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# Impact of ketamine intervention for acute lung injury on RAGE and TLR9

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**Abstract. – OBJECTIVE: The purpose of this study was to explore the benefits of ketamine intervention for acute lung injury (ALI) and its effects on the receptor for advanced glycation end-product (RAGE) and toll-like receptor 9 (TLR9).**

**MATERIALS AND METHODS: Lipopolysaccharide (LPS, 3 mg/kg) was used to induce ALI rat model. Forty healthy Sprague-Dawley rats (6-8 weeks) were assigned into control, model, low ketamine (5 mg/kg), and high ketamine (50 mg/kg) groups. After 24 h, these rats were sacrificed and lungs were collected.**

**RESULTS: The pathological score, lung W/D ratio, the percentage of leukocytes and epithelial in bronchoalveolar lavage fluids (BALF), the expression levels of RAGE, TLR9, and other inflammation markers in serum and lungs were significantly higher in the Model group, indicating a good ALI model. Ketamine intervention restored all these parameters, with more benefits in the High dose group.**

**CONCLUSIONS: The high dose ketamine decreased the degree of ALI by inhibiting the expression of RAGE, TLR9, TNF-α, NF-κB, IL-6 and MPO in tissues.**

Key Words
Ketamine, Acute lung injury, Receptor for advanced glycation end-product, Toll-like receptor 9.

## Introduction

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is a syndrome due to alveolar capillary membrane injury and increased vascular permeability, with a clinical morbidity of 3-15% and a mortality of 10-40%[1]. ALI is reportedly associated with systemic inflammatory response syndrome (SIRS). Current treatment of ALI includes improvement of gas exchange and lung compliance as well as inhibition of the inflammatory response. Ketamine is a good anti-inflammatory agent that can alleviate the injuries to the heart, brains, and lungs[3]. The LPS (lipopolysaccharide)-induced ALI model is widely used in the laboratory. LPS is a component of the cell wall of Gram-negative bacteria that induces a large number of inflammatory factors and thus cause ALI[4]. This study analyzed the benefits of ketamine intervention on treating a rat model of ALI and examined the impact on RAGE and TLR9.

## Materials and Methods

### Materials

Healthy Sprague-Dawley rats (n=40, 6-8 weeks, 220 ± 20 g) were purchased from Shanghai Sangon Health Laboratory Animal Center. This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. The rats were acclimated to the environment in normal cages before the experiment. They received 3 mg/kg LPS i.p. and were anesthetized with 10% chloral hydrate (4 ml/kg) i.p. 24 h later. Eye enucleation was performed to collect 3 ml blood. Then, the animals were sacrificed by cervical dislocation. The cervical trachea was exposed, a catheter was inserted and fixed, 1 ml of normal saline was injected and absorbed three times to collect bronchoalveolar lavage fluid (BALF). Then, the thoracic cavity was open; the bilateral lung tissues were collected and washed with normal saline. The lungs were fixed in 4% paraformaldehyde, paraffin-embedded, and collected into 4 μm thick sections.

### Animals and Histology

Rats were randomly assigned to Control, Model (untreated), Low ketamine dose (5 mg/kg), and High ketamine dose (50 mg/kg) groups. Rats in the Model group received an equal volume of normal saline. Lung injury was observed after Hematoxylin & Eosin staining, and the pathological score was determined based on alveolar septum edema, hemorrhage, fibrin deposition, and cellular infiltration. Three sections were selected randomly. Five fields


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(left, right, upper, lower and central) were selected from each section. The average sum of each score was calculated. To calculate the wet/dry weight ratio of the lung, left and right lungs were placed on filter paper. Wet weight was determined with an electronic scale after the water was absorbed. Then the tissue was placed in a 70°C oven for 72 h, and dry weight was determined. The mean wet/dry weight (W/D) ratio was calculated.

### Cell Count

For counting leukocytes and epithelial cells, BALF was filtered with a 100-mesh nylon filter and centrifuged at 1500 x g. The cloudy lower suspension was harvested and used to prepare smear. After Giemsa staining, 3 slides were randomly selected, and 5 fields (left, right, upper, lower and central field) were analyzed under the microscope. The percentage of leukocytes and epithelial cells were calculated.

### ELISA and Western Blot

We measured the expression levels of receptor for advanced glycation end-product (RAGE), toll-like receptor 9 (TLR9), tumor necrosis factor-alpha (TNF-α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), interleukin 6 (IL-6) and myeloperoxidase (MPO) in serum and lung by ELISA and western blot. The ELISA kit was purchased from Jiangsu Biyuntian Science and Technology, and ELISA was performed according to the manufacturer's instructions. For western blot, lungs were centrifuged at 3,000 x g for 15 min, the concentration of proteins in the supernatant was measured by the BCA method (Zhongshan Golden Bridge Biotechnology, Beijing, China). The protein sample (15 μl) was mixed with loading buffer (Kaiji Biotechnology, Nanjing, China). The mixture was boiled for 5 min and separated by 10% SDS-PAGE electrophoresis (Sigma-Aldrich, St. Louis, MO, USA). Then the protein sample was transferred to PVDF membrane (Invitrogen, Carlsbad, CA, USA) and blocked in 5% skim milk. The membrane was incubated with rabbit anti-mouse RAGE, TLR9, TNF-α, NF-κB, IL-6, and MPO mAbs (R&D, Minneapolis, MN, USA, working concentration 1:2000) at 4°C overnight followed by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. Then, the membrane was washed in PBS, and ECL luminescence solution (Bio-Rad, Hercules, CA, USA) was added to develop a signal. After exposure, the membrane was observed with a Gel Imaging instrument (Media Cybernetics, Rockville, MD, USA).

### Statistical Analysis

SPSS20.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Measurement data were represented by mean ± SD. The inter-group comparison was performed by one-way analysis of variance (ANOVA). The pairwise comparison was performed by Fisher's least significant difference (LSD) *t*-test, $p<0.05$ was considered as statistically significant.

## Results

### Pathological Score of Lung Injury and W/D Ratio

After generating the ALI model with LPS, we examined the effects on the lungs (Table I). Both the pathological score of lung injury and the W/D ratio were significantly higher in the Model group than in the control group, demonstrating the successful generation of an ALI rat model. Then, we examined the benefits of treating the rats with two different doses of ketamine (Table I). Both treatments significantly decreased the pathological scores compared to the model group, but the high ketamine dose showed the most dramatic improvement. These results support the benefits of the anti-inflammatory activity of ketamine in this rat ALI model.

### Leukocytes and Epithelial Cells in BALF

Next, we counted the amount of leucocytes and epithelial cells in BALF in the rat ALI model (Table II). Leukocytes and epithelial cells were significantly elevated in the model group compared to the control group, again supporting the inflammation in the ALI model. Leukocytes and epithelial cell numbers were significantly decreased after ketamine intervention, but the effect of the High dose group was more dramatic (Table II).

### Inflammation Markers in the Lungs

Next, we examined the expression levels of several inflammation markers in the lungs (Table III). We found that the levels of RAGE, TLR9, TNF-α, NF-κB, IL-6, and MPO were significantly higher in the model group compared to the control group, supporting a successful ALI model with local inflammation. Ketamine intervention decreased the levels of all the inflammation markers, but the high dose group showed the largest reductions (Table III).

**Table I.** Pathological score of lung injury and W/D ratio.

| Group | Control | Model | Low dose ketamine | High dose ketamine | F | p |
|---|---|---|---|---|---|---|
| Pathological score of lung injury | 0.8 ± 0.2 | 9.5 ± 1.6 | 7.2 ± 1.3 | 3.6 ± 0.7 | 15.632 | 0.00 |
| W/D ratio | 1.3 ± 0.4 | 4.5 ± 1.2 | 3.2 ± 0.9 | 1.8 ± 0.6 | 6.427 | 0.00 |

**Table II.** Leukocytes and epithelial cells counting in BALF.

| Group | Control | Model | Low dose ketamine | High dose ketamine | F | p |
|---|---|---|---|---|---|---|
| Leukocytes | 46.2 ± 13.4 | 89.7 ± 21.5 | 72.3 ± 22.6 | 52.6 ± 23.7 | 16.427 | 0.000 |
| Epithelial cells | 38.7 ± 11.2 | 82.4 ± 25.8 | 68.7 ± 23.6 | 45.5 ± 24.8 | 18.524 | 0.000 |

**Table III.** Inflammation markers in lung (μmol/L).

| Group | Control | Model | Low dose ketamine | High dose ketamine | F | p |
|---|---|---|---|---|---|---|
| RAGE | 0.12 ± 0.03 | 0.68 ± 0.15 | 0.45 ± 0.12 | 0.26 ± 0.08 | 16.529 | 0.000 |
| TLR9 | 0.23 ± 0.05 | 0.75 ± 0.18 | 0.59 ± 0.13 | 0.34 ± 0.07 | 18.524 | 0.000 |
| TNF-α | 0.08 ± 0.02 | 0.49 ± 0.14 | 0.32 ± 0.08 | 0.17 ± 0.06 | 13.624 | 0.000 |
| NF-κB | 0.06 ± 0.02 | 0.46 ± 0.19 | 0.30 ± 0.13 | 0.15 ± 0.05 | 12.528 | 0.000 |
| IL-6 | 0.24 ± 0.13 | 0.66 ± 0.22 | 0.48 ± 0.16 | 0.35 ± 0.13 | 14.527 | 0.000 |
| MPO | 0.15 ± 0.06 | 0.57 ± 0.24 | 0.38 ± 0.15 | 0.24 ± 0.11 | 13.326 | 0.000 |

### *Inflammation Markers in Serum*

Finally, we determined the expression of inflammation markers in serum (Table IV). The expression levels of RAGE, TLR9, TNF-α, NF-κB, IL-6, and MPO in serum were significantly higher in the Model group than in the control group, supporting systemic inflammation in the ALI model. Ketamine intervention decreased the values for all the inflammation markers, with the High dose showing more dramatic reductions than the Low dose group (Table IV).

## Discussion

Ketamine can alleviate injuries by blocking the sodium channels in alveolar epithelial cells[5]. Yang et al[6] compared the efficacy of 5 and 50 mg/kg ketamine to treat LPS-induced ALI in rats and found that ketamine inhibited NF-κB activation by LPS. The inhibition effects of 50 mg/kg ketamine were more significant on the expression of TNF-α, Il-6, and MPO as well as lung wet/dry weight ratio. Ketamine can exert anti-inflammatory effects

**Table IV.** Inflammation markers in lung (μmol/L).

| Group | Control | Model | Low dose ketamine | High dose ketamine | F | p |
|---|---|---|---|---|---|---|
| RAGE | 24.6 ± 10.2 | 113.2 ± 34.6 | 76.6 ± 22.5 | 45.8 ± 16.7 | 13.264 | 0.000 |
| TLR9 | 15.2 ± 4.3 | 65.9 ± 21.2 | 44.8 ± 16.6 | 26.7 ± 12.3 | 10.258 | 0.000 |
| TNF-α | 6.3 ± 2.4 | 45.8 ± 15.6 | 25.7 ± 5.2 | 13.5 ± 4.6 | 9.658 | 0.000 |
| NF-κB | 7.8 ± 3.3 | 52.3 ± 17.9 | 39.7 ± 6.4 | 16.9 ± 5.2 | 8.659 | 0.000 |
| IL-6 | 56.9 ± 13.3 | 245.6 ±46.5 | 136.4 ± 35.7 | 83.2 ± 23.6 | 14.623 | 0.000 |
| MPO | 34.5 ± 13.9 | 98.7 ± 25.7 | 72.4 ± 14.3 | 55.6 ± 12.8 | 12.326 | 0.000 |

through regulating the release of inflammatory factors by alveolar macrophages, decreasing the accumulation and activation of polymorphonuclear leukocytes, intervening the signal transduction of inflammatory cells, inhibiting the production of NO, and increasing intracellular levels of cAMP[7-9]. Also, ketamine can decrease pulmonary artery pressure and improve lung compliance[10].

RAGE is a multi-ligand receptor and a member of the immunoglobulin superfamily that can be expressed as full-length or soluble RAGE. Full-length RAGE is located in the basolateral membrane of type I alveolar epithelial cells (ACE I) and is a marker of injury of these cells. The increase in serum levels of soluble RAGE is positively correlated to the injury degree of ALI/ARDS[11]. TLRs are important pattern recognition receptors in innate immunity. They not only recognize pathogenic microorganisms and relevant product but also are involved in acquired immunity, and are considered the link between innate immunity and acquired immunity[12]. TLR9 is extensively expressed in immature dendritic cells, macrophages, and monocytes, and plays key roles in the recognition of LPS from Gram-positive bacteria, and the signal transduction pathways that are mediated by heat shock protein 60 and LPS[13,14]. RAGE is a pattern recognition receptor on the cell surface that can bind AGEs and β-amyloid peptides, activate the NF-κB signal transduction pathway, mediate the amplification of the inflammatory response, and, as a result, create a positive feedback loop[15]. TLR9 mediates the myeloid differentiation factor (MyD88) pathway, the TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway, and the activation of NF-κB[16]. TNF-α is an important pro-inflammatory factor in the pathogenesis and development of ALI. After binding to the corresponding receptor in the lungs, TNF-α can cause lysosome damage, activate the release of IL-6 from endothelial cells, monocytes and macrophages, and aggravate lung injuries[17,18]. Ketamine can prevent the production of inflammatory factors through inhibiting the expression of TLR4 and NF-κB[19,20]. The possible mechanism may be that ketamine inhibits the translocation of NF-κB from the cytoplasm to the nucleus through inhibiting the degradation of IκB.

## Conclusions

We showed that the pathological score, lung W/D ratio, the leukocytes and epithelial cells in BALF, and lung and serum inflammation markers were significantly improved after ketamine intervention, particularly in the high dose group. Therefore, high-dose ketamine decreases the degree of ALI associated with inhibiting the expression of several relevant inflammatory markers in the lung and the serum.

### Conflict of Interest:

The authors declare no conflict of interest.

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