

Ketamine alleviates HMGB1-induced acute lung injury through TLR4 signaling pathway

Dong Xu^{1,B,C}, Xang Sun^{2,A,B}, Yang Zhang^{3,E,F}, Li Cao^{1,C,D}

¹ Department of Anesthesiology, Jinan Central Hospital Affiliated to Shandong University, China

² Department of Intensive Care Unit, Jinan Central Hospital Affiliated to Shandong University, China

³ Department of Anesthesiology, Binzhou People's Hospital, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;

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Address for correspondence

Lei Chao

E-mail: qiechengjing58@163.com

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Abstract

Background. Acute lung injury (ALI) is a common critical respiratory disease that seriously threatens human health. Ketamine has good anti-inflammatory and immune-regulating properties that can delay the lung injury process.

Objectives. High mobility group box protein 1 (HMGB1) plays an important role in the occurrence, development and treatment of ALI. Toll-like receptor 4 (TLR4) is the receptor for HMGB1. The aim of this study was to determine the role of the HMGB1 TLR4 signaling pathway in the treatment of ALI using ketamine.

Material and methods. A total of 30 healthy, male, 8-week-old Sprague-Dawley rats were randomly, equally divided into a control group, an lipopolysaccharide (LPS) group and a ketamine group. In order to establish a rat ALI model, 15 mg/kg of LPS was injected into the femoral veins. Ketamine was intravenously injected (10 mg/kg) into the experimental group rats. The rats were euthanized 24 h after modeling and lung tissue samples were collected. Western blot was used to test TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 protein expression in the lung tissue. Real-time polymerase chain reaction (RT-PCR) was performed to detect TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 mRNA levels.

Results. Compared with the controls, the LPS group had significantly higher TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 mRNA and protein levels ($p < 0.05$). These levels were significantly lower after ketamine intervention in comparison with the LPS group ($p < 0.05$). A positive correlation was found between TLR4 and HMGB1 expression in the LPS and ketamine groups ($r = 0.952$, $p < 0.001$; $r = 0.941$, $p < 0.001$).

Conclusions. Ketamine attenuates HMGB1-induced ALI, possibly by regulating the TLR4 signaling pathway.

Key words: ketamine, acute lung injury, TLR4, HMGB1

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Introduction

Acute lung injury (ALI) is a type of pulmonary inflammatory reaction which is caused by severe trauma, shock, severe infections, acidosis, and injuries of capillary endothelial cells and alveolar epithelial cells triggered by inflammatory cell cascade.¹ It may lead to diffuse pulmonary interstitial and alveolar edema, resulting in hypoxia and causing tissue necrosis and dysfunction. Acute lung injury may advance to acute respiratory distress syndrome. It is a common critical disease featuring rapid onset, quick development, poor prognosis, and high mortality.² In recent years, although ALI treatment has achieved certain progress, the mortality of ALI is still higher than 40%.³

Ketamine is widely used in clinical intravenous anesthesia. It has anti-inflammatory and immune-regulating functions, and presents a great inhibitory effect which prevents the production of a variety of inflammatory cytokines, the function of neutrophils and the expression of adhesion molecule.^{4–6} Studies have shown that small doses of ketamine can alleviate the symptom of pulmonary edema,⁷ indicating that ketamine has a therapeutic effect on ALI.^{6,8} In addition, a previous study demonstrated that ketamine can improve the blood gas and pulmonary function index of patients with ALI caused by mechanical ventilation.⁹ However, its mechanism has not been fully elucidated. Therefore, this study investigated the mechanism of ketamine in treating ALI in a rat model.

High mobility group box protein 1 (HMGB1) is widely distributed in the nucleus and cytoplasm of all types of cells. The HMGB1 in immune cells translocates from the nucleus to the cytoplasm and is secreted extracellularly under stimulus.¹⁰ Wang et al. first found in 1999 that HMGB1 was an important inflammatory mediator in sepsis.¹¹ It was reported that HMGB1 was also involved in ALI as an important inflammatory factor.^{12,13} Timely blocking the secretion of inflammatory factors is the key to ALI treatment.^{14,15} Thus, inhibiting HMGB1 secretion can delay the lung injury process. Toll-like receptor 4 (TLR4) also plays a critical role in the inflammatory response. As the receptor of HMGB1, the TLR4 signaling pathway plays pro-inflammatory roles.^{16,17} It was revealed that knockdown of the TLR4 gene can alleviate HMGB1-induced inflammatory response.¹⁸ Therefore, this study tested whether molecules related to the TLR4 signaling pathway and HMGB1 expression are involved in the effects of ketamine on treating ALI, aiming to provide a theoretical basis for using ketamine in the clinical treatment of ALI.

Material and methods

Main instruments and reagents

Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, USA). Ketamine hydrochloride was obtained from Fujian Gutian Pharmaceutical Co., Ltd.

(Ningde, China). A rat HMGB1 ELISA kit was sourced from Shanghai Yaji Biological Technology Co., Ltd. (Shanghai, China), while a rat TLR4 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Shanghai Ricky Biological Technology Co., Ltd. (Shanghai, China). A total protein extraction kit came from BestBio Technologies (Shanghai, China). A Coomassie Brilliant Blue protein detection kit was purchased from MajorBio Technology, Ltd. (Shanghai, China). The SDS-PAGE system, phosphate-buffered saline-Tween (PBST) solution, electrophoresis apparatus, and a GIS-2020D gel image analysis system were obtained from Sigma-Aldrich. HMGB1, TLR4 and GAPDH antibodies came from Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-tagged goat anti-rabbit IgG obtained from Dycen Biotech (Shanghai, China).

Animal modeling

A total of 30 healthy, male, 8-week-old Sprague Dawley rats (180–220 g) were purchased from the Chinese Academy of Medical Sciences Animal Experiment Center (Beijing, China), and randomly, equally divided into a control group ($n = 10$) an LPS group ($n = 10$), and a ketamine group ($n = 10$). Following the study by Gokcinar et al., 15 mg/kg of LPS was intravenously injected to establish an ALI model.¹⁹ Ketamine was intravenously injected (10 mg/kg) into the ketamine intervention group. An equal amount of normal saline was administered to the controls. The rats were euthanized using CO₂ 24 h after modeling, and lung tissue samples were collected and stored at -80°C .

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of China Meitan General Hospital (Beijing, China).

PaO₂ to FiO₂ ratio

The arterial blood gases were analyzed using a Bayer Rapidlab 348 Analyzer (Bayer Diagnostics, Leverkusen, Germany) to calculate the ratio of partial pressure of arterial oxygen (PaO₂) to the percentage of inspired oxygen (FiO₂).

Real-time PCR

Tissue RNA was extracted using TRIzol reagent, and the integrity of the RNA was identified using 1% agarose gel electrophoresis. A total of 1 μg of RNA was reverse-transcribed to cDNA with a kit (Takara, Kyoto, Japan). The real-time polymerase chain reaction (RT-PCR) reaction system contained 5 μL of 2 \times SYBR Green Mixture, 0.5 μL of cDNA, 0.5 μL of Primer (10 μM), and 4 μL of ddH₂O. The reaction conditions consisted of 10 min at 95°C , followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The RT-PCR reaction was performed on a ViiATM 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA). Each sample was set in 3 parallel trials. β -actin was selected as the internal reference.

Western blot

Total protein was extracted from the lung tissue and separated using SDS-PAGE. Next, it was transferred to a nitrocellulose (NC) membrane and blocked in 5% skim milk at room temperature for 2 h. After being washed in PBST 3 times, the membrane was incubated with the primary antibody at 4°C overnight. Then, the membrane was incubated with the secondary antibody in 2.5% skim milk for 60 min. After the chemiluminescence reagent was added, the membrane was developed and analyzed with the GIS-2020D gel image system (Ningbo Sija Lab Equipment Co., Ltd, Ningbo, China). The *GAPDH* gene was selected as the internal reference.

Statistical analysis

The SPSS v. 19.0 software (IBM Corp., Armonk, USA) was used for the data analysis. Data are presented as means \pm standard deviation (SD). Comparison of the mean values was performed using Student's t-test. Correlation analysis was applied using Pearson analysis. A p-value <0.05 was considered statistically significant.

Results

PaO₂:FiO₂ ratio

To confirm the success of the ALI model, we performed a gas test to measure the PaO₂:FiO₂ ratio and

found a significantly lower PaO₂:FiO₂ ratio in the LPS group (156.1 ± 21.3) than in the control group (475.6 ± 39.5) ($p < 0.05$), indicating the successful establishment of an ALI model, which was consistent with the pathological changes of lung as demonstrated by H&E staining (Fig. 1).

TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 mRNA expression in rat lung tissue

Real-time PCR was performed to test TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 mRNA expression in the study groups (Table 1). Compared with the control group, the LPS group showed significantly higher TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 mRNA expression ($p < 0.05$). These levels were notably lower following ketamine intervention in comparison with the LPS group ($p < 0.05$), but were still higher than those in the control group ($p < 0.05$).

TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 protein levels in rat lung tissue

Western blot analysis was performed to detect TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 protein levels, and the findings demonstrate a similar tendency as with mRNA (Table 2 and Fig. 2). The LPS group showed markedly up-regulated TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 protein levels compared with the controls ($p < 0.05$). Ketamine intervention significantly reduced the protein levels, but they were still higher than those in the controls ($p < 0.05$).

Table 1. mRNA expression levels of TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 in rat lung tissue

Group	n	TLR4	MyD88	TRAF-6	LOX-1	HMGB1
Control	1	1.00 ± 0.21	1.00 ± 0.22	1.00 ± 0.18	1.00 ± 0.19	1.00 ± 0.20
LPS	10	3.89 ± 0.31^a	2.96 ± 0.28^a	2.45 ± 0.42^a	1.87 ± 0.25^a	3.24 ± 0.34^a
Ketamine + LPS	10	1.58 ± 0.27^{ab}	2.02 ± 0.25^{ab}	1.85 ± 0.32^{ab}	1.37 ± 0.36^{ab}	1.64 ± 0.23^{ab}

^a $p < 0.05$, compared with the controls; ^b $p < 0.05$, compared with the LPS group; LPS – lipopolysaccharide; n – number.

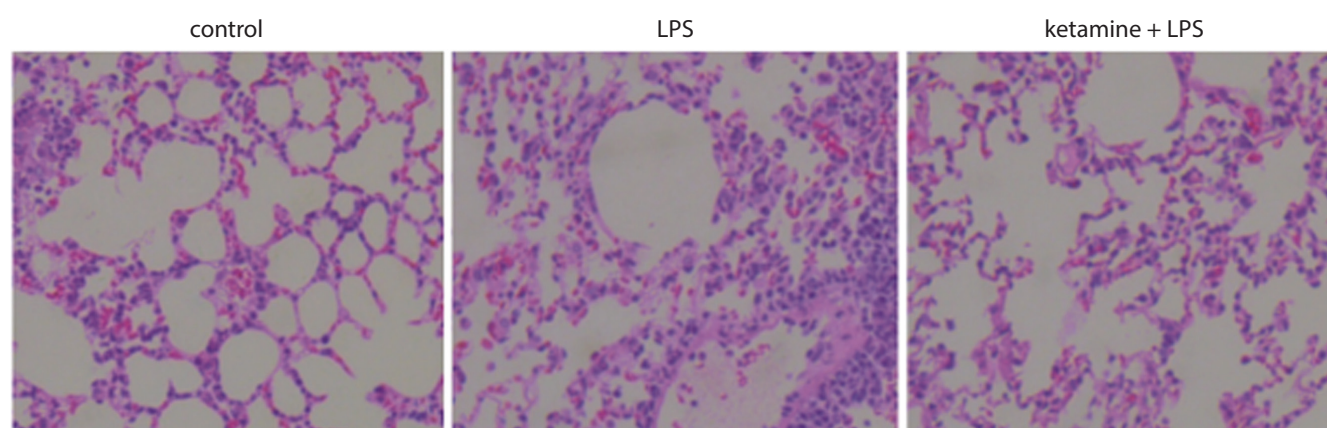


Fig. 1. Histological changes of acute lung injury. In the control group, the pulmonary alveolar, interstitial and bronchial vessels were normal. However, the lung tissues in rats treated with LPS were damaged, with obvious pulmonary edema, positive cell infiltration and visible focal necrosis. Rats receiving ketamine displayed a thick alveolar wall, alleviated hyperemia, reduced exudation in the pulmonary alveolar cavity, and relieved infiltration of inflammatory cells

Table 2. Protein levels of TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 in rat lung tissue

Group	n	TLR4	MyD88	TRAF-6	LOX-1	HMGB1
Control	10	0.81 ± 0.14	0.58 ± 0.12	0.71 ± 0.14	0.51 ± 0.09	0.78 ± 0.15
LPS	10	1.20 ± 0.10 ^a	0.72 ± 0.11 ^a	0.97 ± 0.13 ^a	1.00 ± 0.11 ^a	1.38 ± 0.13 ^a
Ketamine + LPS	10	0.95 ± 0.12 ^{a,b}	0.62 ± 0.10 ^{a,b}	0.81 ± 0.10 ^{a,b}	0.72 ± 0.12 ^{a,b}	1.04 ± 0.13 ^{a,b}

^ap < 0.05, compared with the controls; ^bp < 0.05, compared with the LPS group; LPS – lipopolysaccharide; n – number.

TLR4 and HMGB1 protein level correlation analysis in rat lung tissue

Pearson analysis was performed to test TLR4 and HMGB1 protein correlation in the rat lung tissue (Fig. 3). The results show that there was a positive correlation between TLR4 and HMGB1 expression in the LPS and ketamine groups ($r = 0.952$, $p < 0.001$; $r = 0.941$, $p < 0.001$).

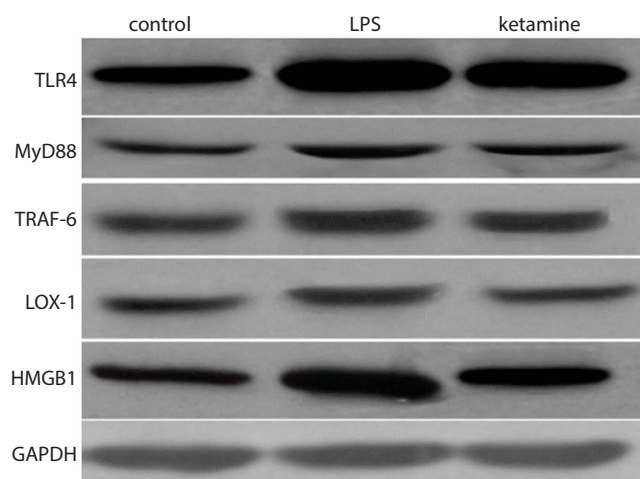


Fig. 2. Western blot analysis of protein levels in lung tissue among the 3 groups. The LPS group showed markedly upregulated protein levels of TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 compared with the controls; ketamine intervention obviously reduced these levels, but they were still higher than in the controls

Discussion

Ketamine, a derivative of phencyclidine, is a noncompetitive N-methyl-D-aspartic acid (NMDA) receptor antagonist and is usually used in anesthesia. Ketamine

can suppress the release of inflammatory factors, such as tumor necrosis factor α (TNF- α), interleukin (IL)-6 and IL-8.²⁰ It was found that ketamine also can inhibit the TLR4 signaling pathway.^{21,22} It was reported that ketamine can delay the progress of ALI. In addition, HMGB1 expression is associated with the occurrence of ALI, and inhibiting HMGB1 expression can ameliorate ALI. TLR4 is the receptor for HMGB1, so suppressing the TLR/NF- κ B signaling pathway can restrain ALI inflammation.²³ Therefore, we have reasons to believe that ketamine may attenuate HMGB1-induced ALI by regulating the TLR4 signaling pathway.

This study used LPS to establish a rat ALI model and tested molecules related to HMGB1 and the TLR4 signaling pathway in lung tissue. The results showed that the TLR4, MyD88, TRAF-6, LOX-1, and HMGB1 mRNA and protein levels were significantly higher in the LPS group than in the control group. This suggests that both HMGB1 and the TLR4 signaling pathway are involved in ALI. Wang et al. demonstrated that HMGB1 was clearly upregulated in ALI, further proving that HMGB1 plays an important role in this disease.²⁴ Abdelmageed et al. reported that the TLR4 signaling pathway was involved in the occurrence of ALI, further confirming the critical role of the TLR4 signaling pathway in ALI.²⁵

As TLR4 siRNA can alleviate HMGB1-induced inflammation,¹⁷ it appears that ketamine may attenuate HMGB1-induced ALI by regulating the TLR4 signaling pathway. This also demonstrates that clinical drug therapy can inhibit inflammation and delay the ALI process by regulating the TLR4 signaling pathway. Similarly, Qin et al. also discovered that ketamine can downregulate HMGB1 and TLR4 protein levels in bronchoalveolar lavage fluid and lung tissue.²⁶ In the present study, we found that ketamine intervention significantly decreased TLR4,

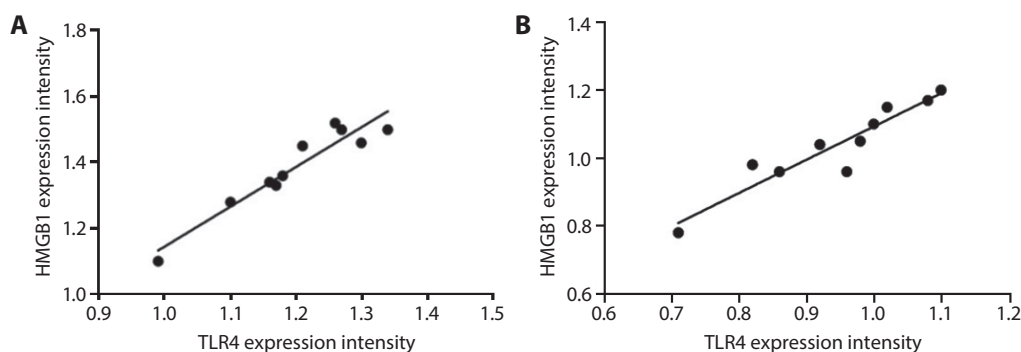



Fig. 3. Correlative analysis of TLR4 and HMGB1 level in rat lung tissue. There was a positive correlation between TLR4 and HMGB1 expression in the LPS (A) and ketamine + LPS (B) groups ($r = 0.952$, $p < 0.001$; $r = 0.941$, $p < 0.001$)


MyD88, TRAF-6, and LOX-1 mRNA and protein expression in comparison with the LPS group. In addition, TLR4 and HMGB1 expression displayed a positive correlation. Our study suggests that ketamine ameliorates ALI, possibly by regulating the TLR4/HMGB1 signaling pathway, thus exerting anti-inflammatory effects. However, future studies are required to confirm this finding.


The mechanism of ALI is complicated, while ketamine may also have an intricate network in treating ALI. This study only found that ketamine may attenuate HMGB1-induced ALI by regulating the TLR4 signaling pathway. Further investigation is needed in order to clarify its specific mechanism.

ORCID iDs

Lei Chao  <https://orcid.org/0000-0001-8247-897X>

Dong Xu  <https://orcid.org/0000-0002-5199-1110>

Xang Sun  <https://orcid.org/0000-0002-3338-5432>

Yang Zhang  <https://orcid.org/0000-0002-4141-6630>

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