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The Protective Effects of Protease Inhibitor MG-132 on Sepsis-Induced Acute Lung Rats and Its Possible Mechanisms

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The aim of the present study was to investigate the protective effects of protease inhibitor MG-132 on sepsis-induced acute lung injury rats.

Material/Methods: Sprague Dawley rats were employed to induce sepsis by cecal ligation and puncture (CLP) method. Rats were divided into 4 groups: control, sham, model (CLP), and MG-132. Histopathology observation was detected by hematoxylin and eosin staining. The ratio of wet lung to dry lung (W/D) was calculated. In addition, the levels of inflammatory factors in bronchoalveolar lavage fluid (BALF) were measured by enzyme-linked immunosorbent assay (ELISA). Also, superoxide dismutase (SOD) and malondialdehyde (MDA) levels were evaluated. Western blotting was performed to measure the expression of hypoxia-inducible factor-1 α (HIF-1 α). In order to assess the role of HIF-1 α , YC-1, the inhibitor of HIF-1 α , was used to treat the rats. The expression of phospho-mTOR (p-mTOR), p-4EBP1, and p-EIF4E were evaluated by western blotting.

Results: Obvious pathological injury and increasing ratio of W/D in the model group were observed. Both pathological injury and W/D were improved in the MG-132 group, and the greatest improvement could be seen in the YC-1+MG-132 group. Furthermore, the MDA levels in the MG-132 group was decreased, accompanied by an increase in SOD levels. The level of HIF-1 α was increased in the model group while a decreased was detected in the MG-132 group. The levels of inflammatory factors were high in the model group, whereas the opposite result was found in the MG-132 group, and the lowest in were in the YC-1+MG-132 group. Furthermore, the expression levels of p-mTOR, p-4EBP1, and p-EIF4E proteins were downregulated in the MG-132 group compared to the model group, and the lowest was in the YC-1+MG-132 group.

Conclusions: Our study suggested that MG-132 was able to protect against acute lung injury via inhibition of HIF-1 α mediated mTOR/4EBP1/EIF4E pathway.

MeSH Keywords: **Acute Lung Injury • Hypoxia-Inducible Factor 1 • Sepsis**

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Background

Sepsis is defined as a systemic inflammatory response to infection which leads to multi-organ dysfunction and is a dominant factor in death in a critical care setting [1,2]. Sepsis has a high morbidity in infections of burns, hypoxias and post-surgeries for systemic inflammatory response syndrome. Despite recent advances in surgical techniques and intensive medicine practices, sepsis is a considerable serious health problem worldwide because of high morbidity and mortality rates in intensive care units [3]. The lung acts as a vulnerable organ in sepsis, which can result in acute lung injury (ALI) [4]. Although the pathophysiology of sepsis is not well understood, an overwhelming response of innate inflammation, which leads to the release of a wide range of pro-inflammatory cytokines, plays an important role in the development of ALI induced by sepsis [5]. Therefore, anti-inflammatory therapy becomes a very direct and effective treatment for sepsis.

MG-132 is an aldehyde peptide proteasome inhibitor, which can inhibit the activation of NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), downregulate the expression of inflammatory factors and reduce the body's inflammatory response [6,7]. It has been reported that MG-132 exerts a protective effect on severe acute pancreatitis and associated lung injury of rats through improvement of edema, cellular damage, and inflammatory activity [8]. Hypoxia-inducible factor 1 α (HIF-1 α) is a nuclear transcription factor that specifically mediates hypoxia in cells. It has been well documented that MG-132 can prevent oxidized low-density lipoprotein-attenuation differentiation phenotype by blocking HIF-1 degradation [9]. The previous study reported that the expression of HIF-1 α was activated accompanied by the presence of lung inflammatory injuries through a HIF-1 α dependent pathway in rats during the acute lung inflammatory damages induced by treatment with septic lymph [10]. Therefore, the role of MG-132 and HIF-1 α in ALI in septic rats is of interests. The present study aimed to study the mechanism of MG-132 on ALI in sepsis-induced acute lung injury rats and determine whether MG-132 can inhibit the inflammatory response by inhibiting HIF-1 α .

Material and Methods

Experimental animals

Adult Sprague-Dawley rats (200–250 g) were obtained from Shanghai SLAC Laboratory Animal Company Limited (Shanghai, China). Rats were fed in a suitable environment at $24\pm 3^{\circ}\text{C}$ and with a 12-hours light/dark cycle. All animals were given access to food and water and were allowed to acclimate to the environment for at least 3 days before the experiment. This study was conducted in strict accordance with the guidelines for

the Care and Use of Laboratory Animals and approved by the Ministry of Science and Technology of China. All of the study protocols were approved by the Ethics Committee on Animal Experiments of Nantong University.

Groups and treatments

All rats were assigned into 1 of 4 groups randomly (n=10 in each group): control, sham, model, and MG-132 groups. Thirty minutes before the establishment of sepsis, the rats in MG-132 group had intraperitoneal injection with 10 mg/kg MG-132. Rats in the other groups were intraperitoneally injected once with normal saline at the same time. In order to investigate the role of HIF-1 α inhibitor, 2 other groups (YC-1 and YC-1+MG-132) were recruited (n=10 in each group). Then 30 $\mu\text{g/g}$ YC-1 (Sigma-Aldrich) was employed to intraperitoneal injection into the rats 3 days before the experiments in the aforementioned 2 groups.

Sepsis induction by cecal ligation and puncture (CLP)

The sepsis model was induced using the cecal ligation and puncture (CLP) method as detailed previously [11,12] for rats in the model group and the MG-132 group. In brief, after 10 hours of fasting but with free access to water, all animals were anesthetized with intraperitoneal injection of 40 mg/kg thio-pental. A small midline incision (1 cm long) was made to expose the cecum and adjacent areas through the skin and peritoneum under aseptic conditions. Subsequently, approximately 1.5 cm of the cecal appendage was ligated midway between the cecal base and the distal pole, using 4-0 silk suture. Then, double punctures were made using an 18-gauge needle and expelling a small amount of feces. Following this, the cecum was reset in its original location before the incision was sutured. After their operation, all the rats were subcutaneously injected with normal saline.

Measurement of cytokine in bronchoalveolar lavage fluid (BALF)

Left lung was obtained, and 0.5 mL saline was employed to lavage from the bronchus alveolar, 3 times. After this, we collected the bronchoalveolar lavage fluid (BALF) which was then centrifuged for 15 minutes (3000 rpm) and the supernatant was retrieved stored at -80°C immediately for the future measurement of inflammatory cytokines. The concentrations of tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-1 β , and intercellular adhesion molecule 1 (ICAM-1) in BALF were evaluated using immune-enzymatic assay kits according to manufacturer's instructions. The enzyme-linked immunosorbent assay (ELISA) kits for TNF- α (F16960), IL-6 (F15870), IL-1 β (F15810), and ICAM-1 (F15700) were all purchased from Shanghai Xitang Biotechnology Co., Ltd. (Shanghai, China).

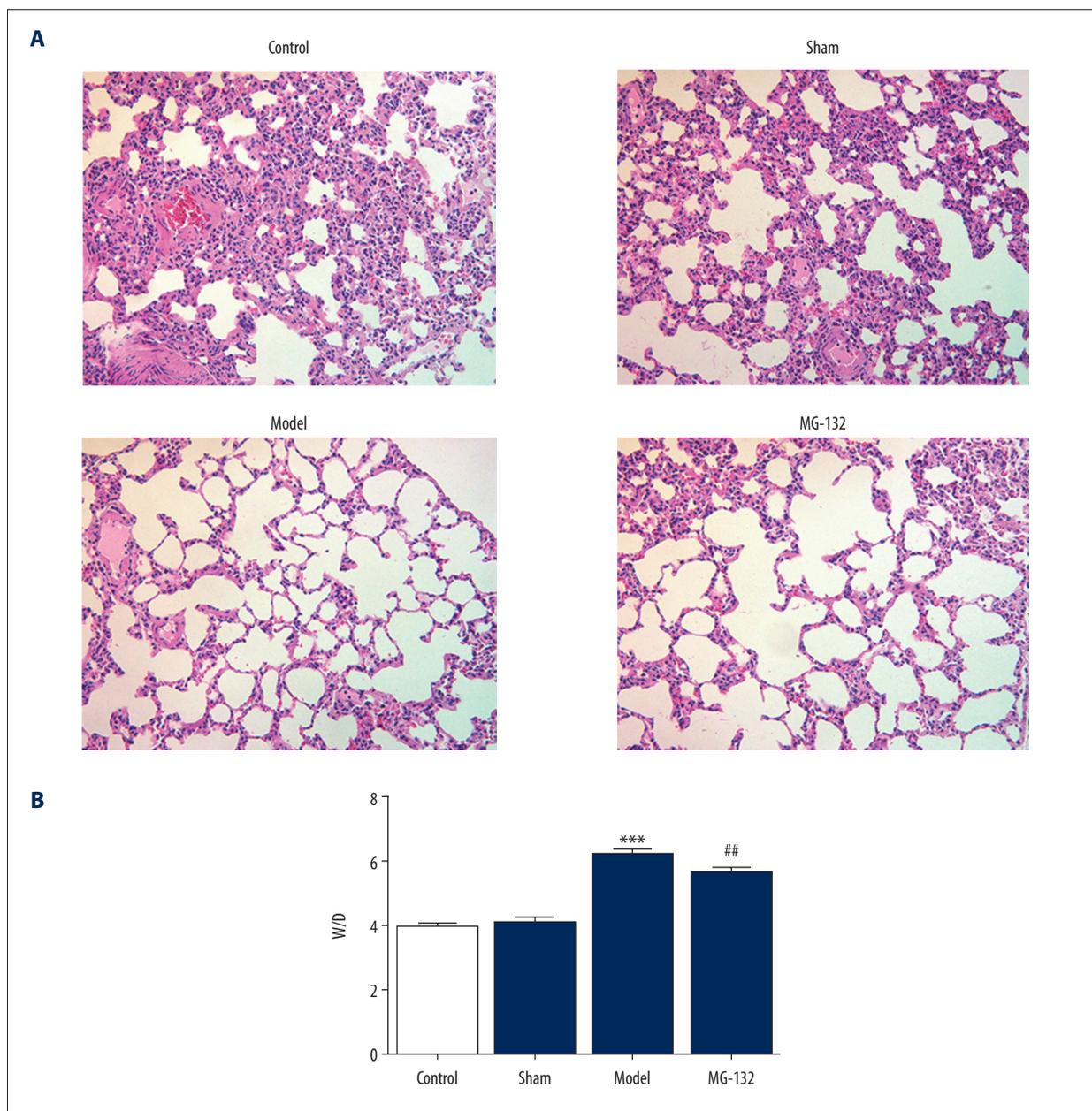


Figure 1. MG-132 improved the lung tissue pathological changes and edema in CLP- induced ALI rats. **(A)** Representative images of hematoxylin and eosin staining from each experimental group at 24 hours after CLP (magnification, 100×). **(B)** MG-132 attenuated lung W/D ratio in CLP-induced ALI rats. *** $P < 0.001$ versus sham; ## $P < 0.01$ versus model. CLP – cecal ligation and puncture; ALI – acute lung injury; W/D – wet/dry ratio of lung.

Wet/dry ratio of lung (W/D)

To assess lung tissue edema, the right upper pulmonary lobe was excised immediately after exsanguination. Then, these tissues were blotted dry and weighed, then placed in a constant temperature oven at 75°C for 48 hours to obtain the lung dry weight. Finally, the ratio of the wet lung to the dry lung (W/D) was calculated.

Histopathology observation

Appropriate lungs tissues were conventionally fixed in 4% paraformaldehyde over night at 4°C and routinely processed in paraffin for sectioning. Tissue was sectioned into 5 μm slices, mounted on slides and stained with hematoxylin and eosin (H&E). Then, all the slide sections were dehydrated with graded ethanol and xylene. The slides (n=8) were examined by an experienced pathologist blinded to the treatments, using a light

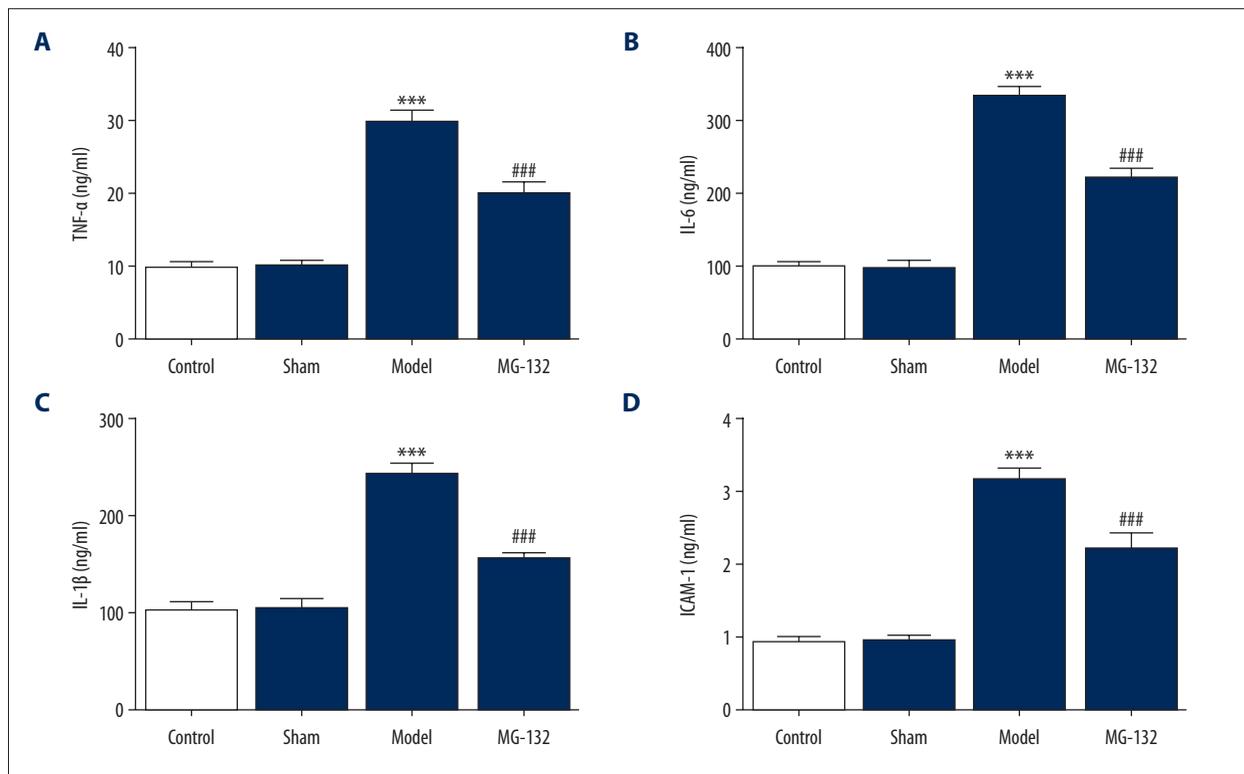


Figure 2. MG-132 decreased the level of inflammatory factors in the BALF of CLP-induced ALI rats. The levels of (A) TNF- α , (B) IL-6, (C) IL-1 β , and (D) ICAM-1 were measured by ELISA. *** $P < 0.001$ versus sham; ### $P < 0.001$ versus model. BALF – bronchoalveolar lavage fluid; CLP – cecal ligation and puncture; ALI – acute lung injury; TNF – tumor necrosis factor; IL – interleukin; ICAM – intercellular adhesion molecule; ELISA – enzyme-linked immunosorbent assay.

microscope (Olympus Corp., Tokyo, Japan) at 200 \times magnification (100 fields per section). Lung tissue lesions were classified as described in a previous study [13].

Tests for superoxide dismutase (SOD) and malondialdehyde (MDA)

The specimens of partial lung tissues were taken and homogenized (10%, w/v) in cold normal saline. The prepared tissue homogenate was used for measurements of superoxide dismutase (SOD) and malondialdehyde (MDA) levels according to the colorimetric methods following the manufacturer's instructions. The assay kits for SOD and MDA were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Western blotting analysis

Lung tissues were lysed on ice in RIPA Lysis Buffer (Beyotime, Shanghai, China). Proteins concentration was measured by Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China). Equal amounts of protein samples (40 μ g) were isolated by SDS-polyacrylamide gels (PAGE), and subsequently electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked

with 5% non-fat milk at room temperature for 1 hour, and then incubated with primary antibodies overnight at 4 $^{\circ}$ C. Subsequently, these membranes were washed with TBST 3 times and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1: 5000; cat. no. ab6721; Abcam, Cambridge, UK) at room temperature for 2 hours. The blots were developed with an enhanced chemiluminescence (ECL) reagent and analyzed by ImageJ software. Anti-HIF-1 α (1: 1000; cat. no. 36169S), anti-phospho-mTOR (p-mTOR) (1: 1000; cat. no. 5536S), anti-mTOR (1: 1000; cat. no. 2983S), anti-p-4EBP1 (1: 1000; cat. no. 2855T), anti-4EBP1 (1: 1000; cat. no. 9644S), anti-p-EIF4E (1: 1000; cat. no. 9741S), anti-EIF4E (1: 1000; cat. no. 2067S), and anti-GAPDH (1: 1000; cat. no. 5174S) antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). The protein expression was normalized to GAPDH levels.

Statistical analysis

All results were confirmed in at least 3 independent experiments and all statistical analyses were conducted using SPSS 14.0 software (Chicago, IL, USA). All experimental results were expressed as mean \pm standard deviation (SD). Statistical comparisons were made by 2-tailed Student's *t*-test or one-way

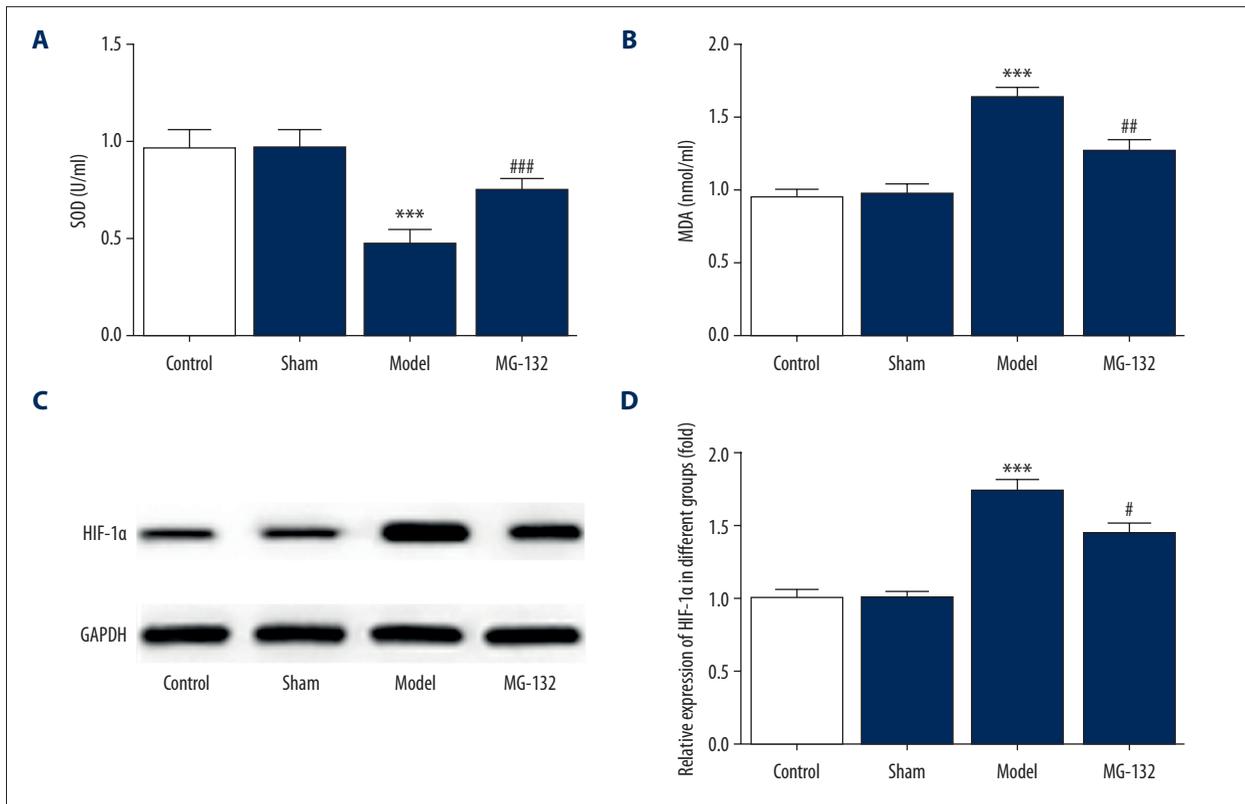


Figure 3. MG-132 decreased the level of ROS and expression level of HIF-1 α in CLP-induced ALI rats. The levels of (A) SOD and (B) MDA were detected by the assay kits. (C, D) The expression of HIF-1 α was measured by western blot. ** $P < 0.01$, *** $P < 0.001$ versus sham; # $P < 0.05$, ## $P < 0.01$ versus model. ROS – reactive oxygen species; CLP – cecal ligation and puncture; ALI – acute lung injury; SOD – superoxide dismutase; MDA – malondialdehyde; HIF-1 α – hypoxia-inducible factor-1 α .

analysis of variance (ANOVA). A significance level of $P < 0.05$ was adopted for all analyses.

Results

MG-132 improved lung tissue pathological changes and edema in CLP-induced ALI rats

Hematoxylin and eosin (H&E) staining was employed to investigate the pathological changes of lung tissue. As presented in Figure 1A, significant differences were found in the model group in comparison to the control and sham groups, which represented that the CLP procedure induced tissue damage and inflammation. After pretreatment with MG-132, the lung tissue damage and inflammation were attenuated, notably. At the same time, the ratio of wet lung to dry lung was calculated. We found that the W/D ratio was increased significantly in the model group compared with the sham group (Figure 1B), indicating that there was severe pulmonary edema in sepsis-induced ALI rats. However, in the MG-132 intervention group, the lung water content was significantly lower than that of the model group. These results indicated that a successful ALI rat

model was established and that MG-132 improved the lung tissue pathological changes and edema.

MG-132 decreased the levels of inflammatory cytokines in BALF

Inflammatory cytokines levels of TNF- α , IL-6, IL-1 β , and ICAM-1 in BALF were measured to study the effect of MG-132 on the secretion of cell cytokine in BALF. As exhibited in Figure 2A, the level of TNF- α in the model group increased obviously compared with the sham group. Speaking of the effect of MG-132 pretreatment, TNF- α level in the MG-132 group was reduced notably in BALF. And the levels of IL-6, IL-1 β , and ICAM-1 showed the same results as TNF- α (Figure 2B–2D). These results indicated that MG-132 could reduce the levels of inflammatory cytokines in BALF.

MG-132 decreased the levels of SOD and MDA in lung tissue

To investigate the level of reactive oxygen (ROS), SOD, and MDA, kits were applied for this analysis and the concentrations in lung tissues of different groups are presented in Figure 3A and 3B.

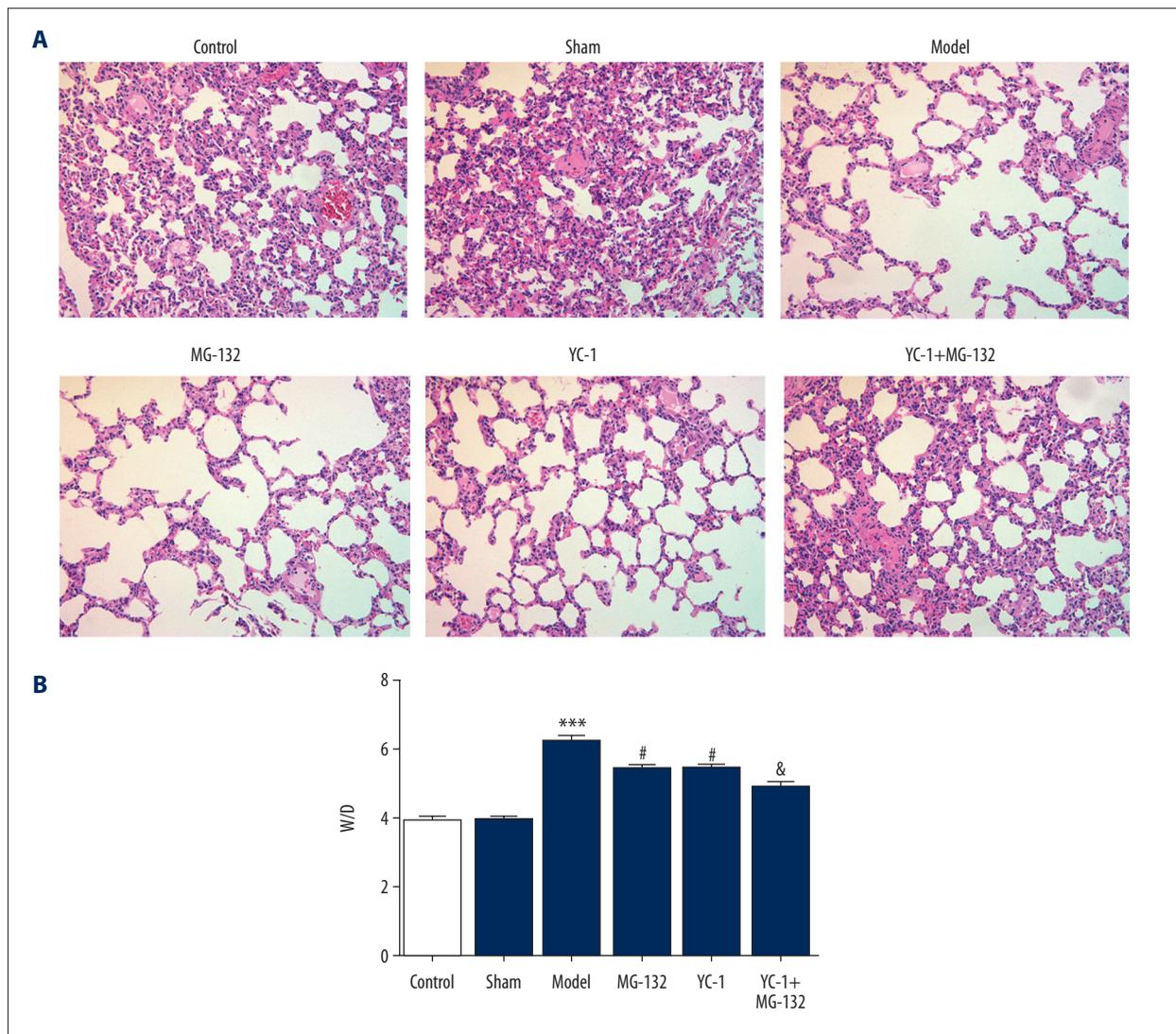


Figure 4. MG-132 and YC-1 improved lung tissue pathological changes and edema in CLP-induced ALI rats. (A) Representative images of hematoxylin and eosin staining from each experimental group at 24 hours after CLP. (magnification, 100×). (B) MG-132 and YC-1 attenuated lung W/D ratio in CLP-induced ALI rats. *** $P < 0.001$ versus sham; # $P < 0.05$ versus model; & $P < 0.05$ versus YC-1. CLP – cecal ligation and puncture; W/D – wet/dry ratio of lung.

CLP in the model group significantly decreased SOD levels while increasing the MDA levels as compared with the sham group. Pretreatment with MG-132 attenuated the CLP-induced decrease in SOD levels and increase in MDA levels compared to the model group. These data indicated that MG-132 could decrease the levels of ROS in CLP-induced ALI.

MG-132 suppressed the expression of HIF-1 α , and HIF-1 α inhibition decreased the levels of inflammatory cytokines

As presented in Figure 3C and 3D, the expression of HIF-1 α was upregulated in the CLP-induced model group compared with the sham group. However, in the MG-132 treatment group, the level of HIF-1 α was decreased significantly. In addition,

YC-1, an inhibitor of HIF-1 α , was employed to treat rats before the experiment started. Figure 4A and 4B, show that we found lung tissue damage was attenuated notably, and accompanied by a decrease in lung water content in the YC-1 group. In the group of both YC-1 and MG-132 treatment, the lung tissue damage and W/D rate was improved compared with YC-1 treatment alone. At the same time, the levels of inflammatory cytokines, including TNF- α , IL-6, IL-1 β , and ICAM-1, had the same aforementioned results (Figure 5A–5D). These data suggested that MG-132 could suppress the expression of HIF-1 α , and that HIF-1 α inhibition could protect rats from CLP-induced ALI.

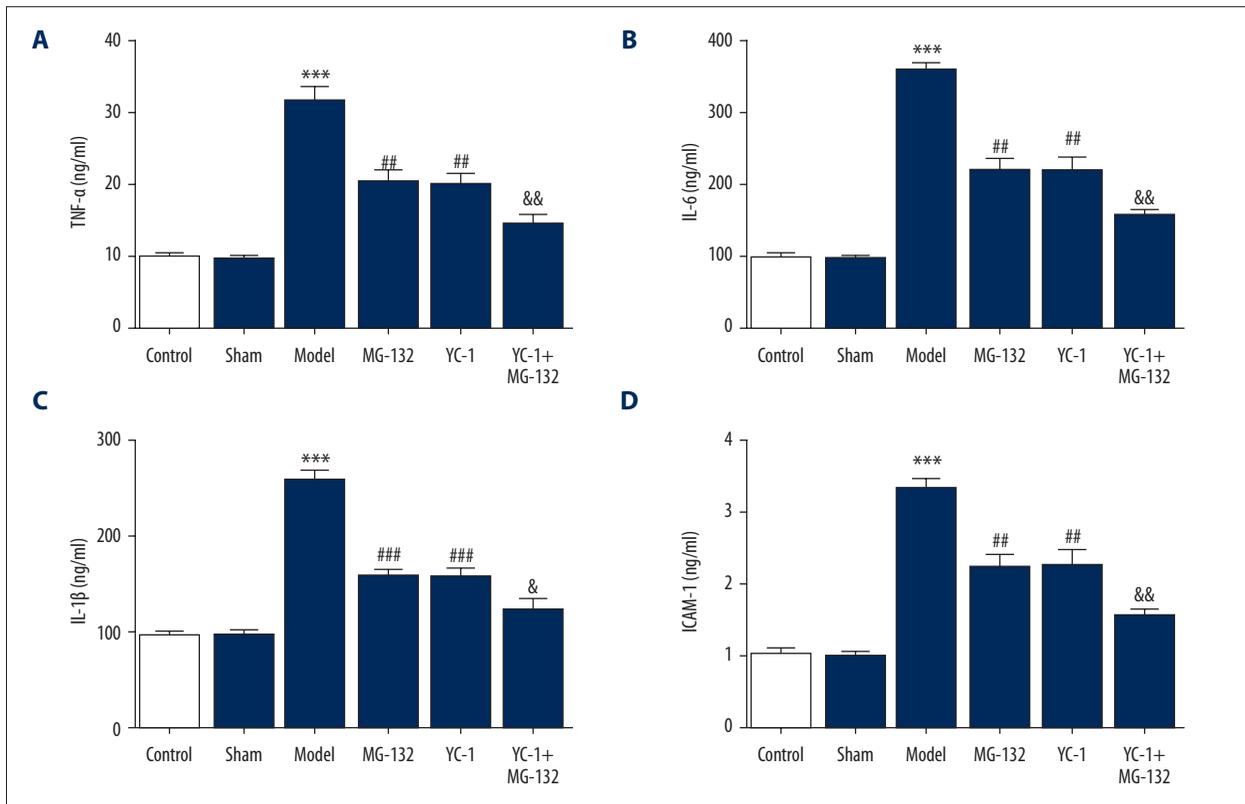


Figure 5. MG-132 and YC-1 decreased the levels of inflammatory factors in the BALF of CLP-induced ALI rats. The levels of (A) TNF- α , (B) IL-6, (C) IL-1 β , and (D) ICAM-1 were measured by ELISA. *** $P < 0.001$ versus sham; ## $P < 0.01$, ### $P < 0.001$ versus model; & $P < 0.05$, && $P < 0.01$ versus YC-1. BALF – bronchoalveolar lavage fluid; CLP – cecal ligation and puncture; ALI – acute lung injury; TNF – tumor necrosis factor; IL – interleukin; ICAM – intercellular adhesion molecule; ELISA – enzyme-linked immunosorbent assay.

MG-132 protected against CLP-induced ALI by inhibiting the mTOR/4EBP1/EIF4E signaling pathway

As shown in Figure 6A–6G, the expression levels of p-mTOR, p-4EBP1, and p-EIF4E were increased in the model group compared with the sham group. However, the levels of the aforementioned proteins were decreased significantly in the MG-132 group. After pretreatment with YC-1, the levels of p-mTOR, p-4EBP1, and p-EIF4E were downregulated. In addition, when treated with both YC-1 and MG-132, the levels of p-mTOR, p-4EBP1, and p-EIF4E decreased compared with the YC-1 only group. These results indicated that MG-132 protected against CLP-induced ALI by inhibiting the mTOR/4EBP1/EIF4E signaling pathway.

Discussion

Sepsis is considered a leading factor for death for intensive care unit patients due to the systemic inflammatory response syndrome which has an extreme high morbidity and mortality rate [14,15]. And lung serves as a vulnerable target organ in

sepsis-induced ALI, which is considered as one of the dominant risk factors for death [16]. Therefore, the aim of the present study was to explore the effect of MG-132 on sepsis-induced ALI and its underlying mechanism, which will have critical significance for the clinical treatment of ALI.

Excessive inflammation is associated with the initial stages of sepsis, and inflammation is considered a prominent characteristic in sepsis-induced lung injury [16,17]. It has been well documented that chronic treatment with MG-132 can be an effective therapy for diabetes-induced aortic oxidative damage and inflammatory response [18]. In addition, MG-132 treatment could attenuate cardiac remodeling and dysfunction following aortic banding in rats via inhibiting the NF- κ B/TGF β -1 pathway [19]. A previous study revealed that MG-132 plays an important role in decreasing foam cell formation and inflammatory cytokine levels [6]. Importantly, MG-132 exerted a protective effect on severe acute pancreatitis and associated lung injury of rats through improvement of edema, cellular damage, and inflammatory activity [8]. Thus, whether MG-132 protected against sepsis-induced ALI was a consideration of our research interests. In the present study, our results demonstrated that

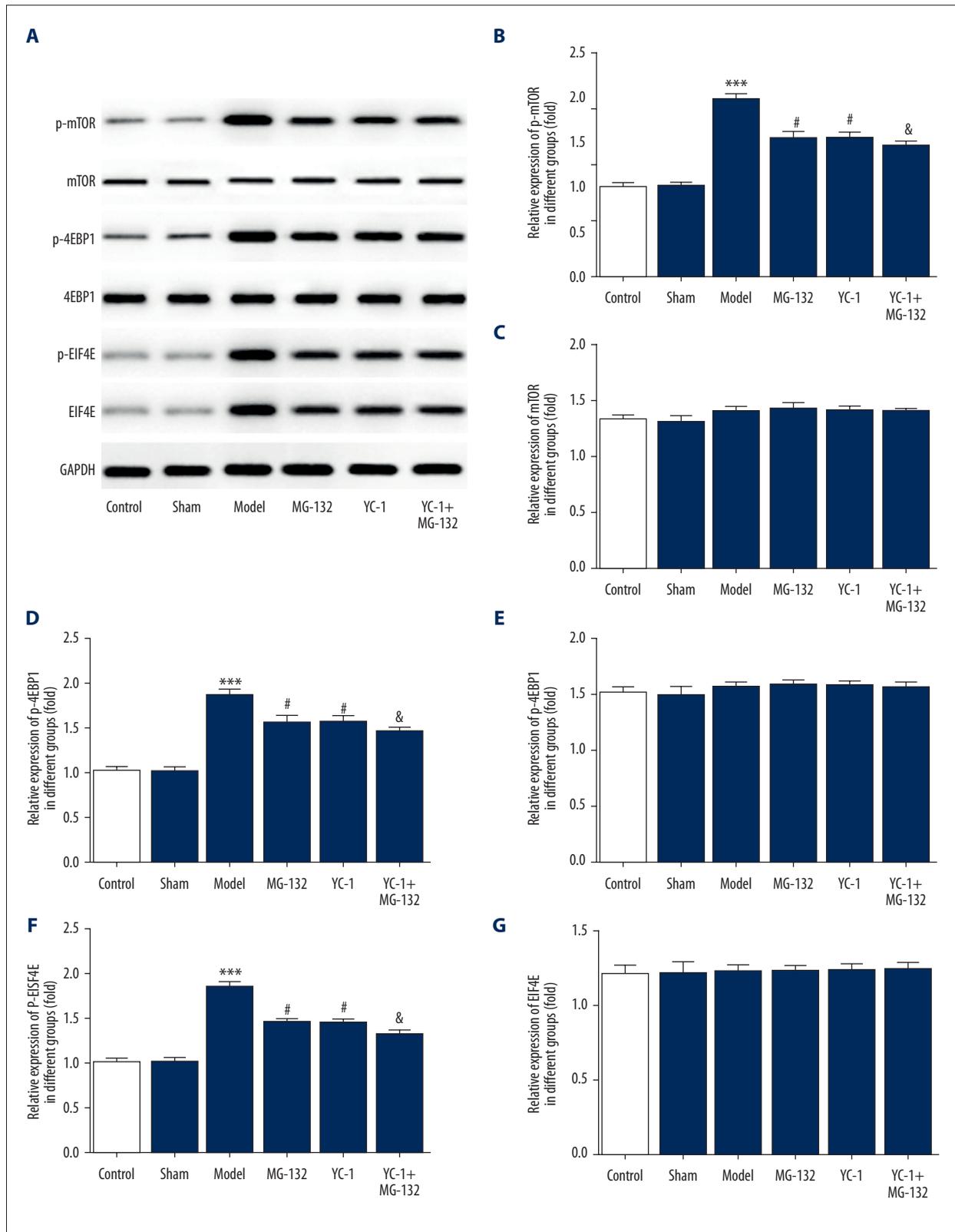


Figure 6. MG-132 and YC-1 inhibited the phosphorylation of the mTOR/4EBP1/EIF4E pathway. (A–G) The expression of p-mTOR, mTOR, p-4EBP1, 4EBP1, p-EIF4E, and EIF4E were measured by western blot. *** $P < 0.001$ versus sham; # $P < 0.05$ versus model; & $P < 0.05$ versus YC-1.

MG-132 improved pathological changes and edema of lung tissue in CLP-induced ALI rats. At the same time, the levels of inflammatory factors TNF- α , IL-6, IL-1 β , and ICAM-1 were decreased significantly.

HIF-1 α exerts a crucial effect in the progress of both hypoxia and inflammatory response. It has been well reported that oxygen metabolic disorders and cascade amplification of inflammatory responses are dominant clinicopathological features in sepsis-induced ALI [20,21]. Accumulating evidence shows that inhibition of HIF-1 α protects against intestinal barrier dysfunction in rats with sepsis [22]. In the development process of acute lung inflammatory damage induced by treatment with septic lymph, the expression level of HIF-1 α was induced and activated in rats [10]. Our present study results revealed that the ROS level, including MDA, was decreased obviously while the level of SOD was increased in the MG-132 group, which indicated that MG-132 was able to improve oxygen metabolic dysfunction of ALI. Importantly, the expression of HIF-1 α was downregulated after pretreatment with MG-132. Emerging evidence supports the notion that MG-132 can prevent oxidized low-density lipoprotein-attenuation differentiation phenotype by blocking HIF-1 degradation [9]. Moreover, the presence of MG-132 can abrogate the suppression of hypoxia-induced HIF-1 α accumulation in a rat model of type 2 diabetes [23]. YC-1, an inhibitor of HIF-1 α , has been shown to alleviate histological damages, decrease lung water content, and reduce the levels of TNF- α , IL-1 β , and IL-6 in ALI rats triggered by trauma and hemorrhagic shock [24], which were in accordance with our present study. We found that with pretreatment with both YC-1 and MG-132, the lung tissue pathological changes and edema, and the level of inflammatory cytokines, were improved significantly compared with YC-1 treatment alone.

A previous study reported that the phosphorylation of the mTOR/4EBP1/EIF4E pathway was activated in hypoxia [25]. It has been reported that the increased expression of HIF-1 α promoted the phosphorylation of mTOR/4EBP1/EIF4E [26]. In our study, the expression of HIF-1 α was downregulated in the MG-132 group and ALI was improved obviously when rats were pretreated with both YC-1 and MG-132 compared with YC-1 treatment alone, which demonstrated that MG-132 could inhibit the expression of HIF-1 α . In addition, the expression levels of p-mTOR, p-4EBP1, and p-EIF4E were decreased notably in the MG-132 group, the YC-1 group, and the YC-1+MG-132 group. The aforementioned results confirmed that MG-132 could inhibit HIF-1 α mediated mTOR/4EBP1/EIF4E pathway.

Conclusions

For the first time, our study investigated the effect of MG-132 on sepsis-induced ALI. Our study results showed that MG-132 protected lung tissue against sepsis-induced oxidative damage and inflammatory responses via inhibition HIF-1 α mediated mTOR/4EBP1/EIF4E signaling pathway, which would significantly increase the survival rate of sepsis-induced ALI rats eventually, and could be of critical significance for the clinical treatment of ALI.

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Conflict of interests

None.

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