Glycyrrhizin attenuates endotoxin-induced acute liver injury after partial hepatectomy in rats

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Abstract

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Massive hepatectomy associated with infection induces liver dysfunction, or even multiple organ failure and death. Glycyrrhizin has been shown to exhibit anti-oxidant and anti-inflammatory activities. The aim of the present study was to investigate whether glycyrrhizin could attenuate endotoxin-induced acute liver injury after partial hepatectomy. Male Wistar rats (6 to 8 weeks old, weighing 200-250 g) were randomly assigned to three groups of 24 rats each: sham, saline and glycyrrhizin. Rats were injected intravenously with lipopolysaccharide (LPS) 24 h after 70% hepatectomy. Glycyrrhizin, pre-administered three times with 24 h intervals 48 h before hepatectomy, prolonged the survival of rats submitted to partial hepatectomy and LPS injection, compared with saline controls. Glycyrrhizin was shown to attenuate histological hepatic changes and significantly reduced serum levels of aspartate aminotransferase, alanine aminotransferase, and lactic dehydrogenase, at all the indicated times (6 rats from each were sacrificed 1, 3, 6, and 9 h after LPS injection), compared with saline controls. Glycyrrhizin also significantly inhibited hepatocyte apoptosis by down-regulating the expression of caspase-3 and inhibiting the release of cytochrome C from mitochondria into the cytoplasm. The anti-inflammatory activity of glycyrrhizin may rely on the inhibition of release of tumor necrosis factor-α, myeloperoxidase activity, and translocation of nuclear factor-kappa B into the nuclei. Glycyrrhizin also up-regulated the expression of proliferating cell nuclear antigen, implying that it might be able to promote regeneration of livers harmed by LPS. In summary, glycyrrhizin may represent a potent drug protecting the liver against endotoxin-induced injury, especially after massive hepatectomy.

Introduction

Extensive hepatectomy is common for hepatic malignancies. Despite improvement in surgical techniques and perioperative management, liver failure remains one of the major complications and involves a high mortality rate since its association with postoperative infections sometimes leads to multiple organ failure and death (1,2). Endotoxemia after massive hepatectomy results in liver injury by oxidative stress (3), free radi-

cal formation (4), inflammatory mediators (5,6) such as tumor necrosis factor-alpha (TNF-α), interleukin-1ß, and cytokine-induced neutrophil chemoattractant, and activation of the transcription factor, nuclear factor-kappa B (NF-κB) (7). Glycyrrhizin, a natural compound extracted from the roots of *Glycyrrhiza glabra*, has been used for more than two decades to treat hepatitis in Japan, and no side effect or toxicity has been observed (8). Its major constituents are glycyrrhetic acid, multiple flavonoids, isoflavonoids, hydroxycoumarins, and sterols (9). Glycyrrhizin inhibits CD4^{+} T cell- and TNF-mediated cytotoxicity (10), and has a membrane-stabilizing effect (11). Because of its anti-inflammatory and antioxidant activities (9), glycyrrhizin has been shown to protect the liver (12) and kidneys (13) from ischemia-reperfusion injury in animal models. Therefore, we designed this study to determine whether glycyrrhizin protects against acute liver injury induced by endotoxemia after massive hepatectomy.

Material and Methods

Animals

Male Wistar rats, 6 to 8 weeks old, weighing 200-250 g, were supplied by the Animal Research Center at the First Clinical Medical School of Harbin Medical University, Harbin, China. The animals were maintained under standard conditions and were fed rodent chow and water. All surgical procedures and animal care were approved by the Institutional Ethics Committee.

Surgical procedures

All surgical procedures were performed by one person in a standardized fashion. The glycyrrhizin injectable solution (2 mg/mL, >95% purity, free of endotoxin) and lipopolysaccharide (LPS, O55:B5) were purchased from Shenzhen Jianan Pharmaceutic Ltd., Shenzhen, China, and Sigma-Aldrich Co., Shanghai, China, respectively. In preliminary experiments, we showed that the effect of 70% partial hepatectomy or LPS treatment alone on rat survival did not differ from the sham operation; thus, these two groups were excluded from the present study. The animals were randomly assigned to sham operation, saline-treated and glycyrrhizin-treated groups of 24 rats each. The rats in the glycyrrhizin-treated group received an injection of glycyrrhizin solution (20 mg/kg body weight) via the penile vein three times (at 48, 24, and 1 h prior to LPS injection; Figure 1). The rats in the saline-treated group received the same volume of physiological saline at the same times. After anesthesia with an intraperitoneal injection of 10% chloral hydrate (300 mg/kg body weight), the animals underwent 70% hepatectomy (14). Twenty-four hours after surgery, LPS was injected into the rats via the penile vein at the dose of 0.5 mg/kg body weight (Figure 1). Animals in the sham operation group underwent laparotomy and the abdominal cavity was closed without partial hepatectomy or LPS injection. At each of the indicated times shown in Figure 1 (1, 3, 6, and 9 h after LPS injection), the rats (6 per group) were randomly sacrificed, and blood and liver samples were collected. Blood samples were centrifuged at 3000 *g* for 10 min to collect serum which was stored at -80ºC.

Biochemical assays

The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and

Figure 1. Experimental protocol. Lipopolysaccharide (LPS, 0.5 mg/kg body weight) was injected intravenously 24 h after 70% hepatectomy. Glycyrrhizin was administered at a dose of 20 mg/kg body weight and samples were collected at the indicated times.
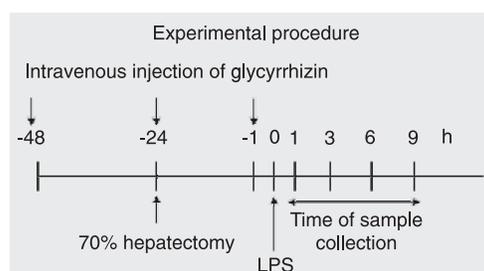
lactate dehydrogenase (LDH) in sera were measured with an automatic biochemical analyzer (Toshiba, Kawasaki, Japan).

Histological examination

Liver specimens were fixed in 10% buffered formalin, embedded in paraffin, stained with hematoxylin/eosin, and examined by light microscopy. The histopathological scoring analysis was performed blindly according to previously described methods (15). The result is reported as the sum of the individual scores from 0 (no findings) to 1 (mild), 2 (moderate), and 3 (severe) for each of the following six parameters: cytoplasmic color fading, vacuolization, nuclear condensation, nuclear fragmentation, nuclear fading, and erythrocyte stasis.

TUNEL assay

Serial 5-μm thick liver sections were prepared. The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL; Roche, Shanghai, China) staining of sections was performed according to manufacturer instructions, and examined by light microscopy. The apoptotic index was calculated as the percentage of stained cells as follows: apoptotic index = number of apoptotic cells x 100 / total number of nucleated cells.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity in liver tissue was determined by a method described in a report by our group (16).

Immunohistochemical analysis

Liver sections (5 μm) were blocked with 2% BSA for 2 h and incubated overnight with antibodies against proliferating cell nuclear antigen (PCNA; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing,

China), or rat NF-κB (Neo Markers Co., Fremont, CA, USA). They were subsequently incubated for 30 min with appropriate secondary antibodies using the PowerVision™ Two-step Histostaining Reagent (Zhongshan Golden Bridge Biotechnology Co.), and developed with Sigma FAST DAB (3,3'-diaminobenzidine tetrahydrochloride) and $CoCl_2$ enhancer tablets (Sigma-Aldrich). Sections were counterstained, mounted, and examined by microscopy. The number of PCNA-positive cells was counted in 30 random high-power fields, and the PCNA-labeling index was calculated according to the following formula: number of PCNA-positive cells x 100 / total number of cells. Similarly, the NF-κB-labeling index was calculated by counting the cells with NF-κB-positive nuclei.

Western blotting

The tissues were excised, minced and homogenized using a motor-driven homogenizer with (for detecting caspase-3) or without (for detecting cytochrome C) protein lysate buffer. Debris were removed by centrifugation at 10,000 g for 10 min at 4ºC. Without protein lysate buffer, the pellets contain the crude mitochondria fraction while the supernatants contain proteins from the cytoplasm, as described previously (17). The supernatants were resolved on 12% polyacrylamide SDS gels and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 3% BSA overnight, and then incubated with primary antibodies against activated caspase-3 or cytochrome C antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and subsequently with alkaline phosphatase-conjugated secondary antibody, and then developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Tiangen Biotech Co. Ltd., Beijing, China). Blots were stained with an anti-tubulin antibody to confirm that each lane contained similar amounts of tumor homogenate.

ELISA

TNF-α levels were measured with a TNF-α ELISA kit (Jingmei Biotech Co. Ltd., Shenzhen, China) according to manufacturer instruction.

Statistical analysis

Survival rate data were analyzed statistically by the log rank test. All other data are reported as means ± SEM and a least significant difference test was used to evaluate statistical significance. P < 0.05 was considered to be statistically significant.

Results

Animal survival

In a preliminary study, glycyrrhizin showed a beneficial survival effect on rats undergoing hepatectomy and LPS treatment. Sham-operated rats had a 100% survival rate. The rats submitted to 70% hepatectomy or LPS treatment alone survived during the monitoring period (data not shown). All animals undergoing 70% hepatectomy and LPS injection and treated with saline died within 36 h of LPS injection, and 30% of them (3/10) died as early as 12 h after LPS injection (Figure 2). In contrast, pre-administration of glycyrrhizin significantly improved the survival rate of rats submitted to 70% hepatectomy and LPS injection (P < 0.05), with the earliest deaths being observed 2 days after LPS treatment, and 50% (5/10) of the rats

survived more than 3 days after LPS treatment (Figure 2).

Liver function and histology

Serum AST, ALT and LDH levels were analyzed to evaluate liver function. LPS significantly increased serum AST (Figure 3A), ALT (Figure 3B) and LDH (Figure 3C) levels in partially hepatectomized rats and these increases were significantly reduced by pre-administration of glycyrrhizin. The results suggest that glycyrrhizin can attenuate the cellular damage that occurs as a result of hepatic damage by LPS after partial hepatectomy.

The serological changes were further confirmed by histological analysis. Histological alteration of the liver from hepatectomized rats treated with LPS was characterized by inflammatory cell infiltration, hemorrhagic change and focal necrosis in the midzone and periportal regions of the liver 6 h after LPS injection (Figure 4). In contrast, pre-treatment with glycyrrhizin markedly attenuated the pathological changes (Figure 4B). The liver injury scores for hepatectomized rats treated with LPS were significantly higher than those for sham-operated rats at all times (P < 0.001 in all cases). Pre-treatment with glycyrrhizin significantly decreased the histological scores compared with the saline-treated group (P < 0.01 in all cases; Figure 4C).

Hepatocyte apoptosis

LPS dramatically increased hepatocyte apoptosis in partially hepatectomized rats (Figure 5B) compared with sham-operated rats (Figure 5A), in which very few apoptotic cells were detected. Pre-treatment with glycyrrhizin markedly reduced the apoptosis of hepatocytes induced by LPS in partially hepatectomized rats (Figure 5C). The apoptotic index in the remnant livers from partially hepatectomized rats was signifi-

Figure 2. Effect of glycyrrhizin on rat survival. A total of 30 rats were randomly assigned to three groups: glycyrrhizin, saline and sham. Glycyrrhizin or saline was pre-administered to the rats in the glycyrrhizin or saline groups, respectively as described in the legend to Figure 1, followed by 70% hepatectomy and lipopolysaccharide (LPS) injection. Rats in the sham group underwent laparotomy and the abdominal cavity was closed without partial hepatectomy or LPS injection. Data indicate percent animal survival (10 rats per group). The experimental protocol is given in Figure 1. PBS = phosphate-buffered saline.
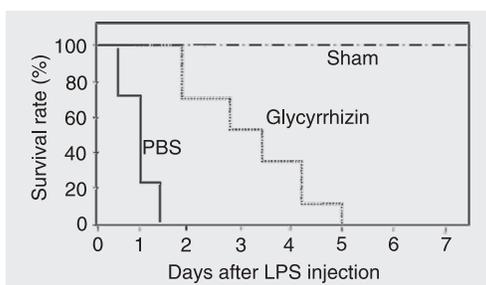
cantly increased at the times indicated after LPS injection compared with sham-operated rats (Figure 5D). Glycyrrhizin significantly reduced the increases in the apoptotic index induced by LPS in remnant livers from hepatectomized rats by 62, 51, 50, and 47%, 1, 3, 6, and 9 h after LPS injection, respectively (Figure 5D).

We further investigated the molecular pathways involved in apoptosis by Western blot analysis of liver homogenates, which demonstrated that LPS up-regulated the expression of activated caspase-3 and increased the expression of cytochrome C in the cellular cytoplasm of partially hepatectomized rats compared with sham-operated rats, and glycyrrhizin attenuated the up-regulation of caspase-3 and the release of cytochrome C into the cytoplasm induced by LPS (Figure 5E).

Inflammatory reaction

LPS dramatically increased liver MPO activity in partially hepatectomized rats compared with sham-operated rats (Figure 6A) and this increase was significantly reduced by pre-treatment with glycyrrhizin. Serum TNF-α levels decreased in a time-dependent manner after LPS treatment in both saline- and glycyrrhizin-treated rats, indicating that TNF-α plays a key role mainly in the early phase of liver injury induced by LPS in partially hepatectomized rats (Figure 6B).

We further investigated the effect of glycyrrhizin on the nuclear translocation of NF-κB, the key upstream factor for various proinflammatory mediators. LPS led to marked overexpression of NF-κB in the cell nuclei of partially hepatectomized rats (Figure 6D) compared with sham-operated rats (Figure 6C). However, pre-treatment with glycyrrhizin inhibited the expression of NF-κB in cell nuclei (Figure 6E). Quantitative data showed that LPS significantly increased the NF-κB-labeling index in partially hepatectomized rats compared with sham-operated

rats (P < 0.001 in all cases), while glycyrrhizin significantly reduced the NF-κB-labeling index increased by LPS at the times indicated (P < 0.01 in all cases, Figure 6F)

Expression of proliferating cell nuclear antigen

We investigated whether glycyrrhizin could improve the regeneration of remnant livers after partial hepatectomy by immunohistochemical analysis of PCNA expression. As shown in Figure 7A and B, pre-administration of glycyrrhizin increased the expression of PCNA in livers from partially hepatectomized rats treated with LPS compared with saline-treated rats. The PCNA-labeling index of hepatocytes was significantly in-
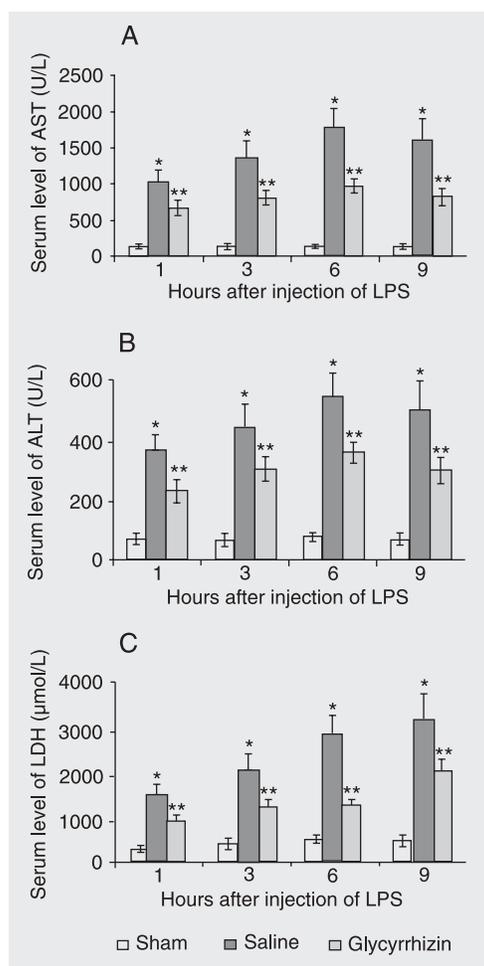

Figure 3. Serum levels of AST (A), ALT (B) and LDH (C). Blood samples were collected at the times indicated after LPS injection from partially hepatectomized rats, which were pre-treated with glycyrrhizin or saline. Sham-operated rats served as control. AST = aspartate aminotransferase; ALT = alanine aminotransferase; LDH = lactate dehydrogenase; LPS = lipopolysaccharide. *P < 0.01 for sham-operated rats compared to saline-treated rats; **P < 0.01 for glycyrrhizin-treated rats compared to saline-treated rats (least significant difference test). The experimental protocol is given in Figure 1.

Figure 4. Histological analysis of the liver. Representative photographs (400X magnification) of liver sections taken 6 h after injection of lipopolysaccharide (LPS) from partially hepatectomized rats, which were treated with saline (A) or glycyrrhizin (B). Arrows point to the necrotic hepatocytes. C, Histopathological scoring of hepatic injury performed as described in Material and Methods. *P < 0.001 for sham-operated rats compared to saline-treated rats; **P < 0.01 for glycyrrhizin-treated rats compared to saline-treated rats (least significant difference test). The experimental protocol is given in Figure 1.
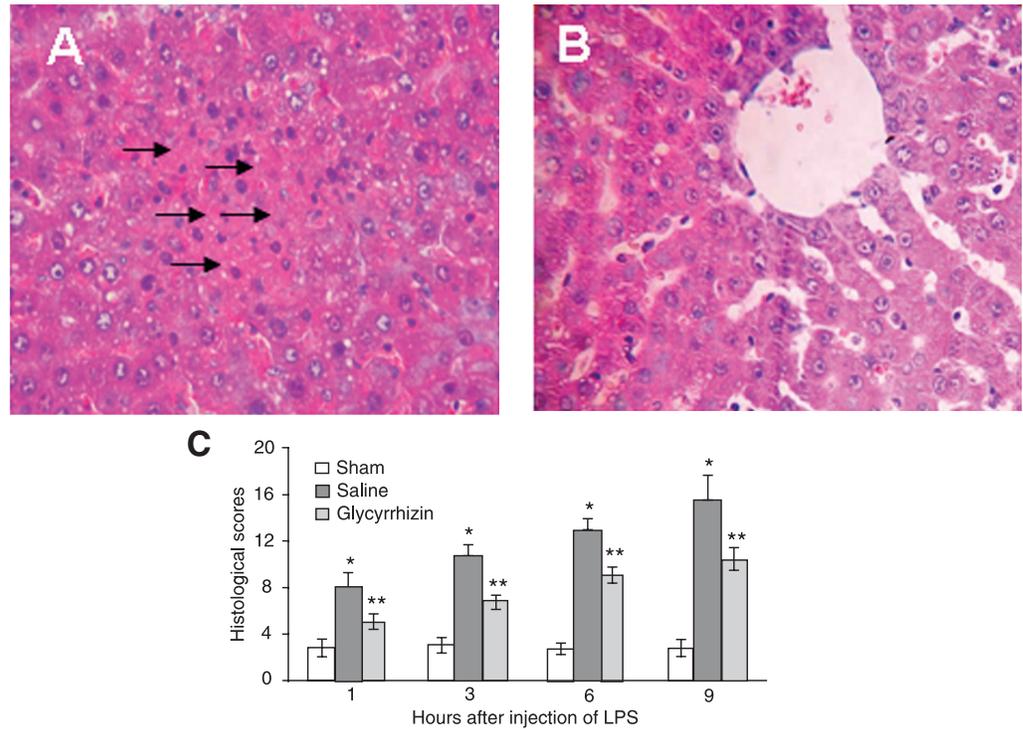

Figure 5. Anti-apoptotic effects of glycyrrhizin. Liver sections stained by TUNEL for apoptotic cells are illustrated. Representative photographs (400X magnification) of liver sections taken 6 h after injection of lipopolysaccharide (LPS) from partially hepatectomized rats, which were treated with saline (B) or glycyrrhizin (C). Sham-operated rats served as control (A). Arrows point to the TUNEL-positive cells. D, TUNEL-positive cells were counted to record the apoptotic index (%). *P < 0.01 for saline-treated rats compared to sham-treated rats; **P < 0.01 for glycyrrhizin-treated rats compared to saline-treated rats (least significant difference test). E, Western blot analysis of cytochrome C in the cellular cytoplasm and of caspase-3 in liver homogenates from sham-operated rats (lane 1), from hepatectomized rats treated with LPS and saline (lane 2), or glycyrrhizin (lane 3). The blot was reacted with an antibody against tubulin, which served as an internal control. The experimental protocol is given in Figure 1.
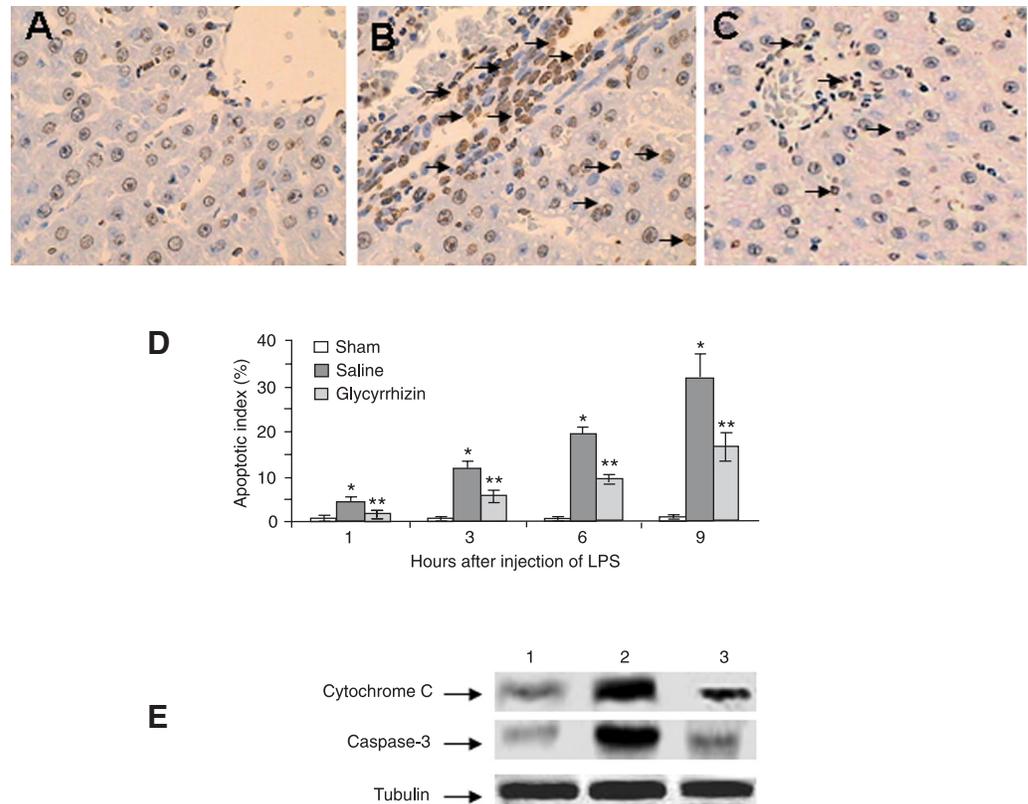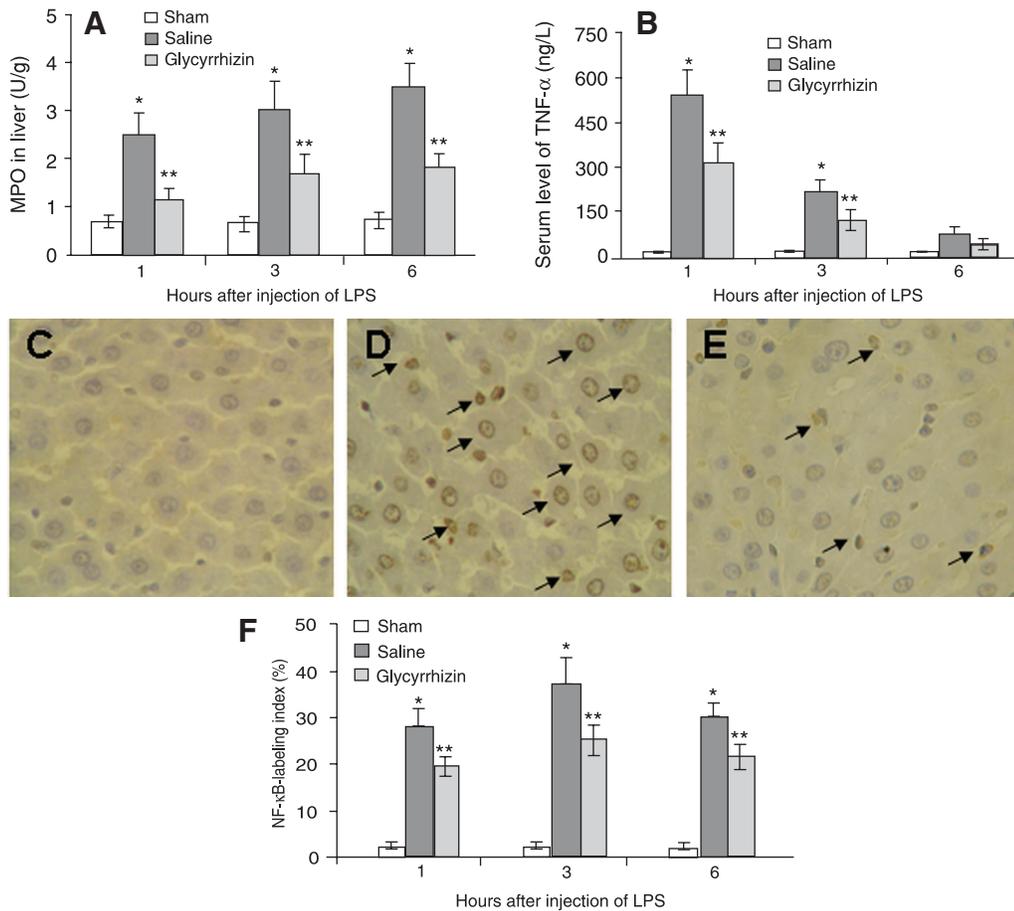
Figure 6. Glycyrrhizin inhibits the inflammatory reaction. At the indicated times after LPS injection, liver (for MPO) and serum (for TNF-α) samples were collected from partially hepatectomized rats, which were pre-treated with saline or glycyrrhizin. Sham-operated rats served as control. The levels of MPO (A) and TNF-α (B) were assessed as described in Material and Methods. C-E, Liver sections taken 6 h after injection of LPS from partially hepatectomized rats treated with saline (D) or glycyrrhizin (E), and sham-operated rats (C), immunostained for NF-κB expression (100X magnification). Arrows point to the NF-κB-positive cell nuclei. F, The cells with NF-κB-positive nuclei were counted to calculate the NF-κB-labeling index. LPS = lipopolysaccharide; MPO = myeloperoxidase; TNF-α = tumor necrosis factor-alpha; NF-κB = nuclear factor-kappa B. *P < 0.001 for saline-treated rats compared to sham-treated rats; **P < 0.01 for glycyrrhizin-treated rats compared to saline-treated rats (least significant difference test). The experimental protocol is given in Figure 1.
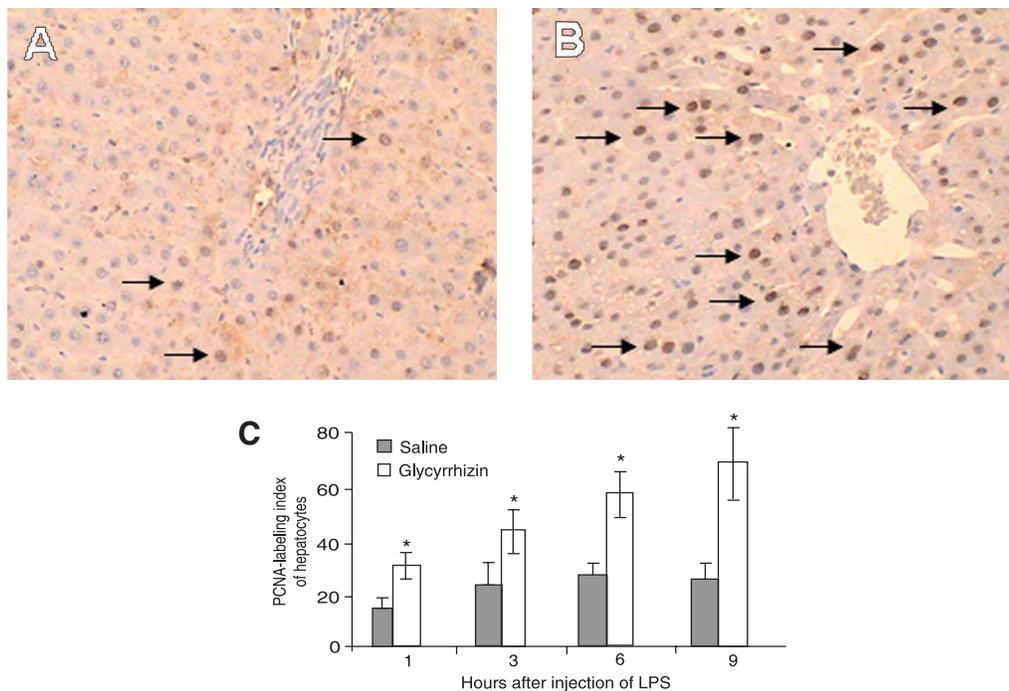

Figure 7. Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA). Liver sections taken 6 h after injection of lipopolysaccharide (LPS) from partially hepatectomized rats treated with saline (A) or glycyrrhizin (B), immunostained for PCNA expression (200X magnification) are illustrated. Arrows point to the PCNA-positive cells. C, PCNA-positive cells were counted to record the PCNA-labeling index for liver sections taken at the indicated times after LPS injection. *P < 0.01 for glycyrrhizin-treated rats compared to saline-treated rats (least significant difference test). The experimental protocol is given in Figure 1.

creased in glycyrrhizin-treated rats compared with saline-treated rats at the times indicated (Figure 7C).

Discussion

Seventy percent hepatectomy is not fatal in rats, but the cytotoxic sensitivity to endotoxin is increased during the early phase after hepatectomy (18). This increased sensitivity is partly associated with the reduced phagocytic function of the reticuloendothelial system after massive hepatectomy (19). Treatment with an even sublethal dose of LPS after hepatectomy induces liver failure (20). The present study demonstrated that the serum levels of AST, ALT, and LDH were significantly increased after LPS injection, and the liver injury was further sustained by pathological alterations including necrosis of the remnant liver in partially hepatectomized rats. Glycyrrhizin inhibited the increases of ALT, AST and LDH, and attenuated the morphological changes. These results indicate that glycyrrhizin prevents endotoxin-induced liver injury, and its protective effect against liver injury has been demonstrated previously in other experimental models (10,12).

Hepatocyte apoptosis was also observed in livers damaged by LPS after partial hepatectomy, in agreement with a previous report (21). Apoptosis is initiated through the release of cytochrome from mitochondria. Cytochrome C binds to the CED-4 homologue Apaf-1, resulting in proteolytic processing and activation of pro-caspase-9. Active caspase-9 then directly cleaves to and activates pro-caspase-3, initiating a cascade of additional caspase activation that culminates in apoptosis (22). The expression of activated caspase-3 and the release of cytochrome C into the cytoplasm were both enhanced by LPS in the present study, and pre-treatment with glycyrrhizin down-regulated this change.

The role of glycyrrhizin in apoptosis is controversial since it has been reported that glycyrrhizin exhibits pro-apoptotic properties, but inhibits the mitochondrial permeability transition, reactive oxygen species generation and cytochrome C release at submicromolar concentration in the same study (23). This difference from the present study was possibly due to the different inducer of cytotoxicity and hepatocyte status in the cited study, in which hepatocyte apoptosis was induced by bile acid *in vitro* (23). On the other hand, apoptosis could also be induced by death domain receptor ligands such as TNF-α and Fas ligand (24). LPS binds to LPS-binding protein (LBP), and the LPS-LBP complex interacts with CD14 to form a ternary complex, LPS:LBP:CD14, which transfers LPS to the toll-like receptor 4 (TLR4) accessory protein MD2 complex, leading to activation of TLR4. Then, LPS-activated TLR4 activates NF-κB (25). Once activated, NF-κB is dissociated from its inhibitor, I-κB, and translocated into the nuclei, where it induces transcriptional up-regulation of various proinflammatory mediators such as TNF-α (26). Glycyrrhizin inhibits the translocation of NF-κB into the nuclei and reduces the levels of TNF-α, thus inhibiting hepatocyte apoptosis via the death domain receptor pathway. Glycyrrhizin has already been demonstrated to attenuate anti-Fas antibody-induced liver injury (27).

During endotoxemia, inflammatory cytokines including TNF-α and neutrophil infiltration play important roles in liver injury (28). TNF-α is a key mediator of the cytokine cascade and tissue injury in sepsis (4), and is involved in the pathogenesis of LPS-induced liver injury (28). By producing oxidative stress, neutrophils activate Kupffer cells and contribute to microvascular dysfunction and edema formation (29). MPO, an enzyme present in neutrophils, is a widely used marker of neutrophil infiltration (30). The present study has demonstrated that LPS treatment increased MPO activity in the liver, suggesting that neutrophils contribute to the

liver damage induced by LPS. Pre-treatment with glycyrrhizin decreases MPO activity, thus inhibiting neutrophil infiltration into the remnant liver and ameliorating the liver injury.

The proliferation of hepatocytes in the remnant liver determines the long-term survival of partially hepatectomized rats. In rats, after 70% hepatectomy, the mass restoration is complete within approximately 7 days and the rate of DNA synthesis in hepatocytes begins to increase after 12 h and reaches a peak at about 24 h after hepatectomy (31). In a previous report, all 70% hepatectomized rats died when they were treated with LPS (18). In the present study, LPS treatment retarded the regeneration of the remnant livers by down-regulating the expression of PCNA, a key marker of cell proliferation. However, pre-treatment with glycyrrhizin up-regulated the expression of PCNA, thus protecting the regeneration of livers harmed by LPS.

The present study demonstrated for the first time the protective activity of glycyrrhizin on endotoxin-induced acute liver injury after partial hepatectomy. Its protective mechanisms may be attributable to its anti-inflammatory activity by inhibiting the release of proinflammatory mediators and neutrophil accumulation. Glycyrrhizin also exhibits anti-apoptotic activity through both the cytochrome and death domain receptor pathways, and pro-regenerative activity. The results indicate that glycyrrhizin may represent a potent drug for liver protection against endotoxin-induced injury, especially after massive hepatectomy.

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