ B signaling pathway in high-fat diet rats. *Biochem Biophys Res Commun* 462: 245-250, 2015.
- Sakaguchi S, Takahashi S, Sasaki T, Kumagai T and Nagata K: Progression of alcoholic and non-alcoholic steatohepatitis: common metabolic aspects of innate immune system and oxidative stress. *Drug Metab Pharmacokinet* 26: 30-46, 2011.
- Uesugi T, Froh M, Arteel GE, Bradford BU, Wheeler MD, Gäbele E, Isayama F and Thurman RG: Role of lipopolysaccharide-binding protein in early alcohol-induced liver injury in mice. *J Immunol* 168: 2963-2969, 2002.
- Su GL, Hoesel LM, Bayliss J, Hemmila MR and Wang SC: Lipopolysaccharide binding protein inhibitory peptide protects against acetaminophen-induced hepatotoxicity. *Am J Physiol Gastrointest Liver Physiol* 299: G1319-G1325, 2010.
- Sun H, Gong S, Carmody RJ, Hilliard A, Li L, Sun J, Kong L, Xu L, Hilliard B, Hu S, *et al*: TIPE2, a negative regulator of innate and adaptive immunity that maintains immune homeostasis. *Cell* 133: 415-426, 2008.
- Gus-Brautbar Y, Johnson D, Zhang L, Sun H, Wang P, Zhang S, Zhang L and Chen YH: The anti-inflammatory TIPE2 is an inhibitor of the oncogenic Ras. *Mol Cell* 45: 610-618, 2012.
- Li D, Song L, Fan Y, Li X, Li Y, Chen J, Zhu F, Guo C, Shi Y and Zhang L: Down-regulation of TIPE2 mRNA expression in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Clin Immunol* 133: 422-427, 2009.
- Li HF, Sun ML, Yu YX and Liu XL: Xuebijing alters tumor necrosis factor- α , interleukin-1 β and p38 mitogen activated protein kinase content in a rat model of cardiac arrest following cardiopulmonary resuscitation. *Neural Regen Res* 6: 2573-2576, 2011.
- Jia P, Wang S, Meng X, Lan W, Luo J, Liao S, Xiao C, Zheng X, Li L, Liu Q, *et al*: Effects of ionic liquid and nanogold particles on high-performance liquid chromatography-electrochemical detection and their application in highly efficient separation and sensitive analysis of five phenolic acids in Xuebijing injection. *Talanta* 107: 103-110, 2013.
- Liu YC, Yao FH, Chai YF, Dong N, Sheng ZY and Yao YM: Xuebijing injection promotes M2 polarization of macrophages and improves survival rate in septic mice. *Evid Based Complement Alternat Med* 2015: 352642, 2015.
- 't Hart NA, van der Plaats A, Leuvenink HG, Wiersema-Buist J, Olinga P, van Luyn MJ, Verkerke GJ, Rakhurst G and Ploeg RJ: Initial blood washout during organ procurement determines liver injury and function after preservation and reperfusion. *Am J Transplant* 4: 1836-1844, 2004.
- Ben Mosbah I, Alfany-Fernández I, Martel C, Zaouali MA, Bintanel-Morcillo M, Rimola A, Rodés J, Brenner C, Roselló-Catafau J and Peralta C: Endoplasmic reticulum stress inhibition protects steatotic and non-steatotic livers in partial hepatectomy under ischemia-reperfusion. *Cell Death Dis* 1: e52, 2010.
- Chen P, Chen Y, Wang Y, Cai S, Deng L, Liu J and Zhang H: Comparative evaluation of hepatoprotective activities of geniposide, crocins and crocetin by CCl₄-induced liver injury in mice. *Biomol Ther (Seoul)* 24: 156-162, 2016.
- Norvell JP: Liver disease after hematopoietic cell transplantation in adults. *Transplant Rev (Orlando)* 29: 8-15, 2015.
- Zhu RZ, Xiang D, Xie C, Li JJ, Hu JJ, He HL, Yuan YS, Gao J, Han W and Yu Y: Protective effect of recombinant human IL-1Ra on CCl₄-induced acute liver injury in mice. *World J Gastroenterol* 16: 2771-2779, 2010.
- Qi F, Liang ZX, She DY, Yan GT and Chen LA: A clinical study on the effects and mechanism of Xuebijing injection in severe pneumonia patients. *J Tradit Chin Med* 31: 46-49, 2011.
- Jiang M, Zhou M, Han Y, Xing L, Zhao H, Dong L, Bai G and Luo G: Identification of NF- κ B Inhibitors in Xuebijing injection for sepsis treatment based on bioactivity-integrated UPLC-Q/TOF. *J Ethnopharmacol* 147: 426-433, 2013.
- Sun ML, Ma DH, Liu M, Yu YX, Cao DB, Ma C, Wang X and Liu XL: Successful treatment of paraquat poisoning by Xuebijing, an injection concocted from multiple Chinese medicinal herbs: a case report. *J Altern Complement Med* 15: 1375-1378, 2009.
- Liu MW, Wang YH, Qian CY and Li H: Xuebijing exerts protective effects on lung permeability leakage and lung injury by upregulating Toll-interacting protein expression in rats with sepsis. *Int J Mol Med* 34: 1492-1504, 2014.
- Gui YG, Chai YF and Yao YM: Effects of Xuebijing injection on tissue factor of monocyte and coagulation parameters in septic rats. *Chin J Exp Surg* 27: 32-34, 2010.
- Lu YQ, Gu LH and Huang WD: Effect of Xuebijing injection on peripheral T-lymphocyte subpopulations in patients with severe trauma. *Chin J Traumatol* 13: 72-76, 2010.
- Chen T, Guo Q, Wang H, Zhang H, Wang C, Zhang P, Meng S, Li Y, Ji H and Yan T: Effects of esculetin on lipopolysaccharide (LPS)-induced acute lung injury via regulation of RhoA/Rho Kinase/NF- κ B pathways in vivo and in vitro. *Free Radic Res* 49: 1459-1468, 2015.
- Brasier AR: The NF- κ B regulatory network. *Cardiovasc Toxicol* 6: 111-130, 2006.
- Killeen MJ, Linder M, Pontoniere P and Crea R: NF- κ B signaling and chronic inflammatory diseases: exploring the potential of natural products to drive new therapeutic opportunities. *Drug Discov Today* 19: 373-378, 2014.
- Zhang Y and Li J: Carbachol ameliorates lipopolysaccharide-induced intestinal epithelial tight junction damage by down-regulating NF- κ B and myosin light-chain kinase pathways. *Biochem Biophys Res Commun* 428: 321-326, 2012.
- Liu MW, Su MX, Zhang W, Wang YQ, Chen M, Wang L and Qian CY: Protective effect of Xuebijing injection on paraquat-induced pulmonary injury via down-regulating the expression of p38 MAPK in rats. *BMC Complement Altern Med* 14: 498, 2014.
- Hong Z, Cabrera JA, Mahapatra S, Kutty S, Weir EK and Archer SL: Activation of the EGFR/p38/JNK pathway by mitochondrial-derived hydrogen peroxide contributes to oxygen-induced contraction of ductus arteriosus. *J Mol Med Berl* 92: 995-1007, 2014.
- Murakami K, Suzuki C, Fujii A, Kobayashi F, Nakano A and Kamizono A: Intravenous immunoglobulin preparation prevents the production of pro-inflammatory cytokines by modulating NF κ B and MAPKs pathways in the human monocytic THP-1 cells stimulated with procalcitonin. *Inflamm Res* 63: 711-718, 2014.
- Freundt EC, Bidere N and Lenardo MJ: A different TIPE of immune homeostasis. *Cell* 133: 401-402, 2008.
- Luan YY, Yao YM, Zhang L, Dong N, Zhang QH, Yu Y and Sheng ZY: Expression of tumor necrosis factor- α induced protein 8 like-2 contributes to the immunosuppressive property of CD4(+)CD25(+) regulatory T cells in mice. *Mol Immunol* 49: 219-226, 2011.
- Das S, Pandey K, Rabidas VN, Mandal A and Das P: Effectiveness of miltefosine treatment in targeting anti-leishmanial HO-1/Nrf-2-mediated oxidative responses in visceral leishmaniasis patients. *J Antimicrob Chemother* 68: 2059-2065, 2013.

35. McKenzie MJ, Goldfarb A, Garten RS and Vervaecke L: Oxidative stress and inflammation response following aerobic exercise: role of ethnicity. *Int J Sports Med* 35: 822-827, 2014.
36. Cheng CY, Kuo CT, Lin CC, Hsieh HL and Yang CM: IL-1 β induces expression of matrix metalloproteinase-9 and cell migration via a c-Src-dependent, growth factor receptor transactivation in A549 cells. *Br J Pharmacol* 160: 1595-1610, 2010.
37. Jeong S, Ledee DR, Gordon GM, Itakura T, Patel N, Martin A and Fini ME: Interaction of clusterin and matrix metalloproteinase-9 and its implication for epithelial homeostasis and inflammation. *Am J Pathol* 180: 2028-2039, 2012.
38. Chang SJ, Wang TY, Lee YH and Tai CJ: Extracellular ATP activates nuclear translocation of ERK1/2 leading to the induction of matrix metalloproteinases expression in human endometrial stromal cells. *J Endocrinol* 193: 393-404, 2007.
39. Tung WH, Tsai HW, Lee IT, Hsieh HL, Chen WJ, Chen YL and Yang CM: Japanese encephalitis virus induces matrix metalloproteinase-9 in rat brain astrocytes via NF- κ B signalling dependent on MAPKs and reactive oxygen species. *Br J Pharmacol* 161: 1566-1583, 2010.
40. Kolosova I, Nethery D and Kern JA: Role of Smad2/3 and p38 MAP kinase in TGF- β 1-induced epithelial-mesenchymal transition of pulmonary epithelial cells. *J Cell Physiol* 226: 1248-1254, 2011.
41. Voloshenyuk TG, Landesman ES, Khoutorova E, Hart AD and Gardner JD: Induction of cardiac fibroblast lysyl oxidase by TGF- β 1 requires PI3K/Akt, Smad3, and MAPK signaling. *Cytokine* 55: 90-97, 2011.
42. Zou W, Roth RA, Younis HS, Malle E and Ganey PE: Neutrophil-cytokine interactions in a rat model of sulindac-induced idiosyncratic liver injury. *Toxicology* 290: 278-285, 2011.
43. Liang Y, Hou C, Kong J, Wen H, Zheng X, Wu L, Huang H and Chen Y: HMGB1 binding to receptor for advanced glycation end products enhances inflammatory responses of human bronchial epithelial cells by activating p38 MAPK and ERK1/2. *Mol Cell Biochem* 405: 63-71, 2015.
44. Hanna DM, Tadros MG and Khalifa AE: ADIOL protects against 3-NP-induced neurotoxicity in rats: possible impact of its anti-oxidant, anti-inflammatory and anti-apoptotic actions. *Prog Neuropsychopharmacol Biol Psychiatry* 60: 36-51, 2015.