Comparison of Pulmonary and Extrapulmonary Models of Sepsis-Associated Acute Lung Injury

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Summary

To compare different rat models of sepsis at different time points, based on pulmonary or extrapulmonary injury mechanisms, to identify a model which is more stable and reproducible to cause sepsis-associated acute lung injury (ALI). Adult male Sprague-Dawley rats were subjected to (1) cecal ligation and puncture (CLP) with single (CLP1 group) or two repeated through-and-through punctures (CLP2 group); (2) tail vein injection with lipopolysaccharide (LPS) of 10mg/kg (IV-LPS10 group) or 20mg/kg (IV-LPS20 group); (3) intratracheal instillation with LPS of 10mg/kg (IT-LPS10 group) or 20mg/kg (IT-LPS20 group). Each of the model groups had a sham group. 7-day survival rates of each group were observed (n=15 for each group). Moreover, three time points were set for additional experimental studying in each model group: 4 hours, 24 hours and 48 hours after modeling (every time point, n=8 for each group). Rats were sacrificed to collect BALF and lung tissue samples at different time points for detection of IL-6, TNF-α, total protein concentration in BALF and MPO activity, HMGB1 protein expression in lung tissues, as well as the histopathological changes of lung tissues. More than 50 % of the rats died within 7 days in each model group, except for the IT-LPS10 group. In contrast, the mortality rates in the two IV-LPS groups as well as the IT-LPS20 group were significantly higher than that in IT-LPS10 group. Rats received LPS by intratracheal instillation exhibited evident histopathological changes and inflammatory exudation in the lung, but there was no evidence of lung injury in CLP and IV-LPS groups. Rat model of intratracheal instillation with LPS proved to be a more stable and reproducible animal model to cause sepsis-associated ALI than the extrapulmonary models of sepsis.

Keywords

Sepsis • Acute lung injury • Cecal ligation puncture • Intratracheal instillation • Tail vein injection

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Introduction

Sepsis is defined as a life-threatening organ dysfunction due to the dysregulation of host response to infection [1]. The annual death rate of septic patients remains stubbornly high [2]. Sepsis leads to multiple organ dysfunction syndrome, including the early occurrence of acute lung injury (ALI) or acute respiratory distress syndrome (ARDS), which is considered to be one of the earliest and most severely damaged organs [3]. Previous studies have shown that sepsis-associated

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ALI/ARDS had a higher rate of mortality [4]. However, the pathogenesis underlying this disorder is not yet entirely clear. One of the principal reasons is that experimental animal models are not stable to replicate human sepsis-associated ALI [5]. So there is an urgent need to determine a stable and reproducible model for the basic research of sepsis-associated ALI.

Traditionally, three types of experimental rat models are commonly used for sepsis study: (1) intratracheal instillation with LPS, (2) cecal ligation and puncture (CLP), (3) tail vein injection with LPS.

CLP model is widely used to study the pathophysiology of sepsis. It may cause secondary lung injury induced by primary bacterial peritonitis [6]. However, due to the complexities of operational procedure and detail, CLP model still remains difficult to control the severity of lesion. As previously reported, many factors could influence the outcome after CLP, and the chief factors of all were the percentage of cecum that was ligated and the number and size of punctures [7]. Thus, in order to evaluate the stability of CLP-associated lung injury, we intended to establish two CLP models with different severity by changing the number of punctures on the premise of cecal ligation performed at the basis of the cecum immediately below the ileocecal valve.

LPS is a type of endotoxin which plays an important role in the progression of sepsis. Tail vein injection with LPS is one of the most frequently used methods to replicate human bacterial sepsis in animal models [8]. Besides, intratracheal instillation with LPS is also a scientific approach to construct sepsis animal models based on direct mechanism of acute lung injury [9]. Researches have shown that both models could lead to damage in the lungs [10]. The dose of LPS dramatically affects the severity and prognosis of septic rats [11]. Therefore, in this study, we established LPS-induced pulmonary or extrapulmonary sepsis models with different severity by giving two different doses so as to verify the stability of these two types of septic ALI models.

Sepsis-associated ALI may exhibit different pathological changes at different time points [12], while many researchers focuse on changes at a single time point. Accordingly, we design the experiment to compare the three types of models based on series of indicators including survival rate, appearance, histopathological characteristic and inflammatory parameters at three different time points. We hope to set up a stable and reproducible animal model for research of sepsis-associated ALI.

Materials and methods

Animals

Animals (Sprague-Dawley male rats, 180-220 g body weight, purchased from Experimental Animal Center of Guangdong Province) were housed under standard conditions (55 % relative humidity, 12-h light/dark cycle, standard chow and water ad libitum) for at least 7 days before modeling. All experiments were approved by the Ethics Committee of Experimental Animal of Guangdong Provincial Hospital of Chinese Medicine (agreement number: 2020009) and performed in accordance with the Guide for the Care and Use of Laboratory Animals. All research protocols were designed to use the minimum number of animals required for statistics and minimize animals' discomfort.

Chemicals and reagents

Lipopolysaccharides (LPS, from Escherichia coli 055: B5) was obtained from Sigma (St. Louis, MO, USA). TNF-α, IL-6 enzyme-linked immunosorbent assay (ELISA) kits were provided by Cusabio Biotech (Wuhan, China). Enhanced BCA protein assay kit was offered by Beyotime Biotechnology (Shanghai, China). Myeloperoxidase (MPO) assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Anti-HMGB1 antibody (Cat. Ab79823) was supplied by Abcam (USA). HRP Conjugated AffiniPure Goat Anti-rabbit IgG (H+L) (Cat. BA1054) was purchased from Boster Biological Technology co.ltd (Wuhan, China). 20G needles and 1ml Syringes were provided by Becton Dickinson (New Jersey, USA). Black-braided silk nonabsorbable surgical spool suture 2-0 USP (Roboz Surgical SUT, cat. no.1066-31).

Experimental design

Base on the different initiating factors, septic models were divided into three categories in our study: (1) intratracheal instillation with LPS, (2) cecal ligation and puncture, (3) tail vein injection with LPS. We also evaluated several parameters which might affect the severity of sepsis by altering the number of punctures (CLP-induced sepsis) or drug dosage (LPS-induced sepsis). On the basis of different treatments, the above three categories of models were subclassed as follows:

CLP1–Rats were subjected to CLP with single

through-and-through puncture.

CLP2 – Rats were subjected to CLP with two repeated through-and-through punctures.

IV-LPS10 – Rats received LPS (dose: 10mg/kg; final volume: 250μL) dissolved in sterile saline by tail vein injection.

IV-LPS20 – Rats received LPS (dose: 20mg/kg; final volume: 250μL) dissolved in sterile saline by tail vein injection.

IT-LPS10 – Rats received LPS (dose: 10mg/kg; final volume: 250μL) dissolved in sterile saline by intratracheal instillation.

IT-LPS20 – Rats received LPS (dose: 20mg/kg; final volume: 250μL) dissolved in sterile saline by intratracheal instillation.

Moreover, three time points were set for experimental studying in each model group: 4 hours, 24 hours and 48 hours after modeling (every time point, n = 8 for each group with different treatments). A group of sham operation animals was simultaneously included as a control in each model. Rats were sacrificed to collect samples at different time points for detection of different indicators.

Extrapulmonary model of sepsis

- *Cecal ligation and puncture*

Rats were fasting for 12 hours before surgery but free access to water. Then anesthetized the rats by injecting intraperitoneally with a solution of 3 % pentobarbital (30 mg/kg body weight). Under sterile conditions, laparotomy with a 2-3 cm longitudinal skin midline incision was practiced after the middle and lower abdomen was shaved using an electric razor and disinfected with alcohol cotton balls to gain entry into the peritoneal cavity (Fig. 1a,b). The cecum was then gently isolated and exteriorized using blunt anatomical forceps (Fig. 1c). The cecal ligation was performed at the basis of the cecum immediately below the ileocecal valve with 2-0 silk thread (Fig. 1d). Made sure not to ligate the ileocecal valve to avoid causing intestinal obstruction.

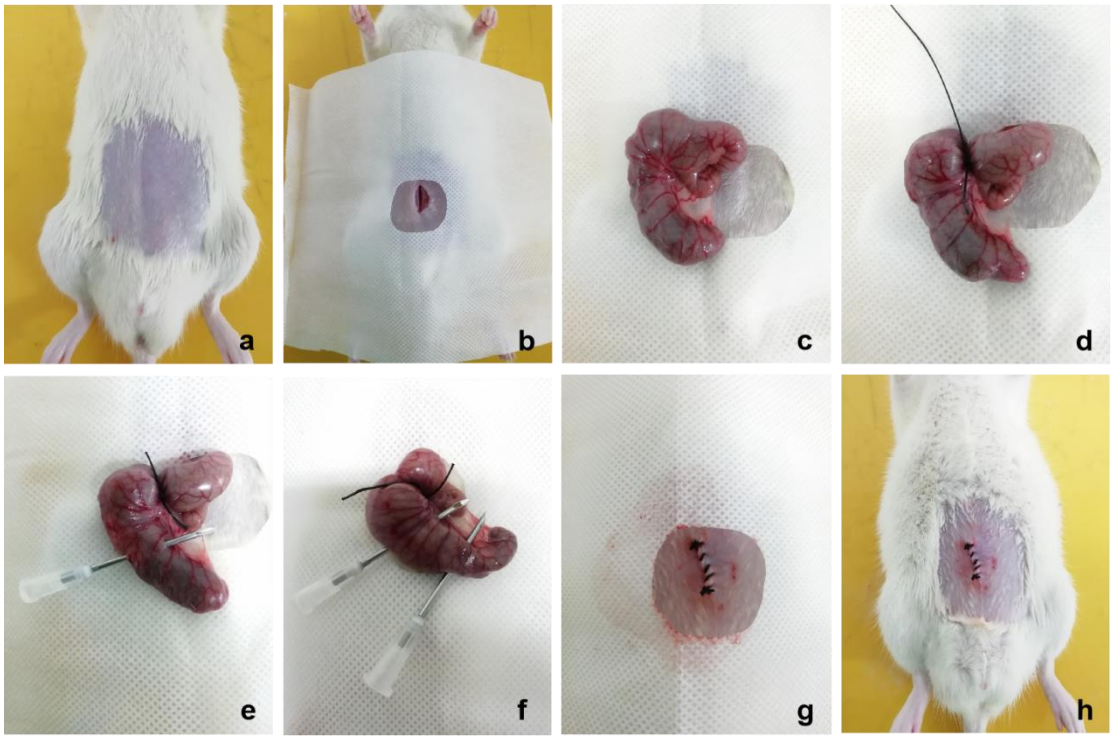

Fig. 1. Crucial steps of the CLP procedure in rats. (**a**) The middle and lower abdomen was shaved using an electric razor and disinfected with alcohol cotton balls. (**b**) Laparotomy with a 2-3 cm longitudinal skin midline incision was practiced to gain entry into the peritoneal cavity. (**c**) The cecum was gently isolated and exteriorized using blunt anatomical forceps. (**d**) The cecal ligation was performed at the basis of the cecum immediately below the ileocecal valve with 2-0 silk thread. (**e, f)** Using a 20-gauge needle, through-and-through puncture was performed once midway between the ligation and the tip of the cecum in CLP1 group, or twice respectively at 1/3 and 2/3 of the ligated cecum in CLP2 group. (**g, h**) The cecum was returned into the abdominal cavity followed by suture of the incision.

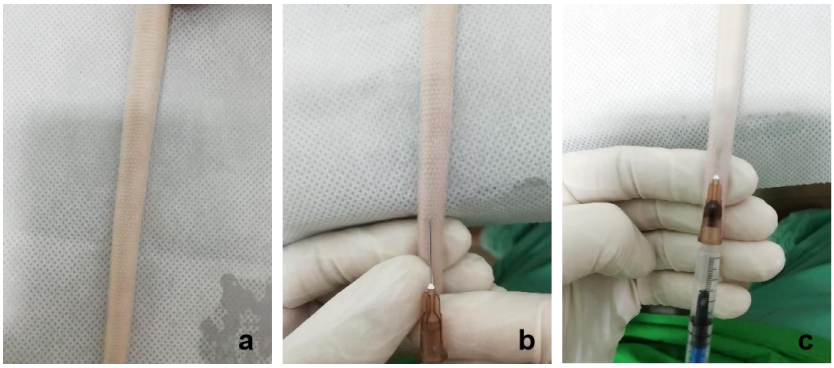

Fig. 2. Key steps of the modeling procedure of tail vein injection with LPS in rats. (**a**) Distal lateral tail vein was wiped repeatedly with alcohol cotton ball to dilate the vessel. (**b**) The needle point of a 1ml syringe was inserted into the distal tail vein. (c) The surefire intravascular position was evaluated by the backflow of venous blood and the subsequent unobstructed injection with 250μl of solutions.

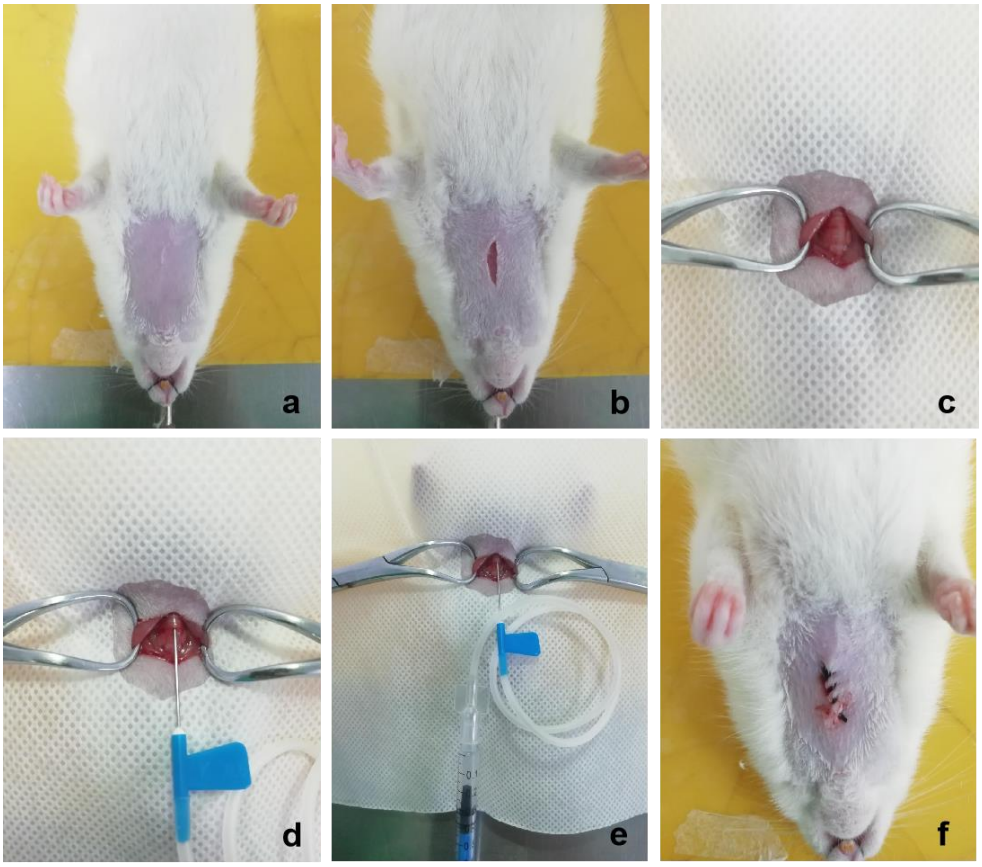

Fig. 3. Key steps of the modeling procedure of intratracheal instillation with LPS in rats. (**a, b**) A cervical ventral midline incision was made from the manubrium sterni to the level of thyroid cartilage. (**c**) Blunt dissection of cervical soft tissue and muscle was performed to expose the trachea. (**d**) A puncture was made between the second and third tracheal cartilaginous rings using a syringe needle. (**e**) 250μl of LPS solutions were instilled. (**f**) The cervical incision was sutured using 2-0 silk suture.

Using a 20-gauge needle, through and through puncture was performed once midway between the ligation and the tip of the cecum in CLP1 group, or twice respectively at 1/3 and 2/3 of the ligated cecum in CLP2 group (Fig. 1e,f). A small amount of excrement was extruded from the perforation sites after removing the needle. Finally, the cecum was returned into the abdominal cavity followed by suture of the incision (Fig. 1g, h). The sham group animals, as control for the experiment, were performed laparotomy with isolation of the cecum rather than ligation and puncture. Postoperative animals were resuscitated instantly by injecting pre-warmed normal saline (3 ml/100g body weight) subcutaneously.

Tail vein injection with LPS

Rats were anaesthetized as described above. Distal lateral tail vein was wiped repeatedly with alcohol cotton ball to dilate the vessel (Fig. 2a). The needle point

of a 1ml syringe was inserted into the distal tail vein (Fig. 2b). The surefire intravascular position was evaluated by the backflow of venous blood and the subsequent unobstructed injection with 250μl of solutions (Fig. 2c). If the puncture failed, insertion of the needle should be tried again in a more proximal position to ensure the administration of a full dose of drugs. The sham group animals were injected with an equal volume of sterilized saline instead of LPS solution.

Pulmonary model of sepsis

- *Intratracheal instillation with LPS*

A cervical ventral midline incision was made from the manubrium sterni to the level of thyroid cartilage (Fig. 3a and Fig. 3b). Blunt dissection of cervical soft tissue and muscle was performed to expose the trachea (Fig. 3c). Thereafter, a puncture was made between the second and third tracheal cartilaginous rings using a syringe needle (Diameter: 0.5mm). Air should be sucked by gently withdrawing the plunger of the syringe after puncture to ensure that the needle tip had entered into the trachea, and then 250μl of LPS solutions were instilled (Fig. 3d and Fig. 3e). Then about 500μl of air was injected following the solutions to facilitate drugs' arrival and even distribution into bilateral lungs. Note here that the injection speed of air should not be too fast to avoid pulmonary barotrauma. Most notably, the puncture wound was extremely small so it hardly affected animal's respiration. Finally, the cervical incision was sutured using 2-0 silk suture (Fig. 3f). In the sham groups, an equal volume of sterilized saline was used instead of LPS solution.

Sample collection

At the designated time points, animals were sacrificed by abdominal aorta bloodletting under 3 % pentobarbital (30 mg/kg body weight, intraperitoneal injection) anesthesia. As previously described [13], the chest was immediately opened to expose the lung, and the whole lung and trachea tissues were dissected out from the chest cavity and photographed. Subsequently, 1 ml of PBS (4 °C pre-cooling, contain 1mM EDTA) was infused into the left lung and recycled using a tracheal cannula after ligation of right hilus. The operation above was repeated 4 times to collect all broncho-alveolar lavage fluid (BALF). Then the BALF was centrifuged at 3000 rpm for 10 min at 4 °C to separate the supernatant for the followed-up experimental detection. The upper lobe of right lung was removed and fixed for Hematoxylin–Eosin (HE) staining. The middle lobe of the right lung tissues were quick-frozen in liquid nitrogen and stored at −80 °C for the subsequent analysis.

Appearance

Appearance of the whole lung tissues was observed by a researcher who was blinded to the experiments. The severity of edema, congestion, swelling, mucus-like secretions on the surface of lung tissues in model rats were observed qualitatively with reference to that in sham-operated rats. Thus the degree of acute lung injury were evaluated initially from appearance.

HE staining

The upper lobes of right lung tissues were fixed with 4 % paraformaldehyde, embedded by paraffin, and made into pathological sections sequentially for HE staining. Lung injury scores (LIS) were graded by a researcher who was blinded to the experiments. Ten random discrete fields of each lung tissue section were captured for pathological evaluation under a light microscope. We used a previously published scoring system (Table 1) to quantify the degree of injury [14]. As shown in Table 1, Each item was respectively graded on a scale from 0 to 2, and the final score was calculated according to the formula below the Table 1.

Table 1. Lung injury scoring system

Parameter	Score per field		
	0	**1**	**2**
A. Neutrophils in the alveolar space	None	1-5	>5
B. Neutrophils in the interstitial space	None	1-5	>5
C. Hyaline membranes	None	1	>1
D. Proteinaceous debris filling the airspaces	None	1	>1
E. Alveolar septal thickening	<2×	2×~ 4×	>4×

Score = [(20 × A) + (14 × B) + (7 × C) + (7 × D) + (2 × E)] / (number of fields × 100)

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IL-6 and TNF-α in BALF were measured by ELISA following the manufacturer's instructions. OD values were measured at 450nm using an ELISA microplate reader. The results were expressed as picogram per milliliter of BALF. Total protein concentration in BALF was also quantified using the Micro BCA protein assay kit.

Myeloperoxidase (MPO) assay

The lung tissue was homogenized and then centrifuged to obtain supernatant for subsequent experimental detection. The activity of MPO in the lung tissue was measured according to the instructions of the manufacturer. The absorbance was measured at 460 nm.

Western blotting

At the specified time points, about 50 mg of lung tissue sample in each group was collected and homogenated in lysis buffer (Shanghai Bocai Biological Technology Co.,Ltd, China). Then, the supernatant was collected after centrifugation (15,000 × g for 30 min at 4 °C and the protein concentration was detected using the BCA protein assay kit. Equal amount of total protein samples were separated by 10 % SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore Biotechnology Inc., USA). The membrane was then blocked in 5 % bovine serum albumin (BSA) for 2 h at room temperature and then incubated with rabbit anti-HMGB1 monoclonal antibody (1 : 1000) overnight at 4 °C. Subsequently, after 3 times of 15min washing with Tris-buffered saline containing 0.5 % Tween 20 (TBST) buffer, the blots were incubated with goat anti-rabbit IgG secondary antibody (1: 10000) at room temperature for 2 h. Enhanced chemiluminescence (ECL) reagents were added to visualize the protein blots. Finally, density analysis of western blot bands was done by using ImageJ software (National Institutes of Health, USA).

Survival studies

In additional, a separate experiment was designed for survival analysis. Same grouping as above, but n=15 for each group. The mortality of rats was observed over a period of 7 days after modeling.

Statistical analysis

The results of the quantitative data were expressed as means ± SD. The differences among experimental rat groups were accessed using one-way analysis of variance (ANOVA) followed by LSD post-hoc test for multiple comparisons. When data failed to obey normal distribution, Tamhane's T2 test was used for post hoc pairwise comparisons. A value of $P < 0.05$ was considered statistically significant. Cumulative survival rates among groups was analyzed by log-rank test and the survival curve with the Kaplan-Meier method. Statistical analysis was performed using SPSS (version 25) statistical software.

Results

Survival rates

The survival rates were carefully monitored every day for up to 7 days after surgery. As a result, all the sham rats survived, however, except for the IT-LPS10 group, more than 50 % of the rats died within 7 days in each model group, as Fig. 4 shows. In contrast, the mortality rates in the two IV-LPS groups as well as the IT-LPS20 group were significantly higher than that in IT-LPS10 group ($P < 0.05$). In order to be close to the clinical situation of high mortality in severe sepsis, the mortality rates of the animal models in this study were required to be 50 % ~ 70 % within a week. So the mortality of IT-LPS10 group obviously fall short of this requirement. Except for the IT-LPS10 group, there was no significant difference in the 7-day survival rate among the rest five groups.

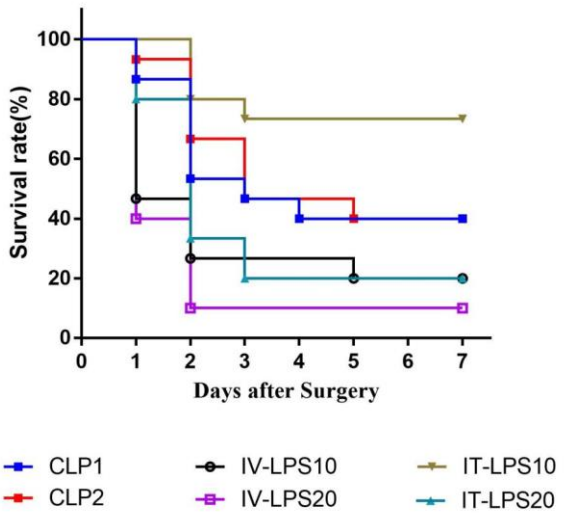

Fig. 4. Kaplan-Meier survival curves of all model groups (n=15 for each group). Within 7 days, the survival rate of rats was observed. Results were expressed as percent survival. The log-rank (Mantel–Cox) test was used to compare the survival rates: chi-square = 22.62, $P = 0.0004$.

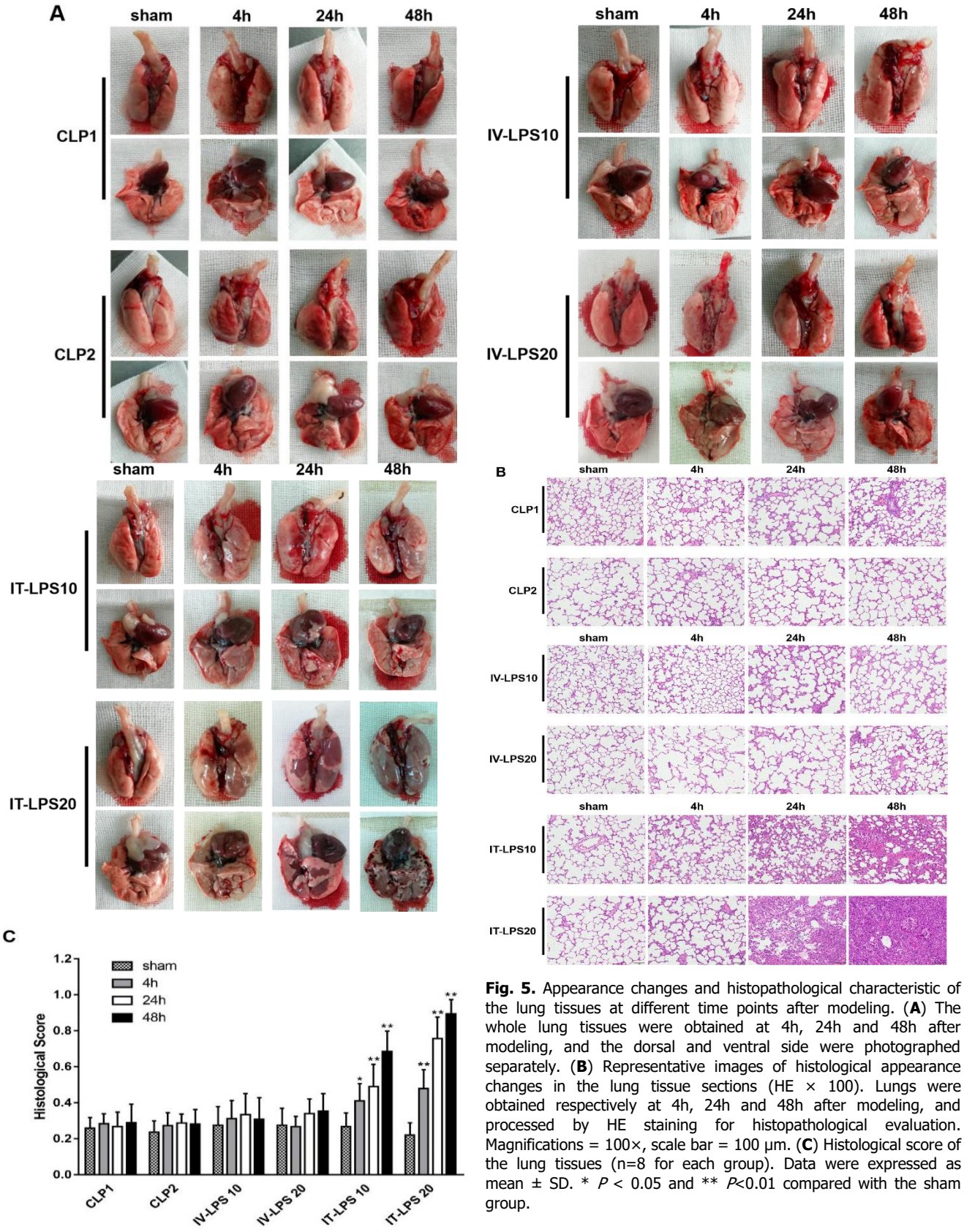

Fig. 5. Appearance changes and histopathological characteristic of the lung tissues at different time points after modeling. (**A**) The whole lung tissues were obtained at 4h, 24h and 48h after modeling, and the dorsal and ventral side were photographed separately. (**B**) Representative images of histological appearance changes in the lung tissue sections (HE × 100). Lungs were obtained respectively at 4h, 24h and 48h after modeling, and processed by HE staining for histopathological evaluation. Magnifications = 100×, scale bar = 100 μm. (**C**) Histological score of the lung tissues (n=8 for each group). Data were expressed as mean ± SD. * $P < 0.05$ and ** $P<0.01$ compared with the sham group.

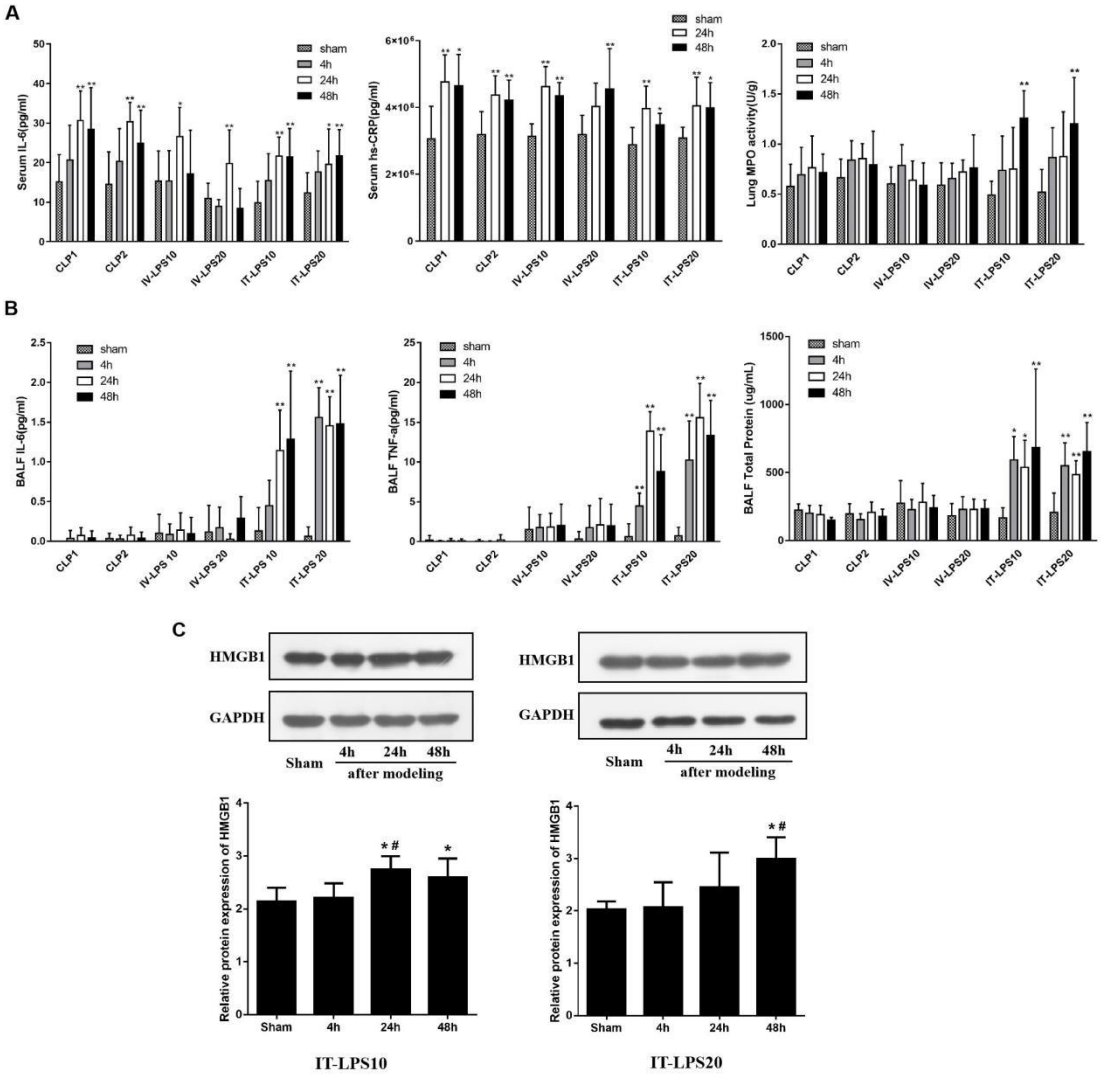

Fig. 6. The intensity of sepsis-associated pulmonary inflammation among three types of models. (**A**) The level of serum IL-6, hs-CRP and MPO activity in lung homogenates. (**B**) The level of IL-6, TNF-α and total protein concentration in BALF. Values are means ± SD (n=8 in each group). (**C**) HMGB1 protein expression in the lung tissues during the development of sepsis-associated ALI in IT-LPS groups. * $P < 0.05$, ** $P < 0.01$ compared with the sham group.

Appearance

The whole lung and heart tissues were obtained at different time points after modeling. As shown in Fig. 5A, compared with sham groups, only mild congestion and swelling could be observed on the surface of lung tissues after modeling in CLP and IV-LPS groups. However, the lung tissues of IT-LPS groups showed more congestive and inflamed, accompanied with massive mucus-like secretions. Moreover, infarct-like bruised areas were observed on the lung surface, which was medically known as red hepatization, especially at 24h and 48h in IT-LPS20 group.

Histopathological characteristic

The upper lobes of right lung tissues were fixed with 4 % paraformaldehyde for HE staining in order to observe the histopathological changes. Morphological study (Fig. 5B) clearly showed that in CLP and IV-LPS groups, the structure of alveoli remained generally complete, only mild congestion, edema and a small amount of inflammatory cells infiltration could be observed after modeling. Whereas in the IT-LPS groups, a large amount of leukocytes and macrophages infiltrated into the alveolar space. What's more, in the stage of red hepatization, normal alveolar structure was barely visible in the lung, to be replaced by abundant neutrophil and lymphocyte infiltrates and proteinaceous deposition. As shown in Fig. 5C, semiquantitative analysis by lung injury scoring achieved essentially consistent results.

Inflammatory parameters

Inflammatory cytokines in serum and BALF

were measured in this study. In all of the model groups, the levels of serum IL-6 and hs-CRP were significantly increased at 24h and 48h after modeling (Fig. 6A). Concentration of IL-6, TNF-α and total protein in BALF exhibited no significant change after modeling in CLP and IV-LPS groups. However, in IT-LPS groups, the values of biomarkers mentioned above significantly increased at 4h after modeling, compared with sham groups ($P < 0.01$). Then, the rise sustained and significant differences were observed at 24h and 48h post-operation ($P < 0.01$) (Fig. 6B). Additionally, we measured the level of MPO activity in lung homogenates and the results (Fig. 6A) showed that CLP and IV-LPS groups exhibited no significant differences at all the time points, while that in IT-LPS groups show a notable increase at 48h after modeling, but no significant changes were observed at 4h and 24h compared to sham groups ($P < 0.01$).

Expression of HMGB1 protein

In order to investigate the inflammatory mechanism of sepsis-associated acute lung injury, the middle lobes of right lungs were collected for the detection of high mobility group box 1 (HMGB1) using western blotting analysis. As shown in Fig. 6C, the expression of HMGB1 proteins was dramatically upregulated at 24h and 48h after stimulation with LPS in IT-LPS10 group ($P < 0.05$), while no significant change was observed at 4h compared to sham group. Also, in IT-LPS20 group, HMGB1 poteins expression was increased markedly at 48h after modeling, but similar discrepancies were not observed at other time points.

Discussion

A stable animal model can go a long way to helping us study the pathogenesis and therapeutics of clinical diseases. Sepsis is believed to be one of the complex clinical diseases and effected by many factors [15]. Despite several experimental animal models have been developed to mimic human sepsis-associated ALI, the stability and repeatability might be doubtful.

Sepsis is a systemic imflammatory response syndrome caused by host infection [1]. Based on the related literature researches [5,11], we summarized the following basic elements that septic animal models for research should possess: (1) Hypermetabolism state; (2) Accompanied with multiple organ function impairment; (3) High natural death rate reached 50 % ~ 70 %; (4) Organ dysfunction or death occurred over 6 ~ 12h after modeling. Because of the condition limitations, we selected the levels of IL-6 and hs-CRP in serum and 7-day mortality as indicators to judge a successful septic rat model. The result showed the levels of serum IL-6 and hs-CRP were significantly increased at 24h or/and 48h after modeling in each of the model group. In terms of 7-day mortality, except for the IT-LPS10 group, more than 50 % of the rats died within 7 days in each model group. Obviously, systemic inflammatory response and natural death rate in each group of the model rats satisfied the basic requirements of septic animal models, except for the IT-LPS10 group.

In this study, we applied a range of indicators such as survival rate, appearance, histopathological characteristic and inflammatory parameters to assess the level of lung damage among three types of sepsis animal model. Our research showed that septic rat models of intratracheal instillation with LPS reproduced the characteristics of sepsis-associated acute lung injury more stably and effectively than the septic models of CLP and tail vein injection with LPS.

CLP model has been considered as the gold standard in the field of sepsis research [5]. Sepsis of the CLP rats was caused by continuous abdominal infection. Serum inflammation cytokines in CLP rats could be detected early and reach a high peak [16]. However, it was less impressive to be associated with pulmonary inflammation [17]. Likewise, our studies showed that it couldn't lead to ALI stably, whether measured by histological changes or expression of inflammatory indicators. As mentioned above, the severity of CLP model mostly depended on the length of ligated cecum and the size and/or number of the puncture [7]. We established two CLP models with different severity by performing through-and-through puncture once or twice on the premise of keeping the cecal ligation performed at the basis of the cecum immediately below the ileocecal valve. Despite their 7-day mortality rate both reached 60 %, none of them could cause obvious lung damage. It seemed to be able to find the marks of lung damage from histological changes at 24h in the CLP groups, this lesion was not as obvious as IT-LPS groups, and the pathological score showed no statistical difference. Some research pointed out, this result might be attributed to a longer process of disease progression for the CLP models [18]. Thereby, CLP rats did not die from respiratory failure. Judging by the route of infection, they were more likely to succumb to intestinal failure. Maybe CLP-induced sepsis led to intestinal failure and deaths before

causing lung injury. Related studies also supported our view on the side [18].

Extrapulmonary sepsis model of systemic administration with LPS by tail vein injection was conducted to mimic human's bloodstream infection. LPS combined with TLR4 and activated subsequent pro-inflammatory factors, such as NF-κB, IL-6, HMGB1 and so on. Leukocyte count decreased firstly and then increased, accompanied by a significant increase of neutrophil count and decreases of lymphocyte and monocytes counts [19]. The disease severity depended critically on the dosage of LPS, so we used two different dosage of LPS to establish sepsis models. Surprisingly, the results revealed that even if the 7-day mortality rates of these two models achieved 80 %-90 %, both of them were unable to cause significant damage in the lung. Previous studies actually also mentioned similar results [20], and our research further confirmed this. Obviously, the model rats did not die of respiratory failure. Considering the primary route of infection, we surmised that the death might be caused by acute circulatory failure, or shock. Maybe LPS-induced sepsis in this model led to severe circulatory failure and deaths before causing lung injury.

In terms of intratracheal administration of LPS, we applied the method of intratracheal instillation on the premise of exposing the trachea surgically, which was distinguished from traditional method of orotracheal instillation. The great advantage of this approach was that it could ensure all the drugs infuse into trachea, while there were difficulties with judging whether the trachea tube was inserted into the airway during orotracheal instillation. Besides, the puncture wound was extremely small so it hardly affected animal's respiration. Certainly, rats possess abundant vascularity in the cervical part, so we should avoid injury of critical nerves and blood vessels during the surgical procedure.

It was reported that pulmonary microvascular injury which was manifested as pulmonary congestion and interstitial edema, neutrophils infiltration and serious lung inflammatory reaction occurred after exposure to LPS [21], especially by the method of intratracheal instillation. Consistent with these studies, our septic rats who received LPS by intratracheal instillation exhibited evident histopathological changes and increased inflammatory biomarkers expression in the lung. Obviously, this method to create a sepsis-associated ALI model was easy to operate and the repeatability was satisfactory.

But the remarkable thing was that the 7-day mortality rate of IT-LPS10 group rats was only 26.67 %, obviously falling short of the requirement of standard septic animal model (50 % ~ 70 %). To sum up, it was our opinion that the septic rat model directly received LPS (dose: 20 mg/kg) by intratracheal instillation was the most stable and reproducible model to cause sepsis-associated ALI.

Due to the nature of heterogeneous changes of lungs in ALI rats, various parameters detected respectively in different parts of lung tissues might not necessarily follow the changes of illness. For example, MPO activity of the right middle lobe might not coincide with pathological damage of the right upper lobe in some individual animals. Even so, this inconsistency had no noticeable effect on the overall tendency.

HMGB1 is an inflammatory mediator involved in sepsis, cancer, autoimmune diseases and so on [22, 23, 24]. It is ubiquitous in the lung, heart, brain and so on. The levels of HMGB1 expression may be associated with the severity of inflammation and tissue damage [25]. Previous studies have demonstrated that HMGB1 might play a potential role as a prognostic biomarker in ALI [26]. So we detected HMGB1 expression in the lung of IT-LPS rats to reveal its significance in assessing the severity of lung damage. Experiment results showed that the expression of HMGB1 proteins was upregulated at 48h after intratracheal instillation with LPS, even at 24h significant increase could be observed in IT-LPS10 group. This results were consistent with the conclusion of above-mentioned indicators.

We also dissected the right lower lobe to measure lung wet/dry weight (W/D) ratio, but there were no statistically significant differences in this indicator. However, took a close look at the data, it seemed to have some similar inclinations synchronous with our findings. Maybe one lung lobe was so light that the base of data was too small to reach statistical significance. Considering the cost and benefit, we had not rerun the experiment to collect more lung tissues for tests and analysis.

It should be noted that compared with own control group, there were no obvious difference in most of the indicators at 4h after modeling. Further referred to some past studies, maybe the time point of 4h after modeling was a little early [27,28]. The damage of vital organs was not apparent and the inflammatory reaction was slight. If time and funds permit, we can set up more time points to conduct researches.

It was a pity that pulmonary function test and arterial blood gas analysis had not been performed due to the restricted experimental condition and complex operation process. However, the model in our study should have satisfied the requirements to reproduce humans sepsis-associated ARDS as recommended by previous study [14].

Limitation

Our results suggested that rat model of intratracheal instillation with LPS proved to be a more stable and reproducible animal model to cause sepsis-associated ALI than the extrapulmonary models of sepsis. However, it was a pity that pulmonary function test and arterial blood gas analysis had not been performed due to the restricted experimental condition and complex operation process.

Conclusion

Based on comprehensive evaluation with multiple parameters, our research suggested that septic rat model of intratracheal instillation with LPS (dose: 20 mg/kg) proved to be a more stable and reproducible animal model to cause sepsis-associated ALI than the models of CLP and tail vein injection with LPS.

Conflict of Interest

There is no conflict of interest.

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References

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, et al. The third international consensus definitions for sepsis and septic shock (sepsis-3). JAMA. 2016;315:801-810. https://doi.org/10.1001/jama.2016.0287
2. Rudd KE, Johnson SC, Agesa KM, Shackelford KA, Tsoi D, Kievlan DR, Colombara DV, et al. Global, regional, and national sepsis incidence and mortality, 1990-2017: analysis for the Global Burden of Disease Study. The Lancet. 2020;395:200-211. https://doi.org/10.1016/S0140-6736(19)32989-7
3. Englert JA, Bobba C, Baron RM. Integrating molecular pathogenesis and clinical translation in sepsis-induced acute respiratory distress syndrome. JCI Insight. 2019;4:e124061. https://doi.org/10.1172/jci.insight.124061
4. Stapleton RD, Wang BM, Hudson LD, Rubenfeld GD, Caldwell ES, Steinberg KP. Causes and timing of death in patients with ARDS. Chest. 2005;128:525-32. https://doi.org/10.1378/chest.128.2.525
5. Korneev KV. Mouse models of sepsis and septic shock. Mol Biol (Mosk). 2019;53:799-814. https://doi.org/10.1134/S0026893319050108
6. Zhao H, Chen H, Xiaoyin M, Yang G, Hu Y, Xie K, Yu Y, et al. Autophagy activation improves lung injury and inflammation in sepsis. Inflammation. 2019;42:426-439. https://doi.org/10.1007/s10753-018-00952-5
7. Dejager L, Pinheiro I, Dejonckheere E, Libert C. Cecal ligation and puncture: the gold standard model for polymicrobial sepsis? Trends Microbiol. 2011;19:198-208. https://doi.org/10.1016/j.tim.2011.01.001
8. Meng PZ, Liu J, Hu PS, Tong F. Protective Effect of Dexmedetomidine on Endotoxin-Induced Acute Lung Injury in Rats. Med Sci Monit. 2018;24:4869-4875. https://doi.org/10.12659/MSM.908887
9. Ehrentraut H, Weisheit CK, Frede S, Hilbert T. Inducing acute lung injury in mice by direct intratracheal lipopolysaccharide instillation. J Vis Exp. 2019;149:10.3791/59999. https://doi.org/10.3791/59999-v
10. Chimenti L, Morales-Quinteros L, Puig F, Camprubi-Rimblas M, Guillamat-Prats R, Gómez MN, Tijero J, et al. Comparison of direct and indirect models of early induced acute lung injury. Intensive Care Med Exp. 2020;8(Suppl 1): 62. https://doi.org/10.1186/s40635-020-00350-y
11. Fink MP. Animal models of sepsis. Virulence. 2014;5:143-153. https://doi.org/10.4161/viru.26083

12. Tucker MH, Yeh HW, Oh D, Shaw N, Kumar N, Sampath V. Preterm sepsis is associated with acute lung injury as measured by pulmonary severity score. Pediatr Res. 2022;10.1038/s41390-022-02218-1. https://doi.org/10.1038/s41390-022-02218-1
13. Baradaran Rahimi V, Rakhshandeh H, Raucci F, Buono B, Shirazinia R, Samzadeh Kermani A, Maione F, et al. Anti-Inflammatory and Anti-Oxidant Activity of Portulaca oleracea Extract on LPS-Induced Rat Lung Injury. Molecules. 2019;24:139. https://doi.org/10.3390/molecules24010139
14. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, Kuebler WM, et al. An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. Am J Respir Cell Mol Biol. 2011;44:725-738. https://doi.org/10.1165/rcmb.2009-0210ST
15. Nedeva C. Inflammation and Cell Death of the Innate and Adaptive Immune System during Sepsis. Biomolecules. 2021;11:1011. https://doi.org/10.3390/biom11071011
16. Chen H, Huang N, Tian H, et al. Splenectomy provides protective effects against CLP-induced sepsis by reducing TRegs and PD-1/PD-L1 expression. Int J Biochem Cell Biol. 2021;136:105970. https://doi.org/10.1016/j.biocel.2021.105970
17. Matute-Bello G, Frevert C W, Martin T R. Animal models of acute lung injury. AJP Lung Cellular and Molecular Physiology. 2008;295:L379-L399. https://doi.org/10.1152/ajplung.00010.2008
18. Puig F, Herrero R, Guillamat-Prats R, Gómez MN, Tijero J, Chimenti L, Stelmakh O, et al. A new experimental model of acid- and endotoxin-induced acute lung injury in rats. Am J Physiol Lung Cell Mol Physiol. 2016;311:L229-L237. https://doi.org/10.1152/ajplung.00390.2015
19. Rossol M, Heine H, Meusch U, et al. LPS-induced cytokine production in human monocytes and macrophages. Crit Rev Immunol. 2011;31(5):379-446. https://doi.org/10.1615/CritRevImmunol.v31.i5.20
20. Szarka RJ, Wang N, Gordon L, Nation PN, Smith RH. A murine model of pulmonary damage induced by lipopolysaccharide via intranasal instillation. J Immunol Methods. 1997;202:49-57. https://doi.org/10.1016/S0022-1759(96)00236-0
21. Mirzapoiazova T, Kolosova IA, Moreno L, Sammani S, Garcia JG, Verin AD. Suppression of endotoxin-induced inflammation by taxol. Eur Respir J. 2007;30:429-435. https://doi.org/10.1183/09031936.00154206
22. Chen J, Li G. MiR-1284 enhances sensitivity of cervical cancer cells to cisplatin via downregulating HMGB1. Biomed Pharmacother. 2018;107:997-1003. https://doi.org/10.1016/j.biopha.2018.08.059
23. De Leo F, Quilici G, Tirone M, De Marchis F, Mannella V, Zucchelli C, Preti A, et al. Diflunisal targets the HMGB1/CXCL12 heterocomplex and blocks immune cell recruitment. EMBO Rep. 2019;20:e47788. https://doi.org/10.15252/embr.201947788
24. Deng C, Zhao L, Yang Z, Shang JJ, Wang CY, Shen MZ, Jiang S, et al. Targeting HMGB1 for the treatment of sepsis and sepsis-induced organ injury. Acta Pharmacol Sin. 2022;43:520-528. https://doi.org/10.1038/s41401-021-00676-7
25. Lee S, Piao C, Kim G, Kim JY, Choi E, Lee M. Production and application of HMGB1 derived recombinant RAGE-antagonist peptide for anti-inflammatory therapy in acute lung injury. Eur J Pharm Sci. 2018;114:275-284. https://doi.org/10.1016/j.ejps.2017.12.019
26. Suda K, Takeuchi H, Ishizaka A, Kitagawa Y. High-mobility-group box chromosomal protein 1 as a new target for modulating stress response. Surg Today. 2010, 40: 592-601. https://doi.org/10.1007/s00595-009-4232-1
27. Qu L, Chen C, Chen Y, Li Y, Tang F, Huang H, He W, et al. High-Mobility Group Box 1 (HMGB1) and Autophagy in Acute Lung Injury (ALI): A Review. Med Sci Monit. 2019;25:1828-1837. https://doi.org/10.12659/MSM.912867
28. Zhao J, Yu H, Liu Y, Gibson SA, Yan Z, Xu X, Gaggar A, et al. Protective effect of suppressing STAT3 activity in LPS-induced acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2016;311:L868-L880. https://doi.org/10.1152/ajplung.00281.2016