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Alprostadil alleviates liver injury in septic rats *via* TLR4/NF-κB pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of alprostadil (Alp) in cecal ligation and puncture (CLP)-induced septic injury in rats and its possible mechanism of action.

MATERIALS AND METHODS: Wistar rats were randomly assigned into three groups, including: Sham group (no CLP was performed), CLP group (CLP was conducted) and Alp group (Alp was injected after CLP). Serum liver function markers, pathological changes in liver tissues, alterations in the level of oxidative stress, activity of the Toll-like receptor 4 (TLR4)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, and release of inflammatory factor tumor necrosis factor alpha (TNF-α) in the liver tissue homogenate were detected in each group.

RESULTS: Compared with Sham group, the rats in CLP group had substantially elevated content of serum liver function markers, increased apoptotic liver cells, upregulated levels of oxidative stress, enhanced activity of the TLR4/NF-κB pathway, and increased release of TNF-α ($p<0.05$). Meanwhile, there were evident pathological changes under microscopic examination in CLP group compared with Sham group ($p<0.05$). In comparison with CLP group, Alp group exhibited significantly decreased concentrations of liver function markers, microscopic findings, such as decreased inflammatory cell infiltration in the interstitum, notably lowered proportion of apoptotic cells, decreased level of oxidative stress, weakened activity of the TLR4/NF-κB pathway and restrained release of TNF-α ($p<0.05$). Furthermore, normal morphology of liver cells was observed in Alp group compared with CLP group ($p<0.05$).

CONCLUSIONS: Alp alleviates liver injury in septic rats by inhibiting the TLR4/NF-κB pathway.

Key Words:
Alprostadil, TLR4/NF-κB, Liver injury.

Introduction

Sepsis is clinically characterized by rapid onset and high lethality rate. The survival rate of patients can be greatly improved by efficacious treatments as early as possible[1-3]. Sepsis occurs when the host fails to control infections and has relevant signs due to the multiple organ failure caused by complex inflammatory disorders[4]. Increased release of interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNF-α) causes cell dysfunction and plays a crucial role in the pathophysiology of sepsis[5-7]. Besides, the overexpression of these inflammatory factors may be responsible for immune system dysfunction, thereby damaging many types of tissues[6].

Sepsis-induced acute liver injury is triggered due to the activation of Toll-like receptor 4 (TLR4) by bacterial products, such as lipopolysaccharide (LPS) or cytokines, such as TNF-α or IL-1. This may further activate the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and enhance the transcription of pro-inflammatory factors, apoptotic factors and other sepsis-associated inflammatory mediators[8,9]. Previous studies[10,11] have shown that oxidative stress is of vital importance in the progression of sepsis-induced acute liver injury. Alprostadil (Alp), a prostaglandin, has versatile pharmacological and biological activities in the treatment of inflammation and cardiovascular diseases. Sepsis-induced inflammation and oxidative stress are the major pathways leading to acute liver injury. In the present study, therefore, it was speculated that Alp could alleviate sepsis-induced acute liver injury by its anti-inflammatory and anti-oxidative effects.

Materials and Methods

Experimental Materials

Wistar rats were purchased from the Animal Experiment Research Center of Tongji Medical College, Huazhong University of Science and Technology, and laboratory rodent food was provided by Purina Mills (St. Louis, MO, USA).

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Alp was obtained from Milwaukee (Milwaukee, WI, USA), and kits for detection of liver function markers were bought from Biodiagnostics (Worcestershire, UK). Antibodies were provided by Abcam (Cambridge, MA, USA), and TRIzol reagent, diethyl pyrocarbonate (DEPC)-treated water, and SuperScript III RT kit were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China).

Study Objects

Wistar rats without undergoing cecal ligation and puncture (CLP), those undergoing CLP, and those injected with Alp after CLP were set as Sham group, CLP group, and Alp group, respectively.

Establishment of Model of Sepsis-Induced Liver Injury in Rats

This investigation was approved by the Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology Animal Center. Male Wistar rats were adaptively fed in an animal environment equipped with air conditioners under a daily 12 h automatic light for at least 1 week. All rats were given free access to rodent food. CLP model was successfully established in rats to induce sepsis as follows. Briefly, Wistar rats were randomly divided into three groups, including: Sham group, CLP group, and Alp group. The rats were anesthetized *via* intraperitoneal injection of the mixture of 10 mg/kg xylazine and 100 mg/kg ketamine hydrochloride. Subsequently, a 2.0 cm-long incision was made along the midline of the abdomen to expose the cecum and ligate the distal ileocecum. Next, the cecum was punctured twice using an 18-gauge needle. CLP was not performed on rats in Sham group. In Alp group, Alp was intraperitoneally injected at a single dose of 60 mg/kg after CLP. After the induction of sepsis and medication, all rats were fixed on heating pads until they regained consciousness.

Determination of Concentrations of Liver Function Markers Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Gamma-Glutamyl Transferase (γ-GT) and Total Bilirubin (TB)

Peripheral blood was first collected from rats and centrifuged at 3,800 rpm for 10 min. Serum liver function markers were determined according to the instructions of liver function test kits.

Immunoassay of Nuclear and Cytoplasmic Proteins

After the rats were sacrificed, the liver was collected and the nuclear extract was prepared. The concentration of proteins was determined using the bicinchoninic acid protein assay kit. Subsequently, 50 μg of nuclear proteins were separated, and the binding to non-specific sites on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) was blocked. After that, the nuclear NF-κB protein was subjected to immunoassay using rabbit polyclonal antibody against mouse NF-κB. The proliferating cell nuclear antigen was used as an internal reference.

Likewise, the cytoplasmic protein TLR4 was extracted from liver tissues. Briefly, liver tissues were collected from rats and placed in 1.15% potassium chloride, followed by centrifugation at 9,000 g for 30 min at a low temperature. The supernatant was harvested, and subjected to protein quantification and immunoelectrophoresis as described above. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference for cytoplasmic protein.

Pathological Examination of Liver Tissues

Liver tissues were first washed using ice brine, fixed in formalin for 24 h, embedded, and sliced into 6 μm-thick sections using a microtome. Hematoxylin-eosin (HE) staining was then performed. Finally, whether there was inflammation was evaluated.

Evaluation of Apoptotic Cell Proportion in Liver Tissue Homogenate

After the rats were killed, liver tissues were collected and gently ground using a glass rod. Pre-cooled medium was added and centrifuged at 1,000 rpm for 10 min. After discarding the supernatant, liver cells were re-suspended and centrifuged twice as above. Liver cell suspension with high purity was harvested. Later, 1 mL of cell suspension was added with an equal volume of binding buffer for re-suspension. Next, 200 μL of cell suspension was added with 10 μL of FITC/PI dye solution, incubated for 10 min in dark, and added with 400 μL of binding buffer. Finally, the resulting cells were detected using a machine.

Quantitative Real Time-PCR Amplification

Total ribonucleic acid (RNA) in liver tissue homogenate was extracted using TRIzol reagent. Subsequently, complementary deoxyribonucleic acid (cDNA) was synthesized using 1 μg of

RNA. Quantitative Real-Time amplification was performed using a thermal cycler. 18S ribosomal RNA (Rn18s) was used as an endogenous reference for TLR4, TNF-α and myeloperoxidase (MPO). The primers used in this study were as follows: Rn18s-F: AGTTGGTGGAGCGATTTGTC, Rn18s-R: GAACGCCACTTGTCCCTCTA; TNF-α-F: ATGTGGAACTGGCAGAGGAG, TNF-α-R: TGGAACTGATGAGAGGGAG; GAPDH-F: CGCTCTCTGCTCCTCCTGTTC, GAPDH-R: ATCCGTTGACTCCGACCTTCAC. With GAPDH served as the internal control, the relative mRNA expression was calculated by using 2^{-DDCt} method[12].

Measurement of Oxidative Stress Level in Liver Tissues

The concentration of reduced glutathione (GSH) was determined in strict accordance with the Ellman's method. The absorbance of the yellow compound produced in the reaction between GSH and Ellman's reagent was measured at 412 nm, and the concentration of GSH was expressed as nmol/mg tissue. The activity of superoxide dismutase (SOD) in liver homogenate was measured by monitoring its inhibition on the self-oxidation of pyrogallol at an alkaline pH. Absorbance at 420 nm were recorded, and the activity was presented as U/g tissues. Additionally, malondialdehyde (MDA), the end product of lipid peroxidation, was measured by the thiobarbituric acid reactive substance assay. Absorbance at 532 nm was determined, and the content was expressed as nmol/g tissues.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Experimental data were represented as mean ± Standard Deviation (SD). The differences between the two groups were analyzed using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Histopathology of Rats with Sepsis-Induced Liver Injury

Microscopically, the rats in the Sham group exhibited intact liver morphology, and normal liver cell size and morphology. However, the rats in CLP group had evident infiltration of hepatic interstitial inflammatory cells, hydropic degeneration of liver cells, and venous congestion and distension. After Alp treatment in septic rats, it was found that the liver structure remained basically normal, with few inflammatory cells. This suggests that Alp can alleviate CLP-induced pathological changes in liver tissues. Subsequently, the proportion of apoptotic cells in liver tissue suspension was analyzed using flow cytometry. The results indicated that CLP group had a substantially higher proportion of apoptotic liver cells than Sham group and Alp group ($p<0.05$) (Figure 1). These findings imply that Alp can inhibit liver cell apoptosis and mitigate liver injury.

Effect of Alp on Apoptosis in Septic Rats

Apoptosis assay was performed to explore the effect of Alp on liver cell apoptosis. It was found that the proportion of apoptotic liver cells in CLP group was considerably higher than that in Sham group and Alp group ($p<0.05$). Compared with

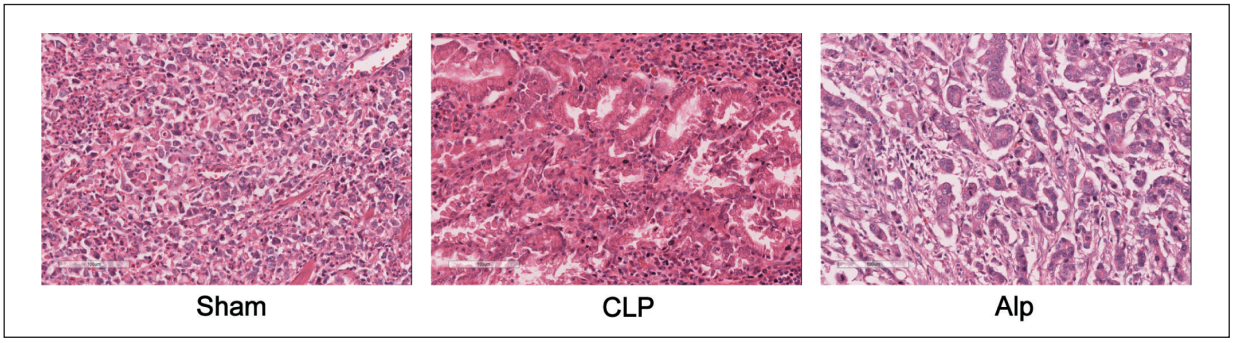

Figure 1. Pathological changes of damaged liver tissues in rats (magnification: 400×). HE staining results indicated that CLP group had increased inflammatory cell infiltration and venous distension, Alp group showed basically normal liver tissue structure and mild inflammatory cell infiltration, and Sham group presented normal liver structure (*$p<0.05$ *vs.* Sham group, $^{\#}p<0.05$ *vs.* CLP group).

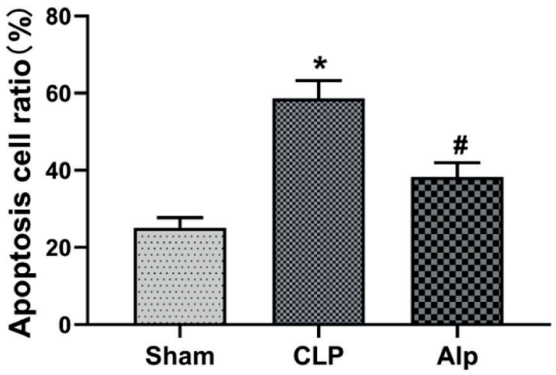

Figure 2. Changes in liver cell apoptosis. The proportion of apoptotic liver cells in CLP group was remarkably higher than that in Sham group and Alp group ($p<0.05$) (*$p<0.05$ *vs.* Sham group, #$p<0.05$ *vs.* CLP group).

CLP group, the proportion of apoptotic liver cells remarkably declined in Alp group ($p<0.05$) (Figure 2). These results suggest that Alp represses the apoptosis in rats with liver injury and promotes the recovery of liver function.

Effect of Alp on Liver Function Changes in Septic Rats

In comparison with Sham group, the parameters of ALT, AST, ALP, γ-GT and TB in the serum of rats with CLP-induced septic liver injury were significantly elevated by 74.5%, 44.6%, 67%, 663.7% and 607.4%, respectively. No statistically significant differences were observed in these parameters between Alp group and Sham group ($p>0.05$). However, they evidently declined in Alp group compared with those in CLP group ($p<0.05$) (Figure 3). Therefore, it can be inferred that the administration of Alp after CLP can significantly prevent the increase of ALT, AST, ALP, γ-GT and TB induced by CLP, thereby alleviating liver function impairment.

Effects of Alp on Changes in Oxidative Stress Markers in Rats with CLP-Induced Liver Injury

Oxidative stress level has been confirmed significantly correlated with liver injury. Therefore, the content of oxidative stress markers was determined in this study. The results demonstrated that the level of GSH decreased by 86.4%, the activity of SOD decreased by 26.9%, and the content of MDA increased by 212.7% in CLP group compared with those in Sham group. After Alp treatment, the rats with liver injury showed the opposite changes in GSH level, SOD activity and MDA content when compared with those in the CLP group (Figure 4). These findings indicate that treatment of Alp can substantially prevent CLP-induced oxidative stress response to relieve liver injury.

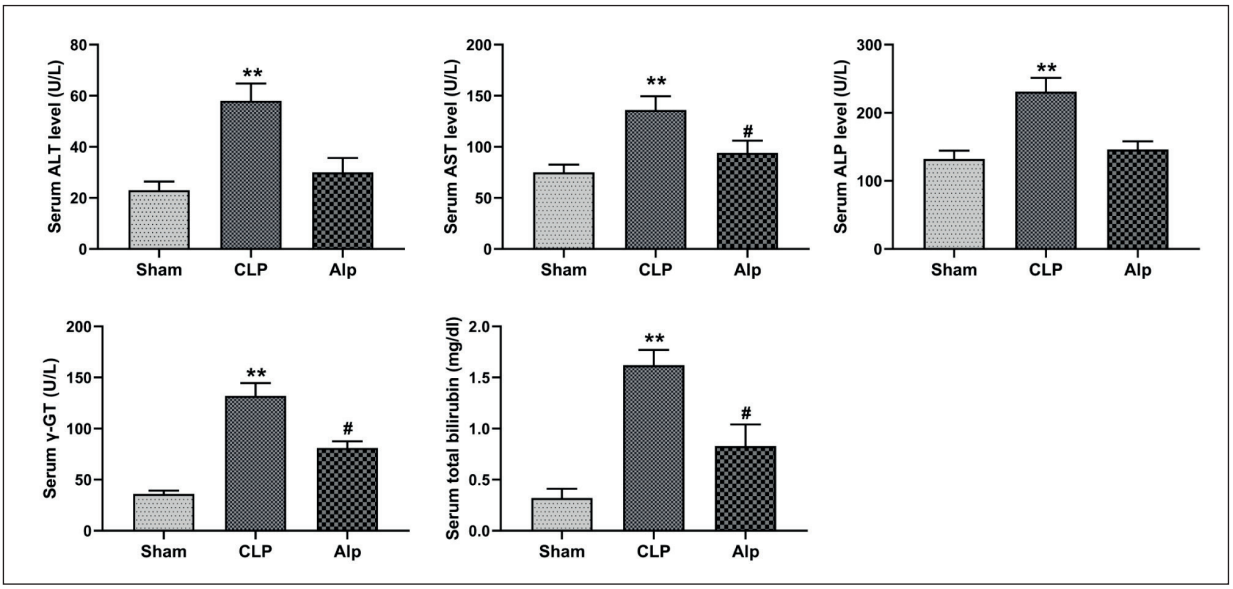

Figure 3. Changes in the content of serum ALT, AST, ALP, γ-GT and TB in rats with sepsis-induced liver injury. The concentrations of serum ALT, AST, ALP, γ-GT and TB in CLP group were notably higher than those in Sham group ($p<0.05$). However, they were not markedly changed in Alp group. Compared with CLP group, Alp group exhibited obviously declined content of serum ALT, AST, ALP, γ-GT and TB ($p<0.05$) (**$p<0.01$ *vs.* Sham group, #$p<0.05$ *vs.* CLP group).

Alp Could Restrain CLP-Induced TLR4 Expression and NF-κB Translocation

The expression of TLR4 and the translocation of NF-κB in the liver of rats were quantified. According to the results, at 24 h after induction of sepsis by CLP, the protein expression of TLR4 rose considerably (p<0.01). Meanwhile, the protein expression of TLR4 induced by CLP expression was significantly reduced after Alp treatment (p<0.01). Through the quantification of TLR4 protein expression, we found that CLP upregulated the protein expression level of TLR4 in the liver two times. However, Alp treatment remarkably repressed CLP-induced enhancement of TLR4 expression in the liver. Likewise, at 24 h after CLP, the level of nuclear NF-κB in the liver was significantly upregulated (p<0.01). However, treatment with Alp markedly suppressed CLP-induced cytoplasmic-nuclear translocation of NF-κB (p<0.01). According to the quantitative analysis results, CLP increased the cytoplasmic-nuclear translocation of NF-κB by 2.5 times, while the translocation triggered by CLP was substantially restrained by Alp (Figure 5).

Alp Repressed Inflammatory Responses in Rats with Sepsis-Induced Liver Injury

NF-κB translocation facilitates the expression of many pro-inflammatory cytokines, of which TNF-α is a leading mediator in systemic inflammation. The mRNA and protein expressions of TNF-α in damaged liver tissues were quantified in the present study. It was discovered that compared with those in the Sham group, the mRNA and protein expressions of TNF-α increased by 2.5 times and 2 times, respectively, in CLP group (p<0.01). However, Alp treatment reversed CLP-induced increases in the mRNA and protein expressions of TNF-α (p<0.05) (Figure 6). These results suggest that Alp inhibits the release of TNF-α to exert an anti-inflammatory effect.

Discussion

Abraham[8] has reported that increased levels of such liver enzymes as AST, ALT, and ALP can indicate sepsis-induced liver injury well. This suggests that cellular leakage, loss of functional integrity of liver cell membranes and cytoplasmic membrane damage occur when liver cells are released into the blood[8]. In the present study, the concentrations of serum liver function markers rose evidently in CLP group, suggesting that CLP caused extensive liver injury. However, Alp treatment decreased the serum concentrations of these markers, implying that Alp could maintain the functional integrity of liver cell membranes. Histopathological examination results corroborated the damage of CLP to the liver as well. Specifically, the rats in CLP group had notable neutrophil infiltration in the liver interstitum, hydropic degeneration of liver cells, venous distension and congestion, and notably increased proportion of apoptotic liver cells. Liu et al[9] have indicated that CLP can induce inflammatory cell infiltration and liver cell swelling and necrosis. In the current study, our findings demonstrated that treatment with Alp mitigated histopathological changes and reduced the proportion of apoptotic liver cells. All these findings confirm that Alp has a protective effect against CLP-induced liver injury.

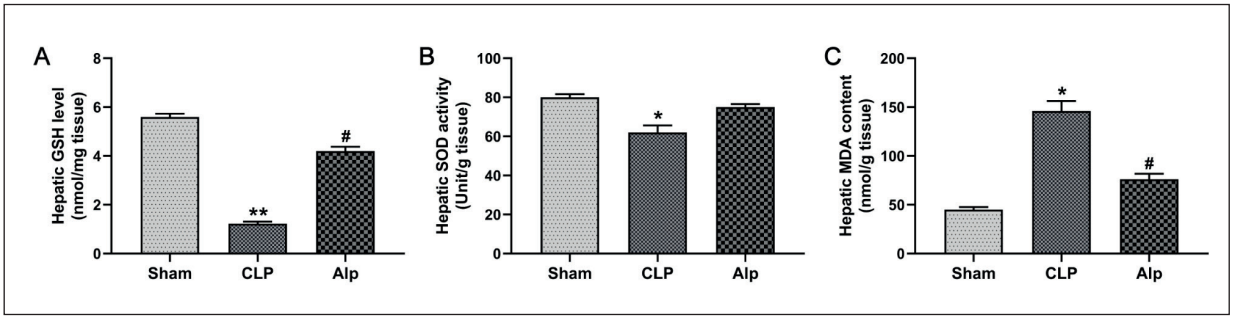

Figure 4. Effects of Alp on changes in oxidative stress markers in rats with liver injury. A-B, Changes in GSH level and SOD activity in rats with sepsis-induced liver injury: CLP group exhibited significant declined SOD activity and GSH level in liver tissues compared with Sham group (p<0.05). SOD activity and GSH level in liver tissues were considerably elevated in Alp group in comparison with those in CLP group (p<0.05). C, Changes in MDA content in rats with sepsis-induced liver injury: CLP group had remarkably higher MDA content in damaged liver tissues than Sham group and Alp group (p<0.05) (**p<0.01, and *p<0.05 *vs.* Sham group, $^{\#}p$<0.05 *vs.* CLP group).

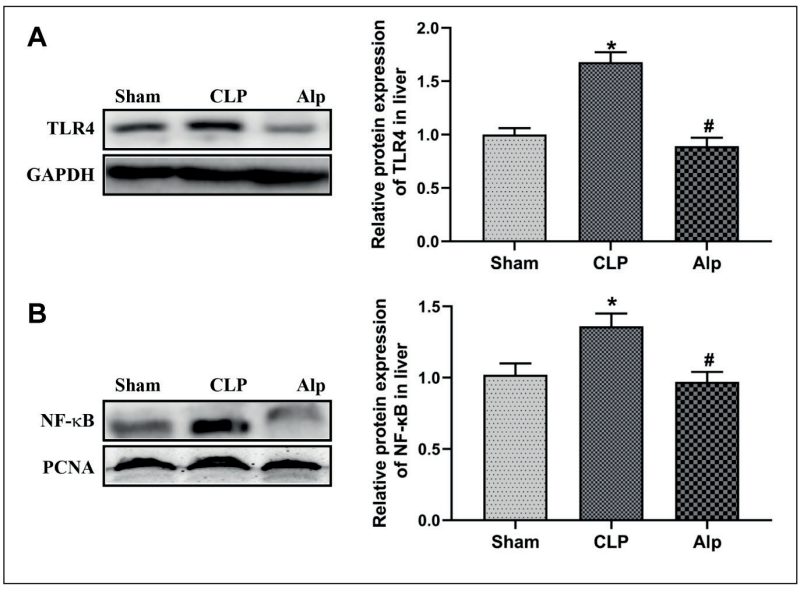

Figure 5. Effects of Alp on TLR4 expression and NF-κB translocation in rats with sepsis-induced liver injury. **A**, Protein expression of TLR4 in liver tissues detected using immunoassay: Compared with that in Sham group, the protein expression of TLR4 substantially rose in CLP group ($p<0.05$). However, no significant difference in the protein expression of TLR4 was observed in Alp group. The protein expression of TLR4 in Alp group was markedly lower than that in CLP group ($p<0.05$). **B**, Changes in NF-κB translocation in liver tissues detected via immunoassay: Compared with that in CLP group, the cytoplasmic-nuclear translocation of NF-κB declined remarkably in Sham group and Alp group ($p<0.05$) (*$p<0.05$ *vs.* Sham group, $^{\#}p<0.05$ *vs.* CLP group).

Oxidative stress is considered as a pathway for acute liver injury. Hausenloy et al[13] have exhibited that activated neutrophils, the potential sources of reactive oxidative species (ROS), will produce hypochlorous acid in the presence of MPO, eventually damaging tissues. In the present study, MDA, an indicator for lipid peroxidation, was found substantially elevated in the liver tissues of rats in CLP group. However, it basically recovered to normal in Alp group, implying that Alp can effectively quench free radicals and possess an anti-oxidative property. It is currently believed that anti-oxidants GSH and SOD are the major defense measures to prevent biological macromolecules from oxidative damage. According to the findings in the present study, CLP weakened the activity of SOD and decreased the level of GSH in the liver. However, Alp treatment enhanced the activity of anti-oxidative enzymes. All these findings suggest that Alp restores the activity of antioxidants to remove ROS and weaken oxidative stress responses, thereby protecting the liver.

In addition to the abnormality in oxidative stress level, inflammatory response is another

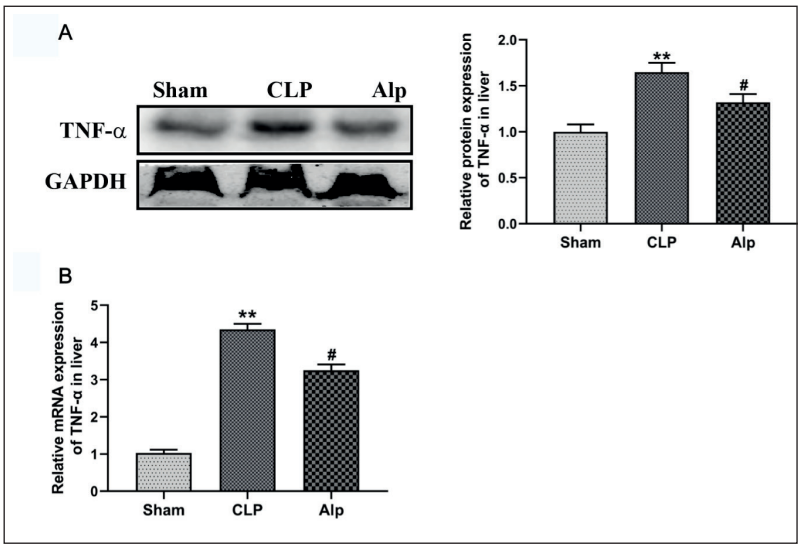

Figure 6. Effect of Alp on changes of the release of TNF-α in rats with liver injury. *A*, MRNA expression of TNF-α in damaged liver tissues detected *via* real-time quantitative amplification: CLP group had a considerably higher mRNA expression level of TNF-α than Sham group and Alp group ($p<0.05$). *B*, Quantified protein expression of TNF-α in damaged liver tissues: Compared with that in Sham group, the protein expression of TNF-α in liver tissues significantly rose in CLP group ($p<0.01$). Meanwhile, Alp group had a markedly lower protein expression level of TNF-α than CLP group ($p<0.05$) (**$p<0.01$ *vs.* Sham group, $^{\#}p<0.05$ *vs.* CLP group).

mechanism of liver injury. Therefore, anti-inflammatory medications are preferred for the treatment of liver injury. TLR4 is one of the most important signal transduction receptors in the diversely structured microbial molecules, which can activate NF-κB to regulate immune responses and expressions of many inflammatory cytokines , as TNF-α[13]. It has been observed that inhibiting TLR4 expression and NF-κB activation is correlated with the inhibition of LPS-induced production of cytokines and prevention of excessive immune response-induced damage to liver tissues[15,16]. In addition, restraining TNF-α expression can inhibit the activation of the TLR4/NF-κB signaling pathway[15]. In the present study, the results revealed that the protein expression of TLR4 and the translocation of NF-κB were enhanced by CLP but repressed by ALP. Yang et al[17] have shown that NF-κB translocation promotes the expression of many pro-inflammatory cytokines, of which TNF-α serves as a major mediator in systemic inflammation. TNF-α not only participates in inducing liver necrosis, but also accelerates sepsis-induced liver failure[18]. CLP upregulated the mRNA and protein expressions of TNF-α in liver tissues, directly damaging liver cells. Meanwhile, Alp treatment reversed such increase, proving that Alp plays an anti-inflammatory role by repressing the TLR4/NF-κB pathway and the related cytokine TNF-α.

Conclusions

The novelty of this study was that Alp not only has an anti-oxidative effect, but also inhibits TLR4 expression, NF-κB translocation and the release of relevant cytokine TNF-α to repress inflammation, thereby alleviating sepsis-induced liver injury.

Conflict of Interests

The authors declare that they have no conflict of interest.

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