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The cyclic hexapeptide AcF attenuates sepsis-induced acute lung injury and mortality in rats

H. DONG, F. SHAN, Q. SUN, B.-X. YANG, C.-P. LI

Department of Critical Care Medicine, The Affiliated Hospital of Qingdao University, Qingdao, China

Abstract. – OBJECTIVE: The purpose of this study was to elucidate the possible beneficial effects of AcF on acute lung injury (ALI) in a rat model of sepsis.

MATERIALS AND METHODS: Male Sprague-Dawley rats were randomly divided into the following four experimental groups (n = 10 per group): animals undergoing a sham cecal ligature puncture (CLP) (Sham group); animals undergoing CLP (control group); or animals undergoing CLP and treated with saline (Saline group) and animals undergoing CLP and treated with AcF (AcF group). At 24h after CLP, blood, bronchoalveolar lavage fluid (BALF) and lung tissue were collected. The lung wet/dry weight ratio, Protein concentration and the count of inflammatory cells or neutrophils in the BALF were determined. The pathologic changes in lungs were examined with the optical microscopy. Myeloperoxidase (MPO) activity, the expression of inflammatory cytokines were measured in lung tissue and BALF respectively. Survival rates were recorded at 120h in the four groups in another experiment.

RESULTS: Histology findings revealed acute lung injury in rats in the CLP group, whereas those in the AcF-treated group had mild lung injury. Treatment with AcF significantly attenuated the CLP-induced pulmonary edema and inflammation, as it significantly decreased lung wet/dry ration, protein concentration and the infiltration of inflammatory cells and neutrophils in the lung tissues. In addition, the secretion of inflammatory cytokines, such as TNF-α, IL-6, IL-1b and macrophage inflammatory protein-2 (MIP-2) was decreased in AcF treated group compared with the control saline treated group.

CONCLUSIONS: AcF administration ameliorates acute lung injury in a rat model of sepsis induced by CLP. AcF can be developed as a novel treatment for severe sepsis-induced ALI.

Key Words:
AcF, Sepsis-induced ALI, C5a, Complement, C5aR pathway.

Introduction

Sepsis can be defined as a systemic inflammatory response syndrome in which there is an identifiable focus of infection[1]. Sepsis is a major cause of morbidity and mortality despite extensive research efforts and improvements in care[2]. Severe sepsis is diagnosed once sepsis becomes complicated by factors, including acute organ dysfunction, tissue/organ hypoperfusion, hypotension, and coagulopathy[3]. Sepsis may lead to end-organ dysfunction, and septic patients are particularly at risk of developing acute lung injury (ALI)[4]. In sepsis, lung dysfunction is the first step in the development of multiple organ failure, and acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are common complications in ICU and take responsibility for significant morbidity and mortality[5]. Acute lung injury (ALI) and its most severe form, acute respiratory distress syndrome (ARDS), are characterized by increased capillary and alveolar permeability, hypoxemia, decreased lung compliance and diffuse bilateral pulmonary infiltrates[6]. Despite recent intensive clinical and basic science advances in the understanding of the themolecular underpinning of sepsis, most of its complications remain refractory to treatment[7], and mortality rates have not changed much over the past few decades[8].

In the ALI model, lung edema, endothelial and epithelial injury are accompanied by an influx of neutrophils into the interstitium and broncheoalveolar space. Neutrophils are considered to play a key role in the progression of ALI and ARDS[9], as activation and transmigration of neutrophils is a hallmark event in the progression of ALI and ARDS. Proof for the importance of neutrophils in ALI comes from clinical data and animal models. In patients with ARDS, the concen-

Corresponding Author: Feng Shan, MD; e-mail: fengshan_2014@126.com

tration of neutrophils in the bronchoalveolar lavage (BAL) fluid correlates with severity of ARDS and outcome[10-12], whereas the severity of lung injury has been reduced by neutrophil depletion in mice[13].

As we know, neutrophils and complement are crucial components of innate immunity. Both are considered among the earliest innate immune effectors that sense incoming inflammatory signals and are rapidly recruited to sites of injury and inflammation[14]. In addition, sepsis triggers complement activation[15]. Excessive activation of complement leads to high levels of complements activation products in the blood, including the potent proinflammatory peptide C5a. C5a is the paramount proinflammatory mediator of the complement cascade, and has been previously thought to act only through a single, G-protein-coupled, C5a receptor (C5aR; also termed CD88). In addition, it is also known that C5aR interact with C5a in a G-protein-dependent manner, which results in increased intracellular Ca^{2+} together with MAPK and Akt activation, followed by a series of functional responses such as chemotaxis, enzyme release, Mac-2 upregulation, degranulation, a respiratory burst, etc. The role of C5aR in the setting of sepsis also seems obvious: C5aR interacting with C5a contributes to adverse events in ALI after deposition of IgG immune complexes and in cecal ligature punture (CLP)-induced sepsis, including the intensified pro-inflammatory state and lethality.

In the present study, we assessed whether treatment with the cyclic hexapeptide AcF[Opd-ChaWR], a C5a-C5aR signal antagonist, can reduce ALI in rats with cecal ligation and puncture (CLP)-induced polymicrobial sepsis. We found that the treatment with AcF could prevent CLP-induced ALI by inhibiting the proinflammatory cytokine response, neutrophils infiltration and the survival rate in rats.

Materials and Methods

Animals

Specific pathogen-free male Sprague-Dawley rats werghing 200 ± 20 g at the beginning of the experiments were used. The animals were maintained at 23°C under a 12h light-dark cycle and were allowed access to food and water ad libitum. All surgery was performed under sodium pentobarbital anaesthesia, and all efforts were made to minimize suffering.

Surgical Procedures and Experimental Protocols

After an overnight fast, we anesthetized animals with intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg). We induced sepsis by CLP. Briefly, after shaving the abdominal fur and preparing the abdominal wall with 10% povidoneiodine solution, we made a 2 cm ventral midline abdominal incision. We then exposed the cecum, isolated it, and ligated it with a 3-0 silk ligature just distal to the ileocecal valve to avoid intestinal obstruction, punctured it in 2 locations with an 18-gauge needle, compressed it gently until the feces were extruded, and then repositioned it. We closed the incision in 2 layers with a 4-0 sterile synthetic absorbable suture. The sham-operated rats underwent laparotomy through a lower-midline incision, and the cecum was manipulated, without being ligated or perforated. At the end of the operation, we immediately resuscitated animals by subcutaneous administration of saline (2 ml/100 g body weight). No antibiotics were administered.

We randomly divided animals into 4 groups (n = 10/group): (1) control: rats were injected ip with 6 mg/kg saline 12h before undergoing a sham operation; (2) CLP: rats were injected ip with 6 mg/kg saline 12h before undergoing CLP; (3) AcF plus CLP: rats were injected ip with 6 mg/ml AcF solution 12h before undergoing CLP. At 24h after the operations, we killed all animals to collect the lung tissues and other samples.

Histology

Lungs were dehydrated in 70% ethanol, processed using standard procedures and embedded in paraffin. Section of 5 um were cut, mounted on slides, and staining with haematoxylin and eosin (H&E). The histopathology analysis was performed with a conventional light microscope (Olympus BX51, Olympus Latin America, Sao Paulo, Brazil) and images were captured with a Nikon DXM1200C digital camera.

Lung Wet/Dry Weight Ratio

Lung edema was estimated by determining lung W/D weight ratios. The fresh upper part of the left lung was weighed and dried in an oven at 80°C for at least 24h, then weighed again when it was dry, to calculate the lung W/D weight ratio.

MPO Activity

In brief, a part of the frozen right lung tissue was thawed and homogenized in 1ml of 0.5% hexadecyltrimethylammonium bromide. The

sample was then freezed-thawed, and the MPO activity of the supernatant was measured as previously described[16]. The enzyme activity was assayed by measuring absorbance changes in the redox reaction of H_2O_2 by spectrophotometry at 450 nm. Results were expressed as MPO units per gram of tissue.

The Measure of Protein Concentration in Lung BALF

The concentration of protein in the BALF was measured using Bradford reagent (Bio-Rad Protein Assay kit., Hercules, CA, USA). Briefly, 160 µl of each standard and sample solution was pipeted into separate microtiter plate wells, and 40 ul of the dye reagent was added to each well and mixed thoroughly. The mixture was incubated at room temperature for at least 5 min before measurement of the OD at 595 nm. Comparison to a standard curve provided a relative measurement of the protein concentration.

Btonchoalveolar Lanage (BAL) Examination

The trachea was exposed and cannulated with a catheter. The left lung was lavaged for 3 times with sterile phosphate buffered saline (PBS) in a volume of 0.5 ml/wash. The fluid recovered after lavage was greater than 90% on average. The BAL fluid (BALF) was centrifuged at 2000 rpm for 10 min at 4°C, and the supernatant was stored at –80°C for cytokine and protein analysis, while the cell pellet was resuspended in PBS for counting the neutrophils.

ELISA

Detection of TNF- and IL-6 amount with ELISA according to the manufacturer's protocol. The experiment was repeated three times and results were shown with the mean value.

Bacterial Load

To determine the bacterial load in the peritoneum, the peritoneal cavity was lavaged with 5 ml of sterile saline. Serial log dilutions were made. To determine the bacterial load in the blood, 100 ml of blood was collected and serially diluted with sterile saline. To determine the pulmonary bacterial load, the lungs were harvested and equal amounts of wet tissue were homogenized and briefly centrifuged to remove gross particulate matter. Serial log dilutions of tissue homogenates were applied. Five hundred microliters of each dilution was then plated on chocolate agar plates (Fisher Scientific, Pittsburgh, PA, USA) and incubate at 37°C for 24 hours under aerobic conditions. Colony-forming units were counted. Results were expressed as colony-forming units per milliliter or milligram of wet tissue.

Survival Study in Rats of Sepsis-Induced ALI

We tested whether pretreatment with AcF would confer protection against sepsis-induced ALI. Rats were randomly divided into three experimental groups (n = 10 per gourp) as mentioned above. And then survival rates were recorded at 120h.

Statistical Analysis

All the data were analyzed using SPSS13.0 software (SPSS Inc., Chicago, IL, USA) and expressed as Means ± SD. Significant differences were assessed by one-way analysis of variance (ANOVA) followed by Fisher protected least significant difference test. A probability value of less than 0.05 was considered to indicate a statistical significance.

Results

Histologic Evaluation of Lung Tissue

Previous studies reported that the AcF could block the C5a-C5aR signal pathway and inhibit the activation of neutrophil and subsequent inflammatory responses. So we hypothesized whether AcF could protect rats from CLP-induced lung injury by inhibiting the activation of C5a-C5aR signal pathway in neutrophils and inflammatory responses in ALI rats. Then we administrated the CLP-induced ALI rats with AcF or saline control via tail vein injection. We found that, compared with the sham control group (Figure 1 A), the CLP-induced lung injury showed diffuse pathological changes characterized by alveolar congestion and inflammatory cell infiltration into the airspace (Figure 1 B). In addition, the pretreatment with AcF improved the histology of the lung (Figure 1 D) compared with the saline-pretreated control group (Figure 1 C) and not treated group (Figure 1 B). These results suggested that these AcF-pretreated rats were resistant to CLP-induced ALI.

Effect of AcF Administration on CLP-Induced Lung Edema Index

To further analyze the effect of AcF on the degree of CLP-induced lung edema index, the lung

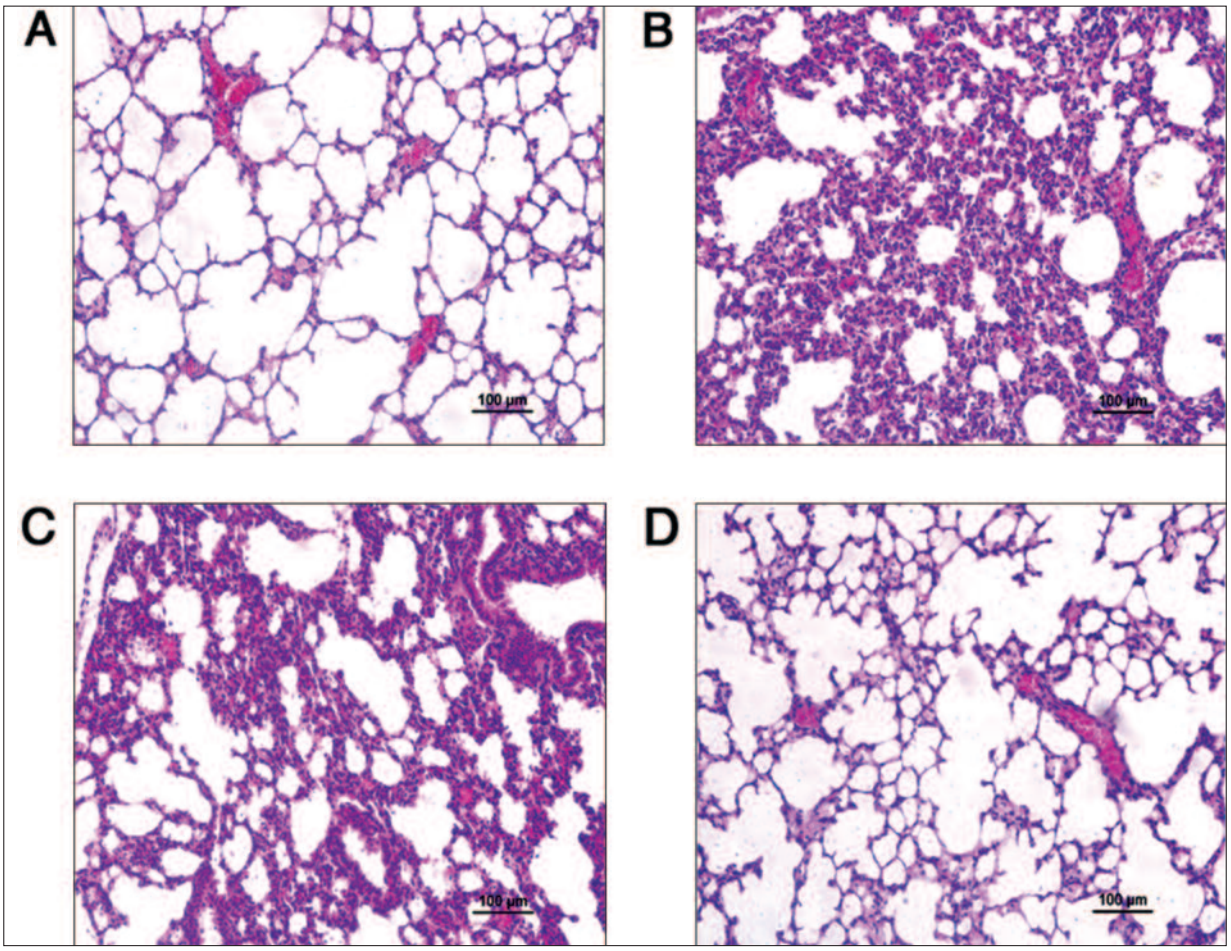


Figure 1. Effect of AcF treatment on histopathologic changes in lungs from septic mice. ***A,*** Sham group. ***B,*** CLP-induced ALI group. ***C,*** Saline-treated control group. ***D,*** AcF-treated group. *$p < 0.05$ compared with the healthy control group mice; **$p < 0.05$ compared with the saline-treated control group.

Wet/Dry ratio was evaluated to indicate the pulmonary edema. As shown in Figure 2, the lung Wet/Dry ratio was found to be significantly higher in the CLP-induced ALI group compared with the sham control group ($p < 0.05$), however the AcF treatment decreased the lung Wet/Dry ratio compared with the saline treated control group ($p < 0.05$). These results demonstrated that the treatment of AcF could decrease the lung edema in CLP-induced lung injury rats.

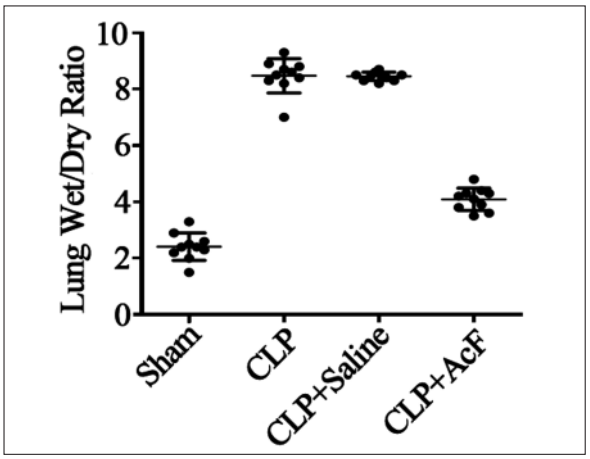
Treatment with AcF Reduced the Activity of MPO and the Protein Concentration in the Injury Lung

The activity of MPO, a peroxidase enzyme most abundant in neutrophil granulocytes was evaluated after the CLP procedure in the lung tissue of rats (Figure 3 A). MPO activity was significantly increased in the CLP group compared with the sham group ($p < 0.05$), however, CLP-induced MPO activity was decreased by the AcF treatment compared with the saline treatment ($p < 0.05$). In addition, as shown in Figure 3 B, CLP caused a significant increase in the BALF protein level compared with the sham group rats ($p < 0.05$), but the protein concentration in the AcF treated-group was significantly lower than the saline treated group ($p < 0.05$) and no treated group ($p < 0.05$).

Treatment with AcF Inhibited the Infiltration of Inflammatory Cells Into Lung Tissue in the CLP-Induced Lung Injury

As shown in Figure 4, exposure to the CLP challenge significantly increased the percentage of the total cells (Figure 4 A) and neutrophils (Figure 4 B) in the lung tissue compared with the

Figure 2. The wet/dry ratio of different treated group of rats. The Wet/Dry ratio of lung tissue from rats with indicated treatment was measured. Data are expressed as mean ± SD of the values of 10 rats of each group. *$p < 0.05$ compared with the sham group; **$p < 0.05$ compared with the saline-treated group.



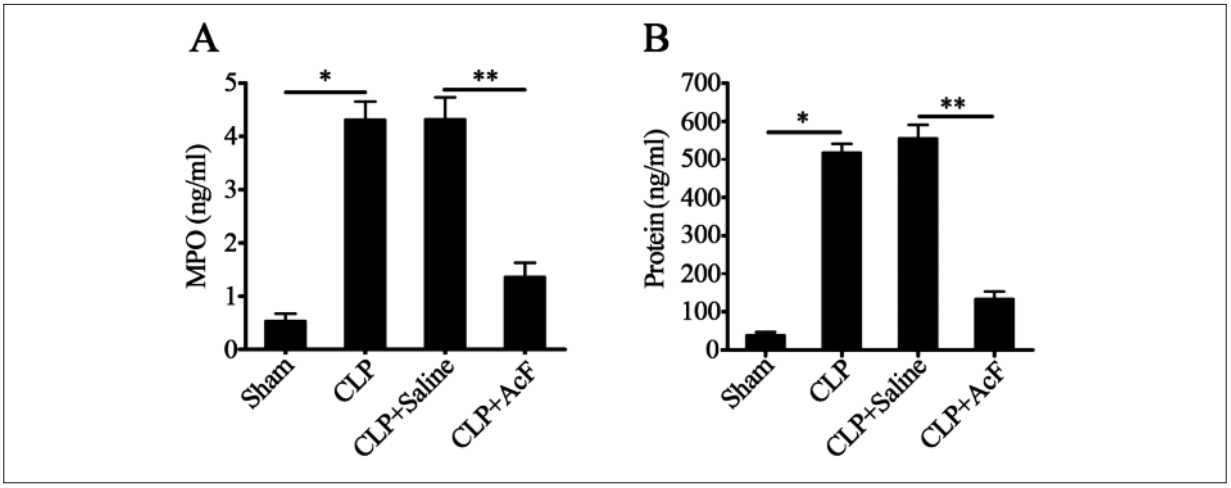


Figure 3. The MPO activity and protein concentration in the lung of septic ALI rats. ***A,*** The MPO activity in the lung homogenates. ***B,*** The protein concentration in the BALF. Data are expressed as mean ± SD of the values of 10 rats of each group. *$p < 0.05$ compared with the sham group; **$p < 0.05$ compared with the saline-treated group.

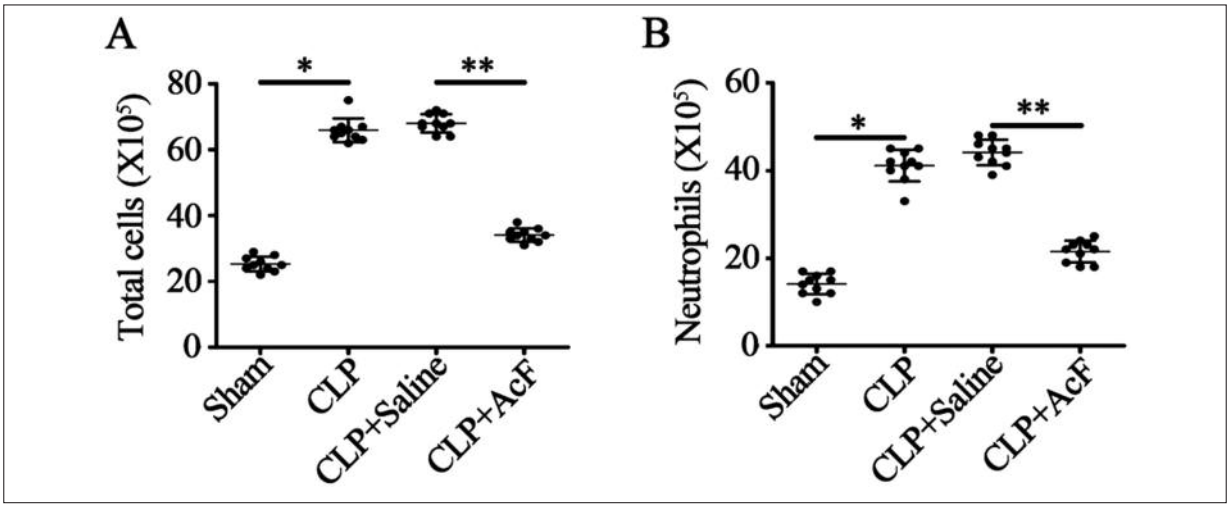


Figure 4. Total cells ***(A)*** and neutrophils ***(B)*** counts in BALF collected from rats in the sham, CLP, CLP + saline and CLP + AcF group 24h after CLP/sham operations. Data are expressed as mean ± SD of the values of 10 rats of each group. *$p < 0.05$ compared with the sham group; **$p < 0.05$ compared with the saline-treated group.

sham control group. However, the infiltration of inflammatory cells (Figure 4 A and B) into injury lung tissue was suppressed in the AcF treated group compared with the saline treated control group and the sham control group. These data indicated that the treatment of AcF could inhibit the inflammatory cells infiltration into the injury lung tissue in the CLP-induced lung injury rats. These results were supported by the data of MPO activity in different treated groups.

Treatment with AcF Suppress TNF-α, IL-1β, IL-6 and MIP-2 Elevation Caused by CLP-Induced Lung Injury

To further explore the effect of AcF on the inflammatory responses in the CLP-induced lung injury rats, we measured the production of several inflammatory cytokines in the lung BALF, such as TNF-α, IL-6, IL-1 and MIP-2. As shown in Figure 5, compared with the sham control group, the concentrations of the inflammatory cytokines, such as TNF-α (Figure 5 A), IL-6 (Figure 5 B), IL-1 (Figure 5 C) and MIP-2 (Figure 5 D), were increased in the CLP-induced lung injury group ($p < 0.05$), this data indicated that CLP induced strong inflammatory responses in lung injury rats. However, the treatment of AcF suppressed the production of inflammatory cytokines induced by CLP surgery compared with the saline treated control group. These results were agreement with the degree of lung injury.

Effect of AcF Treatment on Bacterial Load in CLP-Induced Lung Injury

As indicated in Figure 6, the colony-forming units were determined for peritoneal fluid (Figure 6 A), blood (Figure 6 B), and lungs (Figure 6

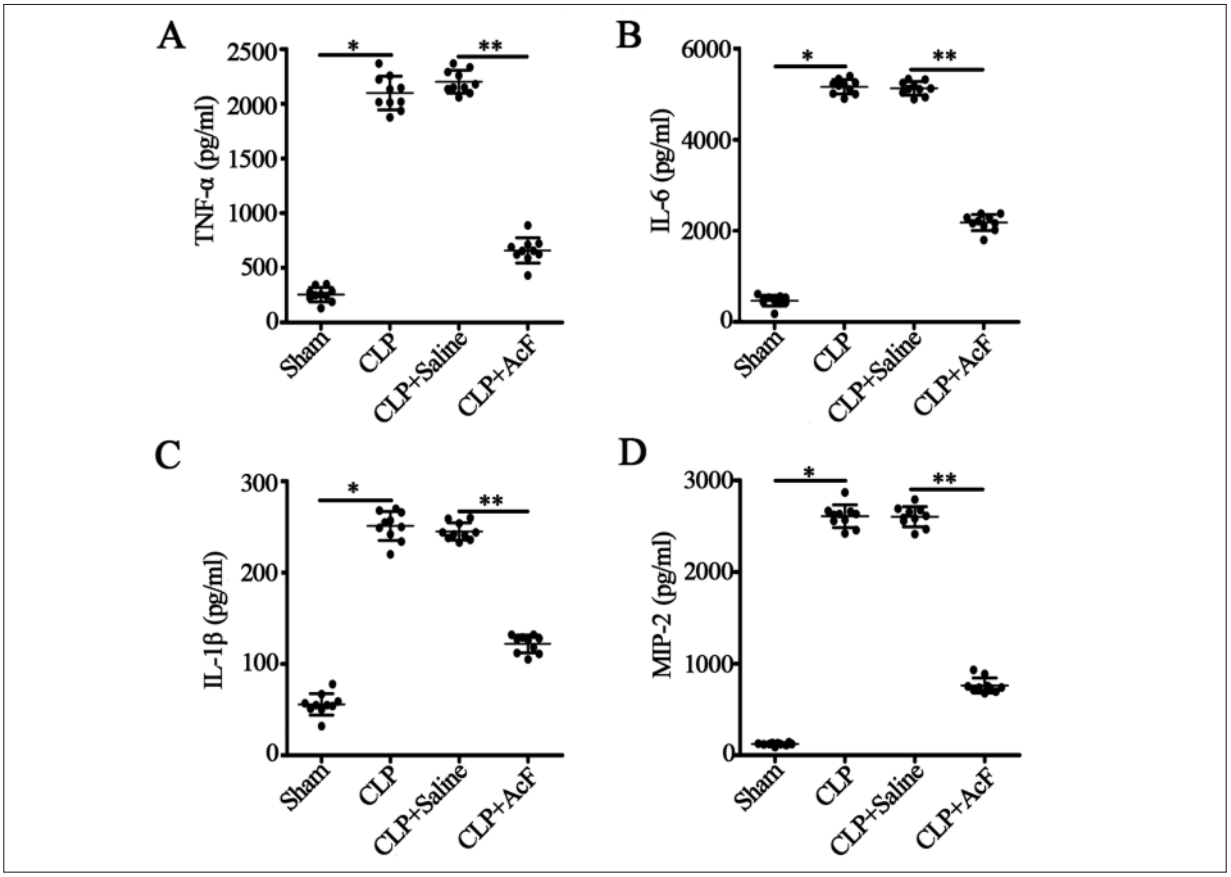


Figure 5. The expression of inflammatory cytokines in BALF of septic ALI rats. ***A,*** The concentration of TNF-α in BALF of rats with indicated treatment was monitored by ELISA. ***B,*** The concentration of IL-6 in BALF of rats with indicated treatment was monitored by ELISA. ***C,*** The concentration of IL-1β in BALF of rats with indicated treatment was monitored by ELISA. ***D,*** The concentration of MIP-2 in BALF of rats with indicated treatment was monitored by ELISA. Data are expressed as mean ± SD of the values of 10 rats of each group. *$p < 0.05$ compared with the sham group; **$p < 0.05$ compared with the saline-treated group.

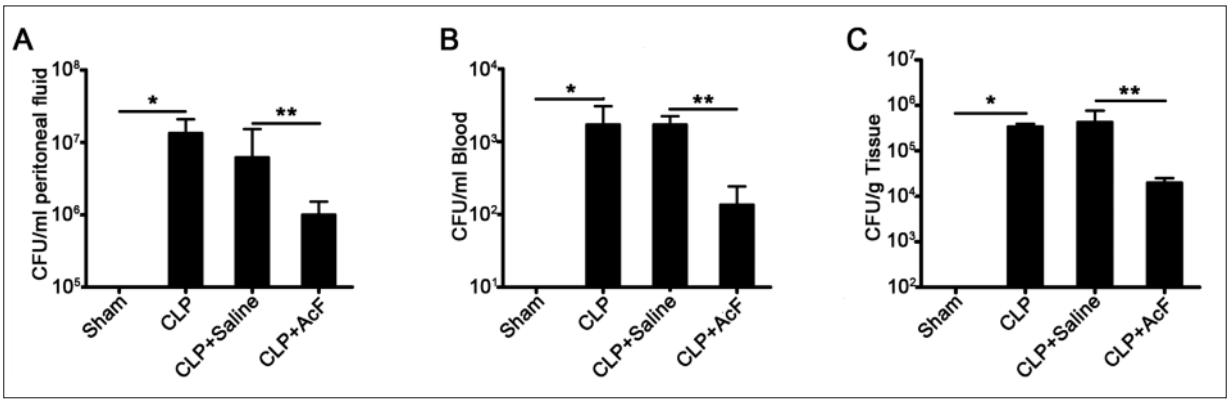


Figure 6. Alterations in bacterial load in the peritoneal fluid ***(A)***, blood ***(B)***, and lungs ***(C)*** in sham-operated rats and septic rats treated with saline or AcF 24 hours after cecal ligation and puncture (CLP). Data are expressed as mean ± SD of the values of 10 rats of each group. *$p < 0.05$ compared with the sham group; **$p < 0.05$ compared with the saline-treated group.

C) in the four groups of rats 24 hours after CLP. We found that the numbers of colony-forming units in the CLP induced group was significantly higher than that in the sham control group. However, the numbers of colony-forming units was significantly decreased in the septic rats receiving AcF treatment compared with the septic rats receiving saline control treatment. These data was also consistent with the degree of lung injury of different treated group.

Effect of AcF Administration on Survival Rate

To further illuminate the therapeutic effect of AcF on the CLP-induced lung injury, we analyzed the survival rate of different treated septic rats. As the data showed, compared with the sham control group, the survival rate of CLP-induced sepsis group was observably decreased ($p < 0.05$). However, the administration of AcF was dramatically improved the survival rate compared with the saline treated group rats. These results suggested that the treatment of AcF could protect septic rats from death.

Discussion

As we know, the complement system not only represents an effective innate immune mechanism of host defense to eliminate microbial pathogens, but it is also widely involved in many forms of acute inflammatory diseases, such ac sepsis, acute lung injury and asthma. C5a is a member of the complement cascade, an important mechanism for host defense against bacteria. As a complement-activated production, C5a displays potent biological activities that lead to inflammatory responses. In addition, as a strong chemoattractant, C5a also involved in the recruitment of inflammatory cells to the sites of inflammation, such as neutrophils and T lymphocytes. So all of which may contribute to the inflammatory responses and tissue damage[17]. Accumulating data suggested that C5a provides a vital bridge between innate and adaptive immune functions, extending the role of C5a in inflammation[18].

Increased complement activation and excessive production of C5a have been implicated in the pathogenesis of several inflammatory disease, such as sepsis, acute lung injury, and considerable effort has gone into developing C5aR antagonists, including organic small molecules and peptide antagonists[19].

As we know, the cyclic hexapeptide AcF[OpdChaWR] is a C5a-C5aR pathway antagonist which could block the activation of C5aR in the neutrophils and reduce the inflammatory responses induced by exvessive activation of C5aR. Previous studies demonstrated that inhibition of the C5aR could decrease the tissue damage induced by inflammation. So we hypothesis that whether the C5aR antagonist AcF could reduce the lung damage in CLP-induced ALI through inhibiting the inflammatory response.

In the present study, we employed the CLP model, because it closely resembles the pathophysiology of human sepsis and represents an indirect insult similar to the pathogenesis of ARDS[20]. Previous studies demonstrated that the CLP rats exhibited marked hypoxemia, increased

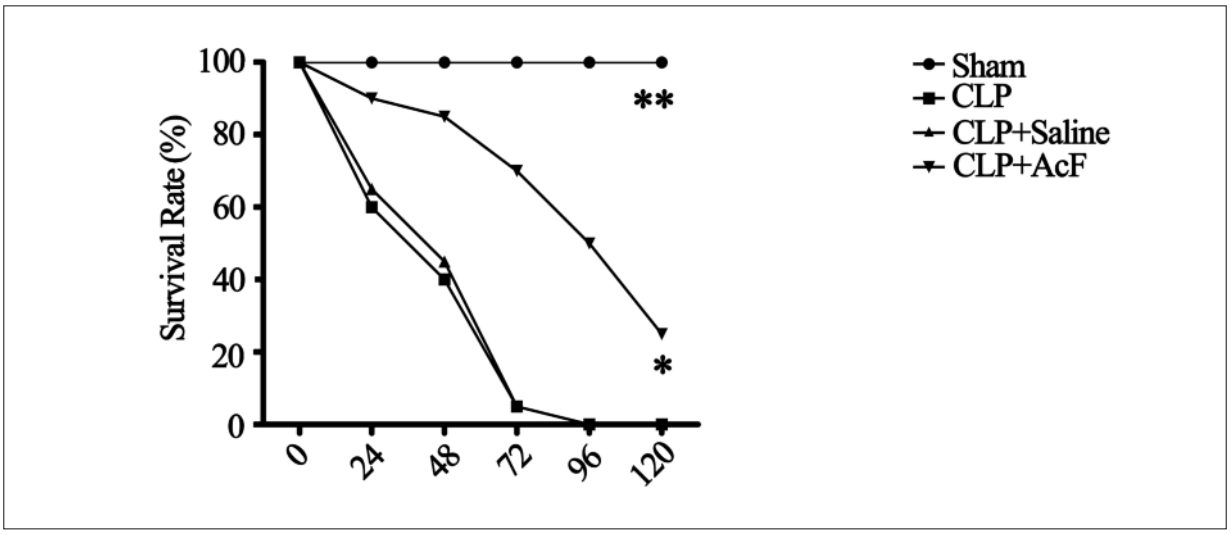


Figure 7. Treatment with AcF delayed the death of rats with CLP-induced lung injury. The Kaplan-Meier survival curves of rats (n=10) with indicated treatment were monitored. Data are expressed as mean ± SD of the values of 10 rats of each group. *$p < 0.05$ compared with the sham group; **$p < 0.05$ compared with the saline-treated group.

lung vascular permeability, and histological damage in lungs, including inflammatory infiltrate and hemorrhage[21]. As the Figure 1 showed, we found that the treatment of AcF (Figure 1 D) dramaticly improved the pathophysiologic consequences of ALI compared with the saline treated control group (Figure 1 C) and not treated group (Figure 1 B). Taken together, these results implicate that the AcF could serve as a novel therapeutic drug in sepsis-induced ALI.

As we know, in the ALI, the process of lung damage is also the process of inflammation activation and neutrophils infiltration. So we measured the activity of MPO, the lung wet/dry ratio, the infiltration of inflammatory cells, the concentration of protein and inflammatory cytokines in the lung BALF. Compared with the saline treated control group, we found that the administration of AcF not only significantly decreased the lung wer/dry ratio (Figure 2), the activity of MPO in the lung tissue (Figure 3 A) and protein concentration (Figure 3 B), but also inhibited the infiltration of inflammatory cells (Figure 4 A) and neutrophils (Figure 4 B) into lung tissue. These results were consistent with the protective effect of AcF on the CLP-induced lung injury (Figure 1).

In addition, we also measured the level of inflammatory cytokines in the BALF of different treated ALI rats. As the data showed, the treatment of AcF observably induced the expression of inflammatory cytokines in lung BALF, such as TNF-a (Figure 5A), IL-6 (Figure 5B), IL-1 (Figure 5C) and MIP-2 (Figure 5D). These data suggested that AcF could really suppress the inflammatory reaction in the CLP-induced ALI rats.

To further evaluated the therapeutic effect of AcF on sepsis-induced lung injury, we determined the bacterial load in the peritoneal fluid, blood and lungs and the survival rate of the different treated CLP-induced septic rats. We found that, the treatment of AcF could reduce the bacterial load in the peritoneal fluid (Figure 6 A), blood (Figure 6 B) and lungs (Figure 6 C) compared with saline treated control group respectively. Moreover, the survival rate of the AcF treated ALI rats was significantly higher than that treated with saline control (Figure 7). These data was consistent with the results of inflammatory reaction in sepsis induced ALI rats.

Conclusions

Taken together, our study demonstrated that AcF have a protective effect on the CLP-induced lung injury and prolong the survival time of septic rats by inhibiting the activation of C5aR in the neutrophils. Thus, AcF can be developed as a novel treatment for severe sepsis-induced ALI.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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