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Original Article

Protective effects of mesenchymal stem cells on acute liver injury via TLR4/NF-kB signaling pathway in sepsis mice

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Abstract: Objective: Sepsis-induced acute liver injury is mediated mainly by TLR4/NF-kB inflammatory pathway. Mesenchymal Stem Cells (MSCs) has been reported to possess anti-inflammatory and immunosuppressive properties. However, the effects of MSCs against CLP-induced liver injury and mechanism involved have been seldom reported so far. Methods: Male C57BL/6 mice underwent cecal ligation and puncture operation to induce sepsis and then received either normal saline or MSCs (1×10^6, intravenously) 3 h after surgery. Results: The results of histopathological changes and serum biochemical analysis showed that MSCs treatment ameliorated sepsis-induced acute liver injury. ELISA assays showed that MSCs suppressed the production of IL-1β, IL-6, and TNF-α. Furthermore, the neutrophils infiltration and MPO activity in liver were significantly decreased by MSCs. TLR4 is an important sensor in sepsis. The result showed that the mRNA and protein expression of TLR4 were all inhibited by MSCs injection. In addition, MSCs injection inhibited NF-kB signaling pathway activation. Conclusion: Treatment with MSCs can attenuate sepsis-induced inflammatory response in mice liver. The protective effects may be mediated by the inhibition of the activation of the TLR4-mediated NF-κB signaling pathway. MSCs may be a potential new therapeutic agent for the prevention or reduction of sepsis-associated acute liver injury.

Keywords: Mesenchymal stem cells, sepsis-induced acute liver injury, TLR4, NF-κB

Introduction

Liver diseases remain one of the serious health problems associated with a high rate of morbidity and mortality throughout the world [1]. Sepsis is a major challenge in intensive care units causing acute liver injury, characterized by the systemic inflammatory response [2]. Despite the treatment of sepsis has steadily improved over the past several decades, but, the mortality and the cost from sepsis is staggering [3]. It is estimated that there are 750,000 cases of sepsis with a mortality rate between 30% and 50% in North America per year [4]. This puts a significant burden on the health care system, and the health care in annual costs in the United States alone estimated $14 billion and much more worldwide [5]. It has been previously reported that attenuation of liver injury and restoration of liver function lowers morbidity and mortality rates in patients with sepsis [6]. Thus, the development of new strategies to reduce the incidence and mortality associated with sepsis induced acute liver injury would be valuable.

Sepsis was associated with overproductions of proinflammatory cytokines, which can lead to the recruitment of leukocytes and tissue damage [7]. Previous studies showed that sepsis is a severe systemic inflammation and associated acute liver injury is largely initiated through activation of TLR4, which leads to activation of NF-κB. In addition, activation of NF-kB results in increased expression of pro-inflammatory and the exaggerated production of pro-inflammatory

cytokines such as TNF-α, IL-6, and IL-1β that contributes to liver damage in sepsis [8]. Recent studies indicate that TLR medicated signals have been involved in almost all liver diseases [9], and many therapeutic agents that abrogate liver injury might transect with the TLR4 signaling pathway [10]. Furthermore, some studies showed that TLR4 was critically involved in the pathogenesis of sepsis-induced liver damage, and there was increasing evidence shown that sepsis-induced liver injury was significantly attenuated via inhibiting NF-kB activation. Therefore, inhibition of the expression of TLR4 and suppressing the activation of NF-kB pathway may well represent therapeutic targets for sepsis-induced liver injury.

Mesenchymal stem cells (MSCs) are multipotent cells that are capable of differentiating into specific cells [11]. MSCs have become a subject of research interest because of their anti-inflammatory, anti-apoptotic, and immunosuppressive factors [12], MSCs exert powerful tissue repair functions via paracrine effects and cell-cell contact-dependent interactions. Accordingly, MSCs-based therapies are being evaluated for treatment of inflammatory, and immunological disorders. Previous studies have shown that injection of MSCs can ameliorate experimental sepsis and sepsis-induced tissue injury including lung and kidney [12, 13]. However, the effects of MSCs in sepsis-induced acute liver injury and its corresponding mechanisms have not been demonstrated yet.

Cecal ligation and puncture (CLP) induced sepsis is a well-established model because it closely resembles the progression and characteristics of human sepsis in which liver injury is mediated through an inflammatory pathway [14]. So, in the present study, we investigated the protective effect of MSCs on acute liver injury by CLP model, and elucidated potential molecular mechanisms of MSCs in sepsis-induced acute liver injury.

Materials and methods

Animals

Male C57BL/6J mice weighing 22 g to 25 g were purchased from the animal center of Qingdao university (Qingdao, China). All mice were housed in an animal facility with 12-h light-dark cycle at 22°C and free access to food and water. They were acclimated for 1 week before conducting experiments. All experiments were approved by the Institutional Animal Care and Use Committee of our university.

Culture of MSCs

C57BL/6J mouse bone marrow-derived MSCs were purchased from Cyagen Biosciences (Sunnyvale, Calif). The culture process was initiated according to the manufacturer's instructions. The sixth, eighth, and tenth passage MSCs were used for experiments.

Mouse model of cecal ligation and puncture

CLP-induced sepsis was performed as described previously [15]. Briefly, mice were anesthetized with pentobarbital and a midline abdominal incision was made. The cecum was mobilized, ligated below the ileocecal valve, and punctured twice with a 22 gauge needle to induce polymicrobial peritonitis. The abdominal wall was closed in two layers. Sham-operated mice underwent the same procedure, including opening the peritoneum and exposing the bowel, but without ligation and needle perforation of the cecum.

After 3 h of CLP, mice were injected 1×10^6 MSCs in 0.2 mL normal saline by tail vein. As a control, the CLP mice were administered only 0.2 mL normal saline. Mice were divided into three groups: sham, CLP, and CLP + MSCs.

Biochemical analysis

Liver injury was assessed by measuring the levels of alanine transaminase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH). The blood samples were placed at room temperature for 30 min and subsequently centrifuged at 3500 rpm for 15 min. Activities of these biomarkers were measured using Hitachi 7600 automatic analyzer (Hitachi, Tokyo, Japan) following the standard protocol.

Histopathological evaluations

The right lobe of liver was rapidly harvested after blood obtaining. Tissue specimens were fixed in 10% formalin for 48 h and then embedded in paraffin and cut into 5-mm sections. The

Table 1. Primers used for real-time PCR

Gene	Forward Primers (5'-3')	Reverse Primer (5'-3')
TLR4	TCATGGCACTGTTCTTCTCC	TCATCAGGGACTTTGCTGAG
NF-κB	GGAGAAGGCTGGAGAAGATG	GCTCATACGGTTTCCCATTT
GAPDH	GCAAGTTCAACGGCACAG	GCCAGTAGACTCCACGACAT

slides were stained with hematoxylin/eosin and then analyzed blindly using Suzuki's criteria of liver damage [16]. In this classification, sinusoidal congestion, hepatocyte necrosis and ballooning are graded from 0 to 4. No necrosis, congestion or ballooning is given a score of 0, whereas severe congestion or ballooning, degeneration and > 60% lobular necrosis are given a value of 4.

Enzyme-linked immunosorbent assay

Animals were anesthetized and killed at 24 h (n = 6 per group). Specific detection of TNF-α, IL-6, and IL-1β in the supernatant of the liver homogenate was accomplished by sandwich enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, Minneapolis, Minn).

Immunofluorescence studies of liver samples

Neutrophil granulocyte infiltration into the liver was evaluated by detection of Ly6G positive cells by immunofluorescence. 5-μm-thick liver sections were cut and incubated overnight with rat anti-Ly6G (1:100, Santa Cruz Biotechnology; Santa Cruz, CA, USA). Secondary antibodies conjugated to CY3 rabbit anti-rat immunoglobulin G (IgG; 1:50, Jackson ImmunoResearch Laboratories; West Grove, PA, USA) were applied for 1 h at room temperature. Nuclei were stained with DAPI (Zhongshan Goldenbridge Biotechnology, Beijing, China). Each tissue section was observed blindness under a confocal laser scanning microscope (Olympus FluoView 1000) at a magnification of × 600.

Determination of hepatic MPO activity

The extent of neutrophils accumulation in the liver was measured by assaying myeloperoxidase (MPO) activity as previously described [17]. Hepatic tissue was homogenized and centrifuged at 10,000 g for 15 min. The pellets were then re-suspended and re-homogenized. The suspension was freeze-thawed three times using liquid nitrogen, and then centrifuged. The assay was started by adding the supernatant to H_2O_2 and TMB solution and was terminated by adding 100 μl of 4 M H_2SO_4 at 4°C. The obtained color was quantified at 450 nm. Results were expressed as a fold change over sham group.

Real-time polymerase chain reaction

Total RNA was extracted from hepatic tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Reverse-transcription was performed using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and a Gene Amp® PCR System 9700 (Applied Biosystems) was used to generate cDNA. Real-time quantitative RT-PCR using 2 μl of cDNA from each sample was performed in duplicates with SYBR Mastermix according to the manufacturer's instructions. All reactions were performed using a 7500 Real-time PCR System (Applied Biosystems). For analysis, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a normalization control. The relative target mRNA expression normalized to GAPDH expression was analyzed using the $2^{-\Delta\Delta CT}$ method. The primers used are shown in **Table 1**.

Western blot analysis

Liver lysates were prepared by homogenization using radioimmunoprecipitation assay buffer, and then centrifuged at 12,000 rpm for 30 min, after which supernatants were collected. A total of 100 μg of protein was separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Anti-TLR4 and -NF-KB-p65 antibodies (Cell Signaling Technology) were used as primary antibodies at 4°C overnight, and a horse-radish peroxidase-conjugated anti-rabbit/mouse IgG (Santa Cruz Biotechnology) was used as the secondary antibody. Densitometry was performed using the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistics

Data are expressed as means ± SEM. Differences were evaluated using unpaired

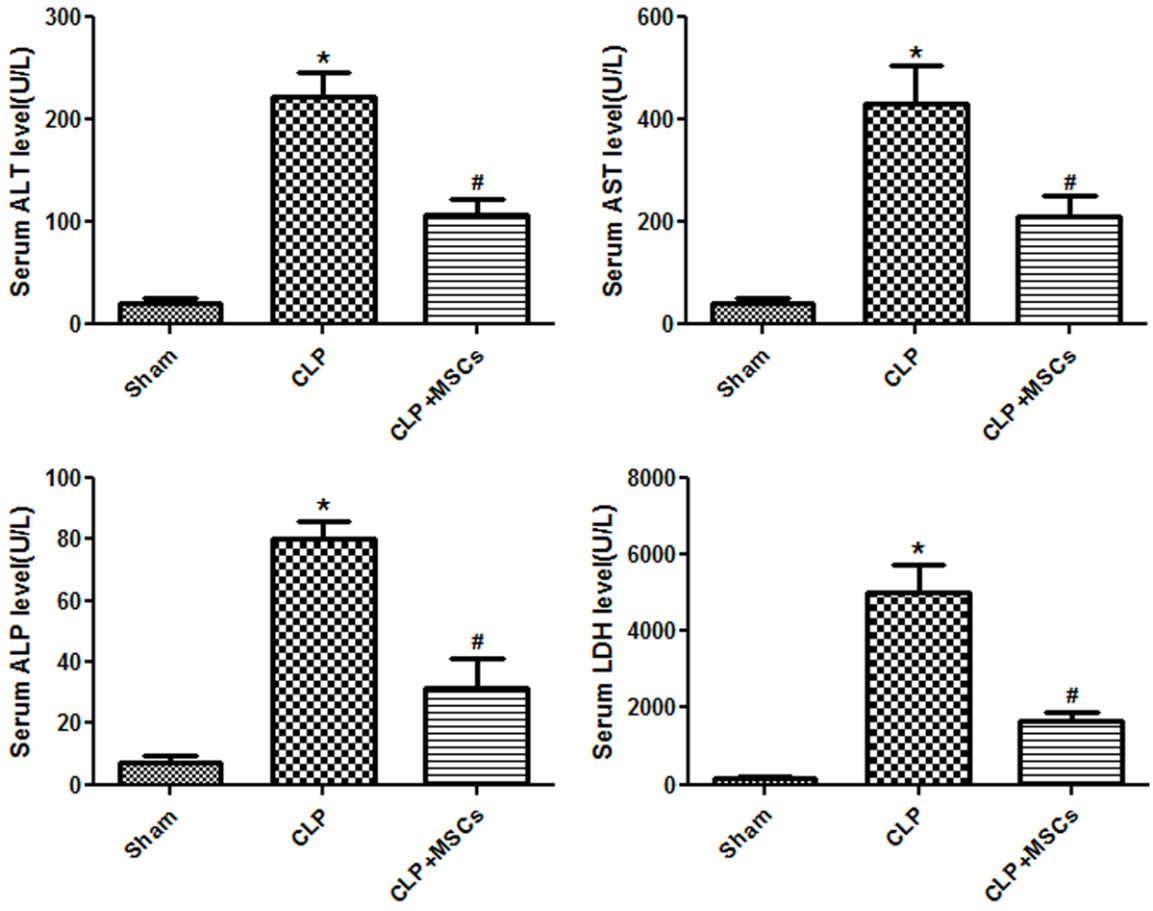

Figure 1. Effects of MSCs on serum biochemical analysis. The serum levels of ALT, AST, ALP, and LDH were determined. Values were presented as the mean ± SD (n = 6). *P < 0.05 compared with sham group. #P < 0.05 compared with CLP group.

Student's t-tests between two groups and one-way ANOVA for multiple comparisons, followed by a post hoc Student-Newman-Keuls test when necessary. And α was corrected by the number of comparisons [2α/n (n-1)] to ensure α = 0.05. mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method. Analyses were performed using SPSS for Windows (version 10.1; SPSS; Chicago, IL, USA). Statistical significance was set at P < 0.05.

Results

Effect of MSCs on CLP-induced changes in liver functions

The severity of CLP-induced liver injury was determined through serum AST, ALT, ALP and LDH levels. Compared to control group, the serum levels of AST, ALT, ALP and LDH drastically increased after CLP surgy at 24 h. And Compared to CLP group, MSCs treatment significantly suppressed the release of AST, ALT, ALP and LDH into the plasma (P < 0.05, **Figure 1**).

Effects of MSCs on histological changes of hepatic tissues

Histopathological examination of the liver using H&E stain in sham mice revealed normal liver architecture (**Figure 2**). CLP caused a significant pathologic change, including hepatocytes necrosis, destruction of hepatic architecture, and inflammatory cell infiltration. Treatment with MSCs ameliorated the morphological damage. Furthermore, the Suzuki's score and necrotic hepatocellular areas were significantly decreased with MSCs injection.

Effect of MSCs on the infiltration of inflammatory cells

To test whether neutrophil infiltration plays a role in our model, we examined the infiltration

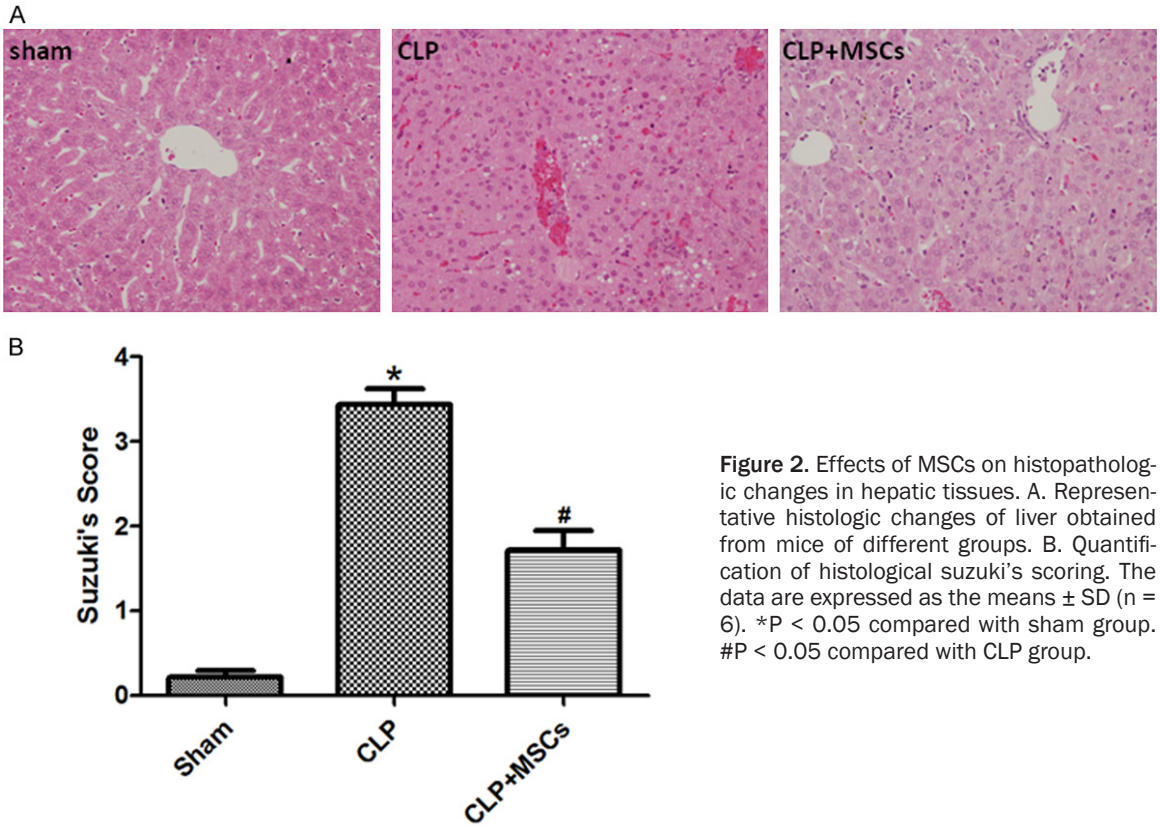

Figure 2. Effects of MSCs on histopathologic changes in hepatic tissues. A. Representative histologic changes of liver obtained from mice of different groups. B. Quantification of histological suzuki's scoring. The data are expressed as the means ± SD (n = 6). *P < 0.05 compared with sham group. #P < 0.05 compared with CLP group.

of neutrophil in liver (**Figure 3**). In the sham group, there was nearly no detectable Ly-6G-positive cell infiltration. In the CLP group, there were many Ly-6G-positive cells in the liver tissue. Compared with the CLP group, the MSCs-treated group exhibited less neutrophil infiltration.

Effect of MSCs on the expression of cytokines in liver

To further investigate the effect of MSCs on the levels of pro-inflammatory cytokines, we analyzed the concentration of TNF-α, IL-6, and IL-1β in the liver tissues by ELISA (**Figure 4**). ELISA results showed that CLP significantly increased the protein expression of TNF-α, IL-6, and 1L-1β. MSCs treatment significantly attenuated the expression of TNF-α, IL-6, and IL-1β.

Effects of MSCs on MPO activity

MPO activity was known as an indirect indicator for the recruitment of neutrophils to the liver tissues. As shown in **Figure 4B**, the biochemical analysis results showed that MPO activity was significantly increased in the CLP group. However, this increase was attenuated by MSCs injection (**Figure 5**).

Effects of MSCs on the mRNA expression of TLR4/NF-kB pathway

We then examined the effect of MSCs on the TLR4/NF-kB signaling pathway in septic liver tissues. The mRNA expression of TLR4 and NF-κB were significantly increased in CLP group, however, the mRNA expressions of TLR4 and NF-κB were decreased after the treatment of MSCs (**Figure 6**), indicating that the anti-inflammatory effect of MSCs is mediated by inhibition of TLR4/NF-κB pathway in CLP-injured mice.

Effects of MSCs on the protein expression of TLR4/NF-kB pathway

To further determine whether the anti-inflammatory mechanism of MSCs on CLP-induced liver inflammation act through the TLR4/NF-kB pathway, western blot assays were carried out. Compared to sham group, the TLR4 and NF-kB p65 proteins were significantly increased in the CLP group. In contrast, the pro-

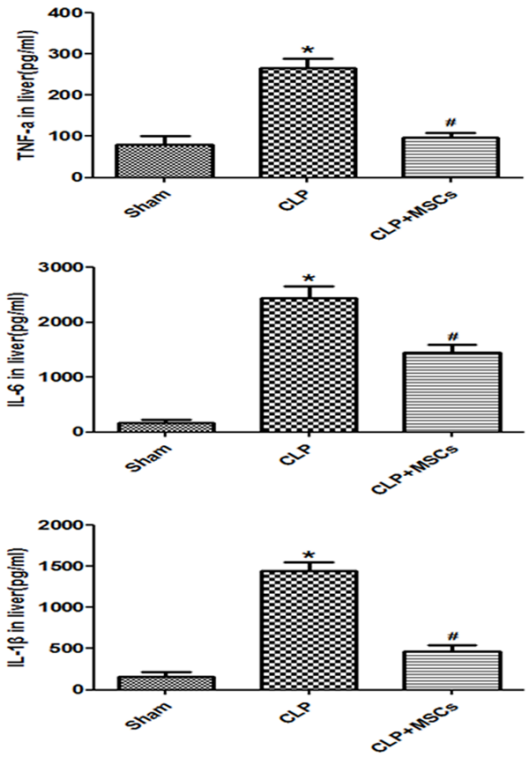

Figure 3. Effect of MSCs on the expression of pro-inflammatory cytokines in hepatic tissues. Enzyme-linked immunosorbent assay was used to quantify the proinflammatory cytokines TNF-α, IL-6, and IL-1β in the liver of each group at 24 h after CLP injury. N = 6 for each group. The data are presented as means ± SD. *P < 0.05 compared with sham group. #P < 0.05 compared with CLP group.

teins levels of TLR4 and NF-kB-p65 were significantly reduced in the MSCs groups (P < 0.05, **Figure 7**).

Discussion

MSCs has been reported to possess anti-inflammatory and immunosuppressive properties. However, the effects of MSCs against CLP-induced liver injury and mechanism involved have been seldom reported so far. In the present study, we observed the effects of MSCs on CLP-induced liver injury.

It has been previously reported that hepatic injury following sepsis is well established by the elevated levels of AST, ALT, ALP and LDH in serum indicating cellular leakage and loss of functional integrity of hepatic membrane as they are released into blood stream when the liver cell plasma membrane is damaged [18]. In the present study, acute liver injury was evidenced by elevated ALT, AST, ALP, LDH and abnormalities in liver structure in histological section which confirming the extensive liver damage induced by CLP. Fortunately, in our study, treatment with MSCs improved survival and inhibited the elevation of serum ALT, AST, ALP, LDH levels, which demonstrated MSCs have protective effect in sepsis associated acute liver injury.

Additionally, histopathological examination confirmed this hepatic damage as CLP resulted in marked inflammatory cells infiltration, hepatocellular necrosis, and central vein dilation and congestion. Similarly, previous study showed that CLP produced inflammatory cells infiltration, swollen hepatocytes and hepatocellular necrosis in rats [19] treatment with MSCs attenuated these histopathological changes confirming the protective effect of MSCs against the CLP-induced liver injury.

It has been reported that sepsis could stimulate the release of TNF-α, IL-6, and IL-1β, and they are regarded as critical cytokines in the inflammatory cytokine family [20]. TNF-α plays a crucial role in exerting variety of biologic effects, which is considered to be an effcient pro-inflammatory factor, IL-6 is necessary to leukocyte recruitment and tissue homeostasis, which is induced by invasion or injury. IL-1β is mainly produced by activated macrophages and is a key factor of inflammation mediator. Therefore, we can reduce inflammation and its symptoms by inhibiting the overproduction and activity of pro-inflammatory cytokines, which has been applied to treat for certain inflammatory diseases, such as sepsis. Our results showed that MSCs protected the mice from sepsis-stimulated liver inflammation through decreasing the secretion of pro-inflammatory mediators.

This systemic inflammatory cascade during sepsis results in neutrophils infiltration to site of injury/infection. And subsequently lead to vascular dysfunction as well as parenchymal cell dysfunction [18]. It has been shown that activated neutrophils secrete MPO [21]. In addition, the MPO activity is often regarded as an early marker for the infiltration of inflammatory cells into tissues, which also predicts the occurrence of inflammatory diseases [22]. Since activity of MPO positively correlated with the number of neutrophils and severity of inflammation, it is more commonly used to

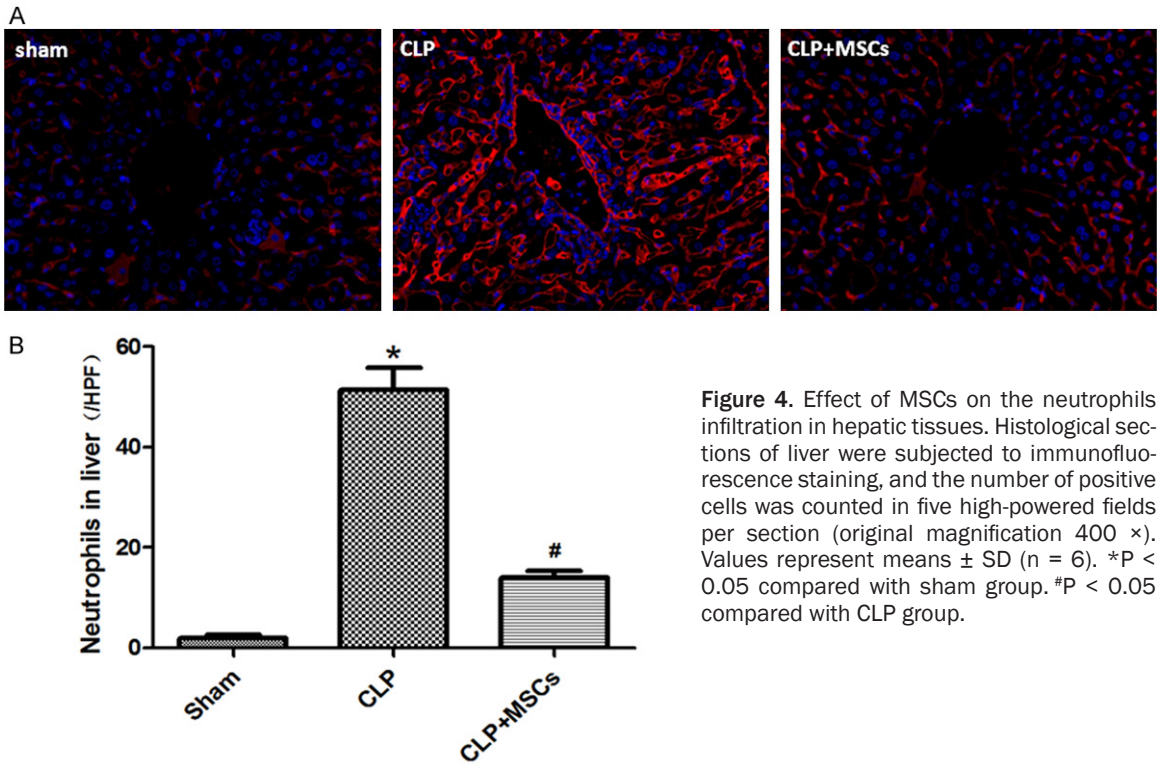

Figure 4. Effect of MSCs on the neutrophils infiltration in hepatic tissues. Histological sections of liver were subjected to immunofluorescence staining, and the number of positive cells was counted in five high-powered fields per section (original magnification 400 ×). Values represent means ± SD (n = 6). *P < 0.05 compared with sham group. #P < 0.05 compared with CLP group.

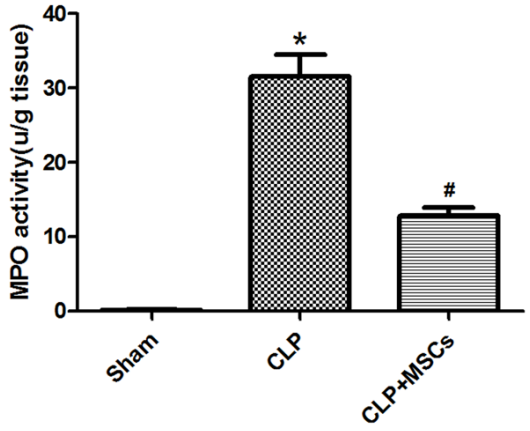

Figure 5. Effect of MSCs on the MPO activity in hepatic tissues. The data are presented as means ± SD (n = 6). *P < 0.05 compared with sham group. #P < 0.05 compared with CLP group.

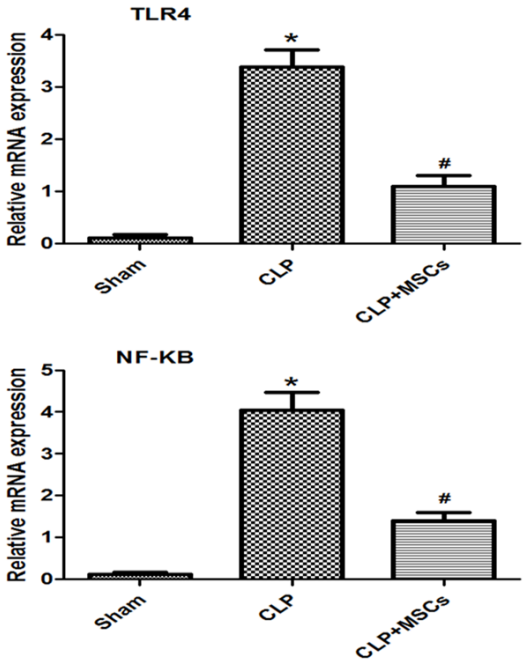

Figure 6. Effect of MSCs on CLP-induced changes in TLR4 mRNA and NF-KB Mrna expression in hepatic tissues. The mRNA levels of TLR4 and NF-KB in liver tissue 24 h after sham or CLP operation (n = 6). *P < 0.05 compared with sham group. #P < 0.05 compared with CLP group.

evaluate tissue neutrophils accumulation in inflamed tissues [23, 24]. It has been previously reported that CLP increased MPO activity in hepatic rats [18]. Thus, weakened MPO activity means decreased anti-inflammatory response. In the present study, MPO activity was markedly increased in CLP mice and injection of MSCs lowered MPO activity. This was further supported by histopathological examination showing decrease in neutrophils infiltration in hepatic tissues. All results suggested that MSCs had a protective action on CLP-induced liver inflammation in mice.

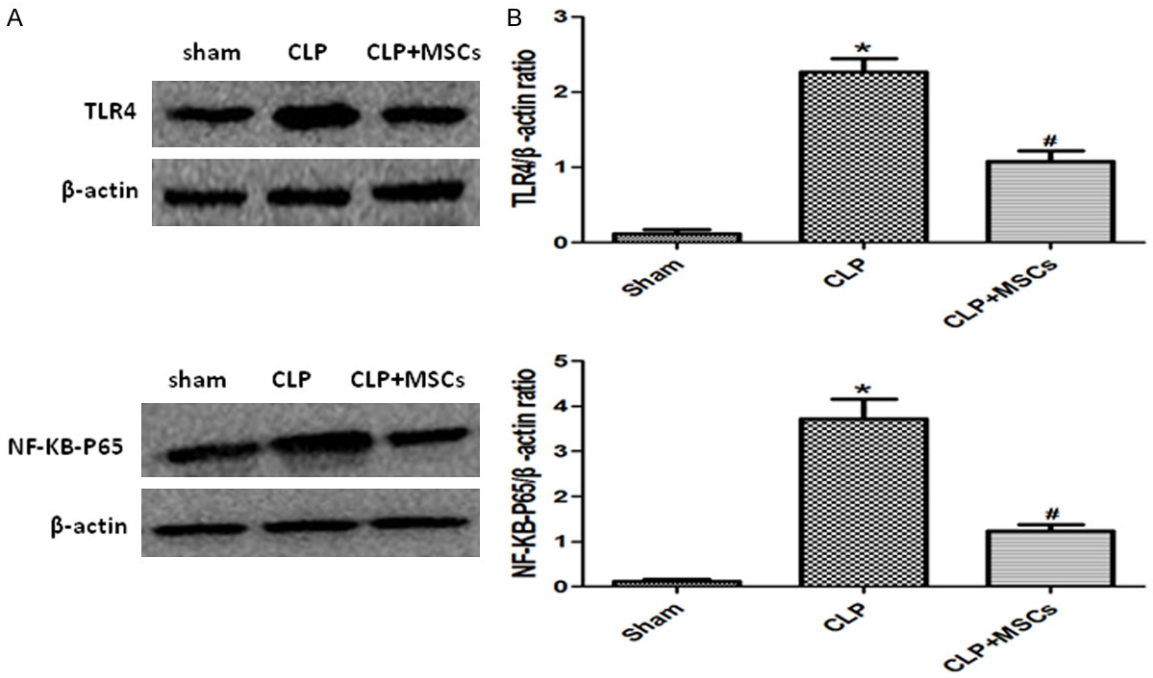

Figure 7. Effect of MSCs on protein expression of TLR4 and NF-KB-P65 in hepatic tissues. A. Western blot results for TLR4 and NF-KB-P65. B. Quantitative analysis of cleaved caspase-3. The protein levels are presented as means ± SD (n = 6). *P < 0.05 compared with sham group. #P < 0.05 compared with CLP group.

TLRs play a great role in the process of liver fibrosis which is caused by many common liver pathogens, including viral, and toxin-induced hepatitis [25]. Many studies have provided convincing evidence that the NF-κB signaling pathway can be activated when LPS triggers the immune response via TLR4 [26, 27]. The previous studies showed that CLP increased TLR4 mRNA expression and NF-κB p65 expression in rats' liver [28]. Some pharmacologic agents exert anti-inflammatory and hepatoprotective effects through the TLR4/NF-κB pathway [29, 30]. To further understand the mechanism by which MSCs exerts its anti-inflammatory property, we investigated the effects of MSCs on the local activities of TLR4 and NF-κB signaling pathway in CLP-induced liver inflammation. In agreement with previously studies, our results showed that CLP injury could upregulate the TLR4 and NF-κB expression. In contrast, MSCs significantly reduced the TLR4 and NF-κB expression. Together, these findings indicate that MSCs exerts its protective effects by inhibiting the activation of proinflammatory factors via the TLR4/NF-κB signaling pathway.

There are some limitations in our study. MSCs did not home to liver, Although we observed decreased levels of TLR4, NF-KB and related cytokines in liver tissues after treatment of CLP mice with MSCs, the mechanism through which MSCs protect sepsis induced acute liver injury remains unknown. Our group has reported that MSCs might interact with splenocytes and protect against ischemic AKI, although the exact crosstalk between MSCs and splenocytes is unclear [31]. Next, our team will further explore the mechanism by in vitro study.

Conclusion

In conclusion, our results demonstrate that MSCs can attenuate sepsis-induced inflammatory response in mice liver. The protective effects may be mediated by the inhibition of the release of inflammatory mediators. In addition, the anti-inflammatory effects of MSCs are associated with inhibiting the activation of the TLR4-mediated NF-κB signaling pathway.

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Disclosure of conflict of interest

None.

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