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# Ketamine alleviates LPS induced lung injury by inhibiting HMGB1-RAGE level

Y. ZHANG[1], M. ZHANG[1], C.-Y. WANG[2], A. SHEN[3]

[1]Department of ICU, Affiliated Hospital of Weifang Medical College, Weifang, China
[2]Department of Medical Equipment, Weifang People's Hospital Affiliated to Weifang Medical College, Weifang, China
[3]Department of Hepatobiliary Surgery, Affiliated Hospital of Weifang Medical College, Weifang, China

**Abstract. – OBJECTIVE: Inflammatory cytokines secretion is an important reason to promote lung tissue inflammation in acute lung injury (ALI). High mobility group box 1 (HMGB-1) and its receptor for advanced glycation end products (RAGEs) play a role in ALI. Ketamine can significantly alleviate ALI, whereas its specific mechanism has not been fully elucidated.**

**MATERIALS AND METHODS: A total of 60 male Wistar rats were equally randomly divided into three groups, including ALI group which was established by 10 mg/kg LPS femoral vein injection, ketamine group which was constructed by 50 mg/kg ketamine femoral vein injection based on ALI model, and control group. Blood gas analysis was applied to detect arterial blood oxygen partial pressure ($PaO_2$) and pH. Lung tissue wet/dry weight ratio (W/D), myeloperoxidase (MPO) and superoxide dismutase (SOD) activity were detected. Real-time PCR and ELISA were used to test HMGB-1 expression in lung tissue and serum. RAGE and NF-κB changes were determined by Real-time PCR and Western blot.**

**RESULTS: Compared with control, ALI group presented decreased $PaO_2$ and PH, elevated W/D, enhanced MPO activity, declined SOD activity, upregulated HMGB-1 mRNA, increased HMGB-1 secretion, and increased RAGE and NF-κB mRNA and protein ($p < 0.05$). Ketamine treatment significantly elevated $PaO_2$ and PH, reduced W/D, declined MPO activity, enhanced SOD activity, inhibited HMGB-1 mRNA and secretion, and downregulated RAGE and NF-κB mRNA and protein ($p < 0.05$).**

**CONCLUSIONS: Ketamine can alleviate LPS induced lung injury through inhibiting HMGB1-RAGE level. It could be treated as a new choice for ALI treatment.**

*Key Words:*
Ketamine, HMGB1, RAGE, ALI, LPS.

## Introduction

Multiple pulmonary internal and external factors except heart can cause acute lung injury (ALI), including shock, infection, and trauma[1,2]. ALI is the early stage of acute respiratory distress syndrome (ARDS), which mainly manifests as hypoxemia and respiratory distress. Although medical technology continues to advance, ALI treatment fails to progress significantly, leading to perennial high morbidity and mortality[3,4]. The main pathological feature of ALI is alveolar capillary permeability increase, a large number of inflammatory cytokines and neutrophil infiltration, pulmonary capillary endothelial cells structural damage, and alveolar epithelium cell dysfunction and integrity damage, resulting in alveolar fluid clearance obstacle[5,6]. Among various pathogenic factors, lipopolysaccharide (LPS)-induced endotoxemia is a common cause of ALI[7]. Inflammatory cytokines release is a critical factor in promoting the pulmonary inflammatory reaction, increasing alveolar-capillary permeability, and leading to alveoli liquid clearance obstacle[8].

As an important inflammatory factor, high mobility group box 1 (HMGB-1) expresses in all eukaryotic cells. It is a type of non-histone chromosome binding protein that involves in cell growth, proliferation, differentiation, migration, nerve growth, tumor, autoimmune disease, cardiovascular disease, and inflammation[9,10]. HMGB-1 can play its role by binding with its specific receptor RAGE. It plays a part in stabling chromosome structure and regulating transcription and translation by binding with DNA[11,12]. Cells pathological damage, apoptosis, or necrosis trigger a large amount of HMGB-1 release, leading to immune system activation and inflammatory damage[13]. RAGEs accumulation may lead to metabolic imbalance and cause a variety of pathological damages[14]. Ketamine has many pharmacological effects. It can significantly alleviate ALI, whereas its specific mechanism is not fully elucidated[15]. This study aimed to analyze ketamine impact on ALI through a HMGB1-RAGE level based on LPS induced rat ALI model.


*Corresponding Author:* An Shen, MD; e-mail: anshen123@yeah.net

## Materials and Methods

### Experimental Animals

A total of 60 healthy male Wistar rats, two-month-old and weighted 200 ± 20 g, were purchased from Weifang Medical College experimental animal center and fed in SPF laboratory.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Affiliated Hospital of Weifang Medical College.

### Reagents and Instruments

Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane was from Pall Life Sciences (Westborough, MA, USA). EDTA was purchased from Hyclone (Thermo Fisher Scientific, Waltham, MA, USA). Myeloperoxidase (MPO) and superoxide dismutase (SOD) detection kits were got from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China). Western blot related chemical reagents were from Beyotime (Suzhou, China). ECL reagent was bought from Amersham Biosciences (Marlborough, MA, USA). Rabbit anti-rat RAGE and NF-κB monoclonal antibodies, and goat anti-rabbit horseradish peroxidase (HRP) tagged IgG secondary antibody were bought from Cell Signaling (Danvers, MA, USA). ABI 7700 Fast fluorescence quantitative PCR reaction apparatus was from ABI (Thermo Fisher Scientific, Waltham, MA, USA). HMGB-1 ELISA kit was from RD (Minneapolis, MN, USA). Other common reagents were purchased from Sangon (Shanghai, China). The Lab System Version 1.3.1 microplate reader was got from Bio-Rad (Hercules, CA, USA). Automatic blood gas analyzer was from Nova Biomedical Company (Waltham, MA, USA).

## Methods

### Animal Modeling and Grouping

After fed for 2 weeks, the rats were randomly equally divided into three groups, including control group, ALI group, and ketamine group. According to the report[16,17], ALI model was established by 10 mg/kg LPS femoral vein injection, while ketamine group which was constructed by 50 mg/kg ketamine femoral vein injection based on ALI model.

### Sample Collection

At 6 h after LPS or ketamine injection, the rats were anesthetized with 10% chloral hydrate. The blood was collected from the abdominal aorta and centrifuged at 2000 rpm for 10 min. The serum was stored at -20°C. Next, the rat was killed, and the right upper lobe was used to test W/D. The left lung tissue was stored at -80°C.

### Blood Gas Analysis and W/D Detection

$PaO_2$ and PH were detected by blood gas analyzer. After sipped up the water on the surface, the wet weight of right upper lobe was weighted. Then, the lobe was dried in an oven for 24 h to test dry weight. W/D ratio was calculated, and pulmonary edema level was evaluated.

### Myeloperoxidase (MPO) Activity Detection

According to the instruction, 100 mg cryopreserved lung tissue was homogenized on the ice together with potassium phosphate buffer. After centrifuged at 4°C and 30,000 g for 30 min, the tissue was resuspended in 50 mmol/L potassium phosphate buffer containing 0.5% HTAB. After incubated in 60°C water bath for 2 h, the tissue was centrifuged at 4°C and 40,000 g for 130 min. The supernatant was added with reaction liquid and tested at 460 nm wavelength. MPO activity (U/g) was calculated by absorbance × 13.5/lung wet weight (g).

### SOD Activity

SOD activity was tested according to the manual. The lung protein was extracted and water bathed at 95°C for 40 min. Then, it was cooled and centrifuged at 4000 rpm for 10 min. At last, the protein was treated with ethanol-chloroform mixture (5:3, v/v 5:3) and the ethanol phase was used to test LDH and total SOD activity.

### Real-time PCR

Lung tissue mRNA was extracted by TRIzol and reverse transcribed to cDNA (Table I). Real-time PCR was applied to test target gene expression. Reaction condition: HMGB1, 52°C for 1 min, followed by 35 cycles of 90°C for 30s, 58°C for 50s, and 72°C for 35s. RAGE and NF-κB, 55°C for 1 min, followed by 35 cycles of 90°C for 30s, 62°C for 50s, and 72°C for 35s. PCR reaction was performed on ABI 7700 Fast amplifier. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal reference. $2^{-\Delta Ct}$ was applied to calculate relative expression level.

**Table I.** Primer sequence.

| Gene | 5′-3′ | 5′-3′ |
|---|---|---|
| GADPH | AGTGCCAGCCTCGTCTCATAG | CGTTGAACTTGCCGTGGGTAG |
| HMGB-1 | CAGCGGATCTCTAAACGGAAT | TGACATCTCTGGCGCTCCGTA |
| RAGE | TGCTCGCAGAAACTGTCAACTG | TCGCCTCAGGGGATTAAAGCTC |
| NF-κ B | GATATCCAGGGAGTTTGGGA | ATGTCCAGGGTTCATTGGTCTGT |

### ELISA

Serum was collected in Eppendorf tube and stored at -80°C. The HMGB-1 level was tested by ELISA kit according to the instruction. A total of 50 µl diluted standard substance was added to 96-well plate to prepare a standard curve. Meanwhile, 50 µl samples were added to the well with three replicates. After washed by the buffer for 5 times, 50 µl enzyme-labelled reagents were added, and the plate was incubated at 37°C for 30 min. After washed for 5 times, the plate was added with 50 µl color agent A and 50 µl color agent B at 37°C for 10 min. At last, the plate was treated with 50 µl stop buffer and read at 450 nm. The linear regression equation of standard curve was calculated according to the standard substance concentration and OD value. Sample concentration was calculated according to the standard curve.

### Western Blot

The tissue was cracked on ice for 15-30 min and ultrasonicated for 4 × 5 s to extract protein. After centrifuged at 10,000 g and 4ºC for 15 min, the protein was moved to a new Eppendorf tube and store at -20ºC. The protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred to PVDF membrane. After blocked by 5% skim milk for 2 h, the membrane was incubated in RAGE primary antibody at 1:1000 and NF-κB primary antibody at 1:500 overnight. Then, the membrane was incubated with secondary antibody at 1:2000 for 30 min and washed with PBST. At last, the membrane was treated with a chemiluminescent agent for 1 min and imaged on X-ray. Protein image processing system and Quantity one software were used for data analysis. All experiments were repeated for four times.

### Statistical Analysis

All the statistical analyses were performed on SPSS16.0 software (SPSS Inc., Chicago, IL, USA). Measurement data was presented as $\bar{x} \pm s$. One-way ANOVA was used for mean comparison. LSD was performed for the post-hoc test. $p < 0.05$ was considered as statistically significant.

## Results

### Blood Gas Analysis and W/D Comparison

$PaO_2$ and PH index in each group were tested, while W/D was calculated to evaluate pulmonary edema degree. LPS induced ALI significantly decreased $PaO_2$ and PH values compared with control ($p < 0.05$). Ketamine treatment enhanced $PaO_2$ and PH value compared with ALI group ($p < 0.05$). The rats in ALI group presented markedly upregulated W/D ratio compared with control ($p < 0.05$). Ketamine significantly reduced lung tissue W/D ratio compared with ALI group ($p < 0.05$) (Table II). The results indicated that ketamine can alleviate gas exchange function and pulmonary edema in ALI group.

**Table II.** Blood gas analysis and W/D comparison ($\bar{x} \pm s$).

| Group | $PaO_2$ (mmHg) | PH | W/D |
|---|---|---|---|
| Control | 117.15 ± 12.24 | 7.37 ± 0.05 | 4.25 ± 0.43 |
| ALI group | 82.32 ± 9.73* | 7.11 ± 0.02* | 6.85 ± 0.75* |
| Ketamine group | 99.71 ± 11.25*# | 7.32 ± 0.06# | 5.14 ± 0.57# |

*$p < 0.05$, compared with control; #$p < 0.05$, compared with ALI group.

**Table III.** Ketamine impact on oxidative stress index.

| Index | Control | ALI | Ketamine |
|---|---|---|---|
| MPO (U/g) | 1.71 ± 0.31 | 6.69 ± 0.75* | 4.53 ± 0.22*# |
| SOD (U/mg) | 32.10 ± 5.26 | 16.15 ± 3.21* | 21.23 ± 6.17*# |

*$p < 0.05$, compared with control; #$p < 0.05$, compared with ALI group.

### Ketamine Impact on Oxidative Stress Index

MPO and SOD content in lung tissue were tested. The results showed that MPO activity increased, while SOD content declined in LPS induced ALI compared with control ($p < 0.05$). Ketamine treatment markedly inhibited MPO activity and elevated SOD content compared with ALI group ($p < 0.05$) (Table III). The results revealed that ketamine can play a protective effect on ALI through regulating oxidative stress.

### HMGB1 mRNA Expression in Lung Tissue

Real-time PCR was applied to test HMGB-1 mRNA expression in lung tissue. It was demonstrated that HMGB-1 mRNA expression significantly upregulated in LPS induced ALI group compared with control ($p < 0.05$). Ketamine suppressed HMGB-1 mRNA upregulation in ALI group ($p < 0.05$) (Figure 1).

### Ketamine Impact on Serum HMGB1 Secretion

ELISA was used to detect ketamine impact on serum HMGB1 secretion. The results showed that similar with HMGB-1 mRNA expression in lung tissue, serum HMGB1 level elevated in LPS induced ALI compared with control ($p < 0.05$). Ketamine markedly inhibited serum HMGB1 level increment in ALI model ($p < 0.05$) (Figure 2).

### Ketamine Impact on RAGE and NF-κB mRNA Expression in Lung Tissue

Real-time PCR was performed to measure RAGE and NF-κB mRNA expression in lung tissue. It was revealed that RAGE and NF-κB mRNA expression significantly elevated in lung tissue after ALI ($p < 0.05$). Ketamine significantly downregulated RAGE and NF-κB mRNA elevation in ALI model ($p < 0.05$) (Figures 3 and 4).

### Ketamine Impact on RAGE and NF-κB Protein Levels in Lung Tissue

Western blot was applied to detect RAGE and NF-κB protein levels in lung tissue after ALI. The rats in ALI model presented evident increased RAGE and NF-κB protein levels in lung tissue compared with control ($p < 0.05$). Ketamine treatment markedly declined RAGE and NF-κB protein elevation in ALI group ($p < 0.05$) (Figures 5 and 6). The results suggested that ALI can activate HMGB-1-RAGE signaling pathway, leading to NF-κB mRNA and protein overexpres-

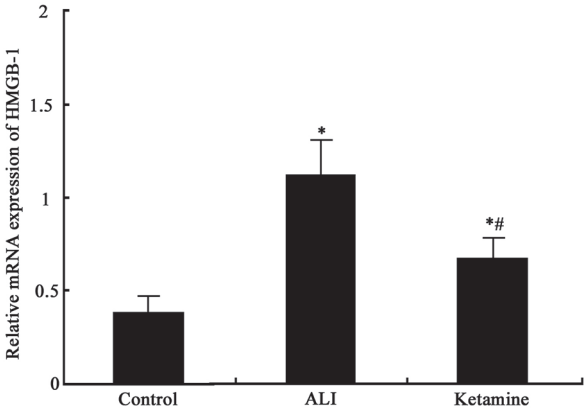


**Figure 1.** HMGB-1 mRNA expression in lung tissue. *$p < 0.05$, compared with control; #$p < 0.05$, compared with ALI group.

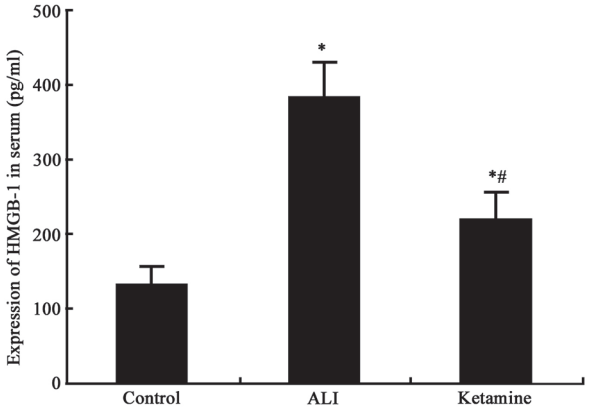


**Figure 2.** Serum HMGB1 secretion in each group. *$p < 0.05$, compared with control; #$p < 0.05$, compared with ALI group.

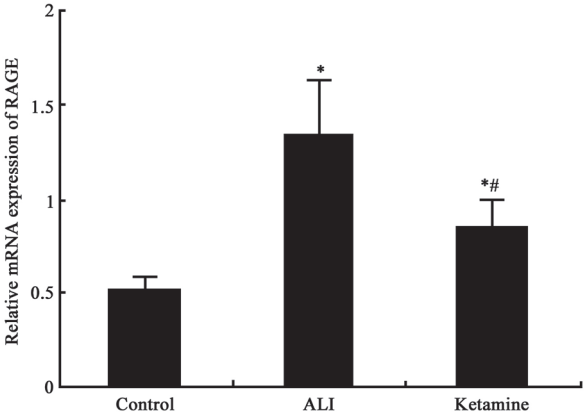


**Figure 3.** Ketamine impact on RAGE mRNA expression in lung tissue. *$p < 0.05$, compared with control; #$p < 0.05$, compared with ALI group.

sion and triggering inflammation. Ketamine can alleviate ALI mainly through HMGB-1-RAGE signaling pathway, thus downregulating NF-κB expression.

## Discussion

ALI mainly presents diffuse lung damage, leading to lung tissue structure changes, alveolar capillary permeability elevation, and lung edema fluid aggregation that unable to be effectively removed[6]. Lung tissue inflammation and edema are the main pathological changes of ALI, causing alveolar wall collapse and pulmonary ventilation dysfunction. As LPS, the main basis of endotoxin, caused lung damage similar to human ALI[7], this research showed that LPS induced rat ALI

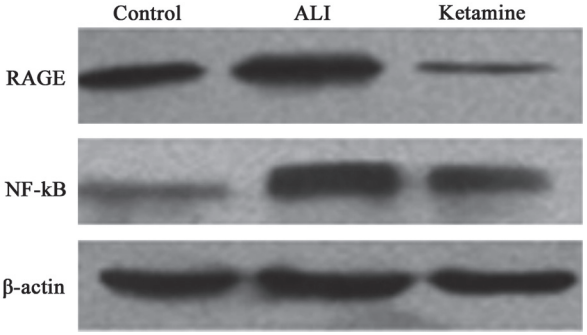


**Figure 5.** Ketamine impact on RAGE and NF-κB protein levels in lung tissue.

model appeared blood gas dysfunction and W/D ratio elevation. It suggested that LPS induced ALI may aggravate pulmonary edema and pulmonary ventilation dysfunction.

Ketamine has multiple pharmacological effects, especially anti-inflammation. It can inhibit inflammatory cytokines production to resist inflammatory damage to important viscera[18]. The study suggested that ketamine can effectively alleviate ALI, whereas it was also reported that ketamine may aggravate lung injury[15,19]. SOD is an important antioxidant enzyme in the lung tissue to clear the oxygen free radicals, which plays a vital role in the balance of oxidation and anti-oxidation. Its activity indirectly reflects the ability to remove oxygen free radicals. MPO is neutrophils lysosomal enzyme that specifically reflects the cluster degree of neutrophils in the lung tissue[20,21]. Our ionvestigation confirmed that ketamine can improve pulmonary ventilation dysfunction and pulmonary edema in ALI rat. Ketamine can

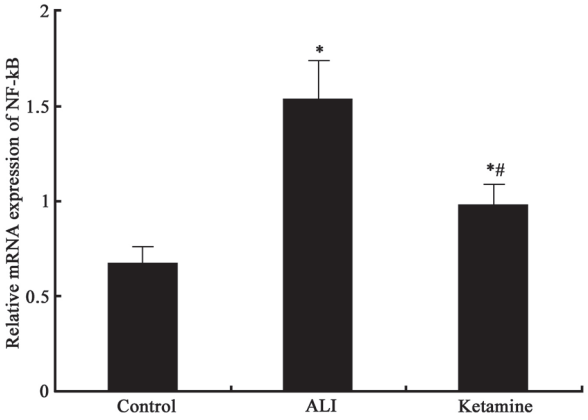


**Figure 4.** Ketamine impact on NF-κB mRNA expression in lung tissue. *$p < 0.05$, compared with control; #$p < 0.05$, compared with ALI group.

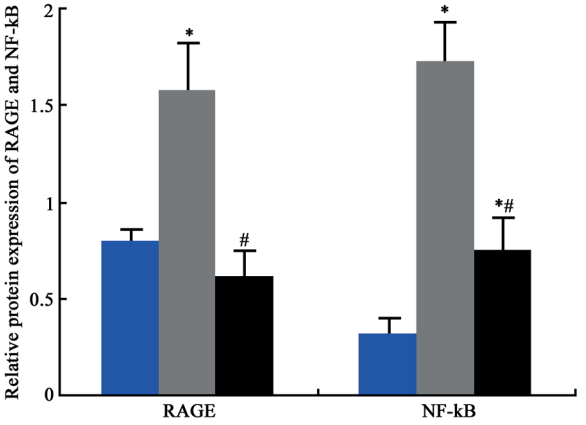


**Figure 6.** Ketamine impact on RAGE and NF-κB protein level analysis in lung tissue. *$p < 0.05$, compared with control; #$p < 0.05$, compared with ALI group.

increase SOD activity and reduce MPO activity, thus regulating neutrophil aggregation. As is known to all, the critical mechanism of ALI occurrence is inflammatory cytokines release, while NF-κB plays an important role in inflammatory factors transcription. NF-κB can regulate various inflammatory cytokines and adhesion molecules that closely associated with ALI in the process of ALI occurrence and development[22]. HGMB-1, a type of late-stage inflammatory factor, can trigger intracellular signaling pathways after binding with its related receptor RAGE. It can act on NF-κB to transform it to the nucleus, thus promoting inflammatory cytokines, chemokines, and colony stimulating factor expression and secretion, leukocyte adhesion, immune cells maturation and migration[23]. This study proved that ALI can significantly activate HMGB-1-RAGE signaling pathway, thus promoting NF-κB mRNA and protein overexpression, leading to inflammation activation. Ketamine can alleviate ALI through HMGB-1-RAGE signaling pathway to downregulate NF-κB expression.

## Conclusions

Ketamine can alleviate LPS induced lung injury by inhibiting HMGB1-RAGE level. It could be treated as a new choice for ALI treatment.

### Disclosure of Conflict of Interest

The authors declare no competing financial or commercial interests in this manuscript.

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