

Budesonide inhalation ameliorates endotoxin-induced lung injury in rabbits

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Abstract

Acute respiratory distress syndrome (ARDS) is a serious clinical problem that has a 30–50% mortality rate. Budesonide has been used to reduce lung injury. This study aims to investigate the effects of nebulized budesonide on endotoxin-induced ARDS in a rabbit model. Twenty-four rabbits were randomized into three groups. Rabbits in the control and budesonide groups were injected with endotoxin. Thereafter, budesonide or saline was instilled, ventilated for four hours, and recovered spontaneous respiratory. Peak pressure, compliance, and PaO₂/FiO₂ were monitored for 4 h. After seven days, PaO₂/FiO₂ ratios were measured. Wet-to-dry weight ratios, total protein, neutrophil elastase, white blood cells, and percentage of neutrophils in BALF were evaluated. TNF- α , IL-1 β , IL-8, and IL-10 in BALF were detected. Lung histopathologic injury and seven-day survival rate of the three groups were recorded. Peak pressure was downregulated, but compliance and PaO₂/FiO₂ were upregulated by budesonide. PaO₂/FiO₂ ratios significantly increased due to budesonide. Wet-to-dry weight ratios, total protein, neutrophil elastase, white blood cells and percentage of neutrophils in BALF decreased in the budesonide group. TNF- α , IL-1 β , and IL-8 levels decreased in BALF, while IL-10 levels increased in the budesonide group. Lung injuries were reduced and survival rate was upregulated by budesonide. Budesonide effectively ameliorated respiratory function, attenuated endotoxin-induced lung injury, and improved the seven-day survival rate.

Keywords: Budesonide, endotoxin, lung injury

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Introduction

Acute respiratory distress syndrome (ARDS) is a serious clinical problem that has a 30–50% mortality rate,¹ and currently has no effective treatment.² In clinical practice, sepsis is the highest risk factor for ARDS progression with a 40% morbidity rate.^{3,4} ARDS is characterized by abnormalities in ventilation perfusion ratio, decrease in compliance, lung edema, severe hypoxemia, increase in lung permeability, and impaired gas exchange.^{5,6} These features are caused by the infiltration of neutrophils, protease activation, increased endothelial and epithelial permeability, and secretion of cytokines in lungs due to nuclear factor- κ B (NF- κ B) activation.^{7–10} Therefore, effective control of inflammation could be the best treatment for ARDS.

Glucocorticoids have been used to treat ARDS via the inhibition of NF- κ B and inflammation.^{11–14} However, systemic use of glucocorticoids may cause immunosuppression¹⁵ and spread infection. Compared with systemic administration, inhaled glucocorticoids provide more

effective local anti-inflammation and less systemic immunosuppression. Moreover, compared with severe lung injury and high mortality associated with ARDS, the possibility of less immunosuppression of inhaled glucocorticoids is acceptable, if it can ameliorate lung injury and severe inflammation. In addition, severe lung injuries could be clinically treated with antibiotics, decreasing the dosage and reducing the complications of glucocorticoids. Budesonide can attenuate lung injuries induced by chlorine gas, surfactant-depletion, aspiration or LPS in short-term experiments.^{16–19} However, long-term outcome of budesonide applications in endotoxin-induced lung injuries remains unclear. Indeed, long-term application of glucocorticoids to treat ARDS remains controversial. In this study, we established an ARDS rabbit model via intravenous endotoxin injection, and investigated whether budesonide applications could provide positive outcomes and safety for ARDS in clinical therapy.

Methods

Animals

Twenty-four male New Zealand white rabbits (2.0–2.5 kg) were anesthetized with ketamine (35 mg/kg, i.m.) and xylazine hydrochloride (0.3 mg/kg, i.m.). Intubation was performed using a 3.5 mm cuffed endotracheal tube through the glottis. Anesthesia was maintained with infusion of ketamine at 30 mg/kg·h and vecuronium bromide (0.05 mg/kg·h). Under local anesthesia, catheters were inserted into the left ear vein for infusion of saline or endotoxin, and into the right femoral artery for blood pressure monitoring and arterial blood analysis. Rabbits were mechanically ventilated with 40% oxygen for 20 min. Tidal volume was 10 ml/kg and respiratory rate was controlled to maintain an arterial carbon dioxide tension (PaCO₂) of 35–45 mm Hg. Positive end-expiratory pressure was 3 cm H₂O. Rabbits were placed on a heating pad to keep body temperature between 36.5°C and 37.5°C at the esophagus. After 20 minutes of ventilation, blood gas was analyzed and peripheral blood was collected. The 24 rabbits were then randomized into three groups: sham group, control group, and budesonide group. Rabbits in the sham group were injected with saline. Rabbits in the control and budesonide groups were injected with endotoxin (500 µg/kg, *Escherichia coli* endotoxin, 0111:B4, Sigma, Saint Louis, Missouri, USA) via ear vein over 30 min to induce lung injury. After saline or endotoxin injection, rabbits in the sham and control groups received 5 ml saline for instillation, while rabbits in the budesonide group received budesonide (0.5 mg/kg, AstraZeneca Pharmaceuticals, Baldock, Hertfordshire, UK)¹¹ in 5 ml saline, which were instilled via the trachea. After instilling saline or budesonide, all rabbits were ventilated for 4 h. Arterial blood gas analysis and peripheral blood were obtained for further analysis before injecting saline or endotoxin (T0), and at 1 (T1), 2 (T2) 4 (T3) h after saline or budesonide instillation.

After 4 h of ventilation, all the rabbits were allowed to spontaneously recover their breath from anesthesia, and then extubated. Survival in each group was assessed every 24 h for seven days. During the seven days, incisions of all rabbits were locally anesthetized with lidocaine for pain relief. Moribund animals were defined as bradycardia with a heart rate lower than 40 beats per minute, severely lethargic, and unresponsive to painful stimulation.

Moribund and survivor rabbits (after seven days) were euthanized by thiamylal overdose. Then, lungs were collected for histological examination or bronchoalveolar lavage fluid (BALF) was performed. Arterial blood analysis was performed and peripheral blood was collected for analysis.

Airway pressure and dynamic compliance

Peak pressure and dynamic compliance were monitored with Datex/Ohmeda S/5 (Datex Instrumentation, Helsinki, Finland).

Arterial blood gas analysis

Arterial blood gases were analyzed at all time-points during ventilation, and seven days with Bayer Rapidlab 348 (Bayer Diagnostics, Germany). PaO₂/FiO₂ ratio was calculated.

Pulmonary alveolocapillary permeability

At day 7, part of the right lobe was weighed and dried to a constant weight for 48 h at 60°C. Wet/dry weight (W/D) ratio was calculated.

Local and systemic inflammation

After euthanasia, BALF was harvested from the left lung by infusing 4°C of saline (15 ml/kg) containing (EDTA)-2Na, and withdrawal for five times. A cytocentrifuged spin preparation (CF-RD, Sakura, Tokyo, Japan) of BALF was stained for cell differentiation. BALF was centrifuged at 1000 g for 15 min at 4°C. After centrifugation, lavage fluids were immediately stored at –80°C. White blood cells and percentage of neutrophils in BALF were counted using a cell counter. Concentration of neutrophils elastase was analyzed. Then, TNF-α, IL-1β, IL-8, and IL-10 levels in peripheral blood and BALF were analyzed (Sigma-Aldrich, St. Louis, MO, USA). The sensitivity of these kits was: TNF-α, 3.12 pg/ml; IL-1β, 2.8 pg/ml; IL-8, 6.1 pg/ml; and IL-10, 2.9 pg/ml.

Histopathologic analysis of lung tissue

HE stain was performed to assess histopathologic injury. The right lower lobes were fixed with 10% formalin. Lungs were embedded in paraffin, and 4-µm sections were stained with hematoxylin and eosin. Two independent pathologists, who were unaware of the experimental groupings, analyzed and scored the lung injuries under light microscopy (0, minimum damage; 1, mild damage; 2, moderate damage; 3, severe damage; and 4, maximum damage) according to the assessment of alveolar congestion, edema, neutrophil infiltration in airspace or vessel wall, hemorrhage, alveolar wall thickness and hyaline membrane formation.

Statistical analysis

All normally distributed data were presented as mean and SD, which were analyzed using SPSS version 11.0 statistical software (SPSS, Chicago, IL, USA). Normally distributed data were analyzed using an unpaired *t*-test for a single time-point, non-normally distributed data were analyzed using the Mann-Whitney rank sum test, and histologic data were analyzed with the Wilcoxon U-test. Survival curves were derived using the Kaplan-Meier method, and differences were evaluated by log-rank tests. *P* < 0.05 was considered statistically significant.

Results

Budesonide improved respiratory function

Compared to the sham group, after injecting endotoxin in both control and budesonide groups, peak pressure was

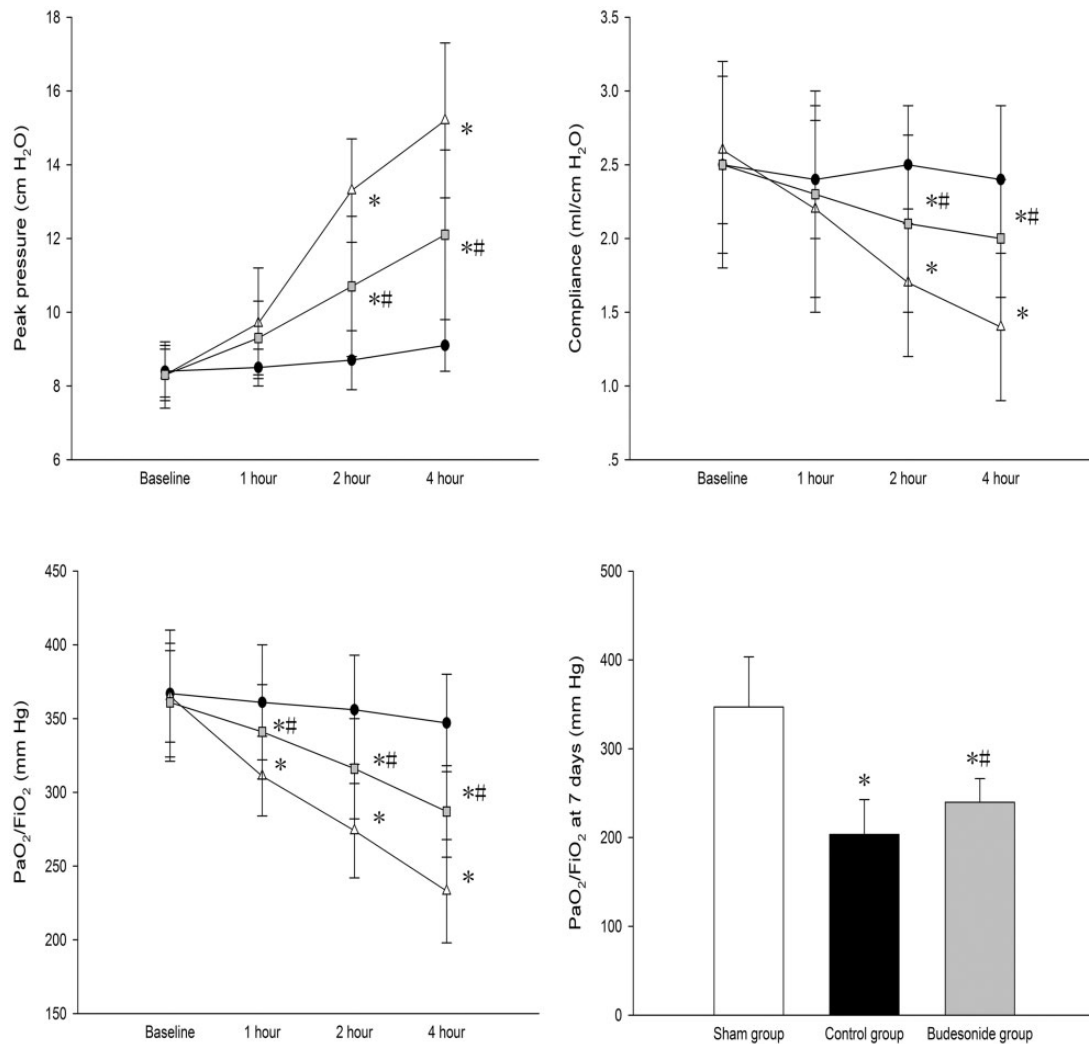


Figure 1 Comparison of peak pressure, dynamic compliance, and PaO₂/FiO₂ among different groups is shown. **P* < 0.05, compared with the sham group; #*P* < 0.05, compared with the control group. (●, Group S; ▲, Group C; ■, Group B)

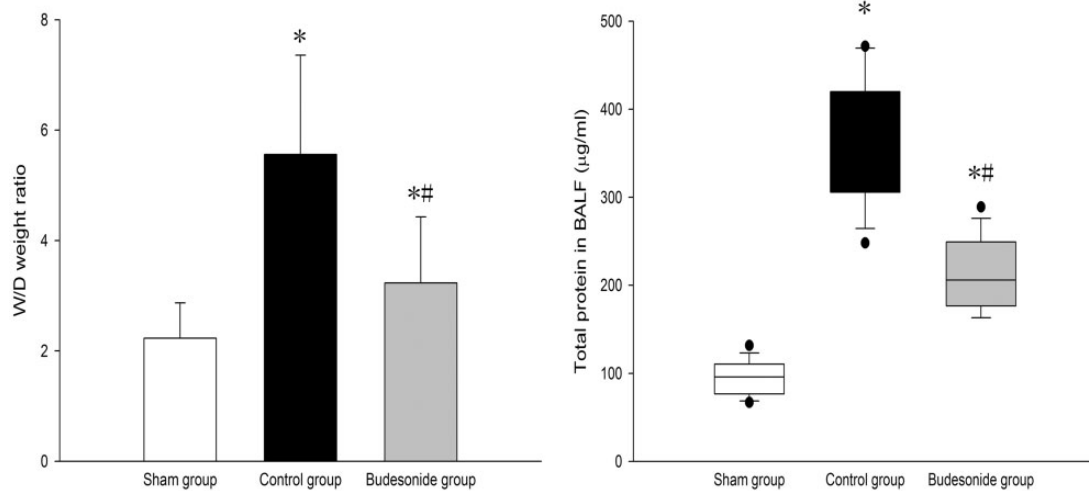


Figure 2 Comparison of W/D weight ratio and total protein in BALF among the three groups is shown. **P* < 0.05, compared with the sham group; #*P* < 0.05, compared with the control group. (□, Group S; ■, Group C; ▒, Group B)

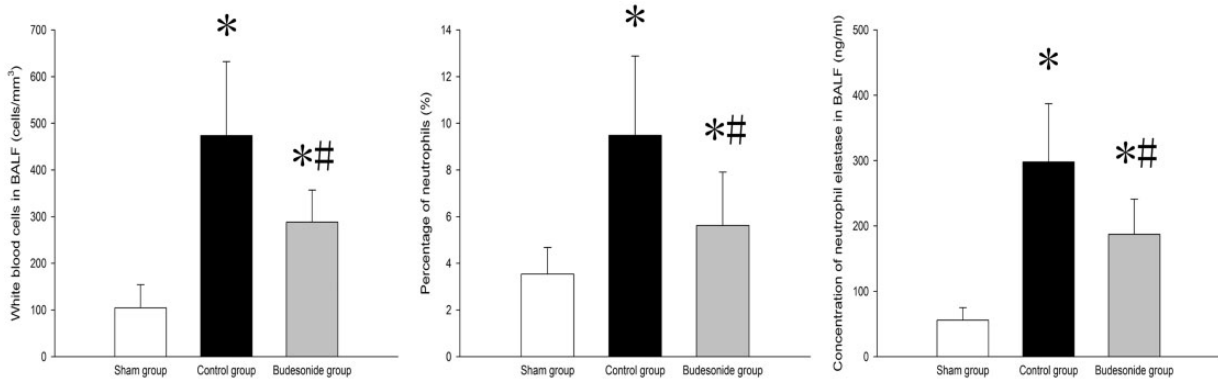


Figure 3 White blood cell count, percentage of neutrophils and neutrophil elastase levels in BALF among different groups is shown. * $P < 0.05$, compared with the sham group; # $P < 0.05$, compared with the control group. (□, Group S; ■, Group C; ▒, Group B)

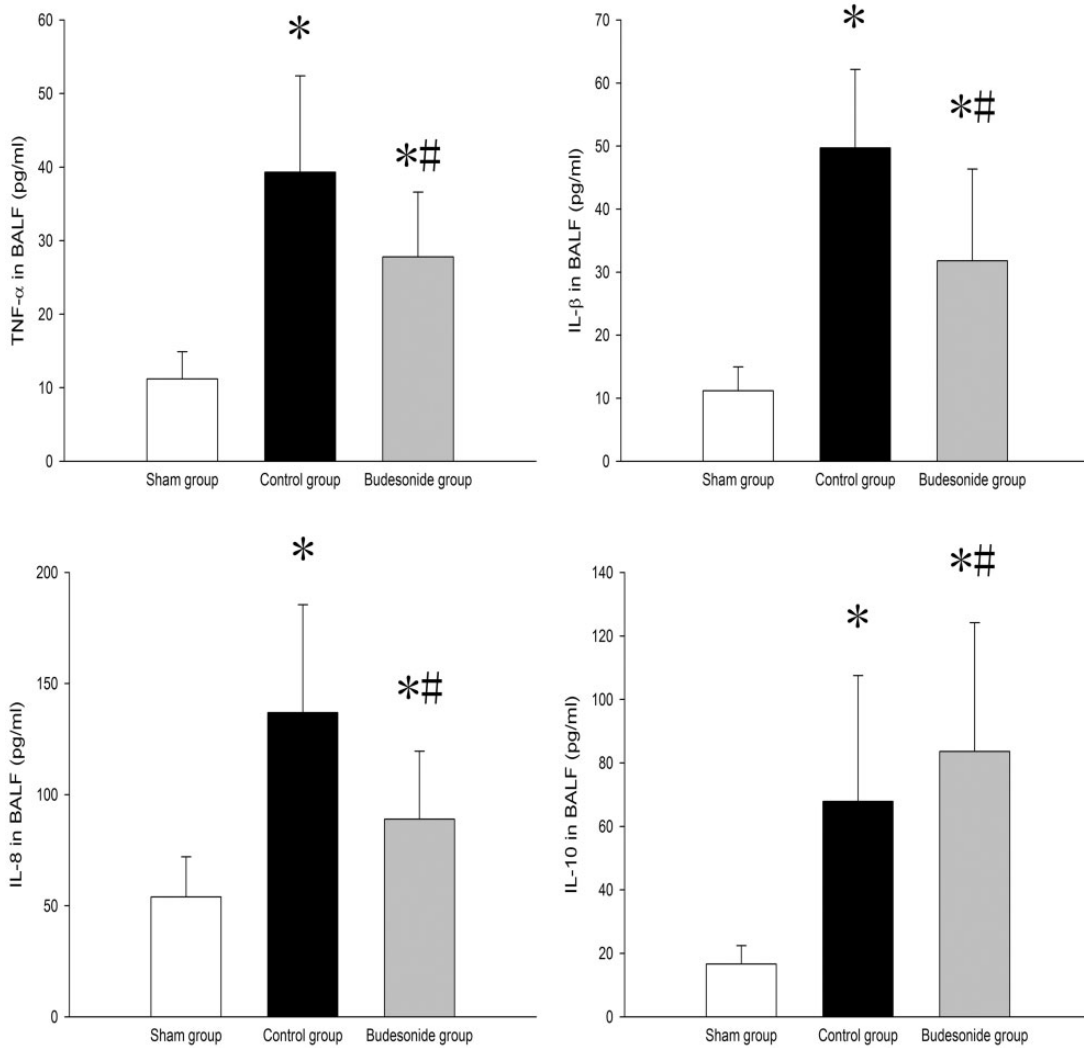


Figure 4 TNF-α, IL-1β, IL-8, and IL-10 levels in BALF among different groups are shown. * $P < 0.05$, compared with the sham group; # $P < 0.05$, compared with the control group. (□, Group S; ■, Group C; ▒, Group B)

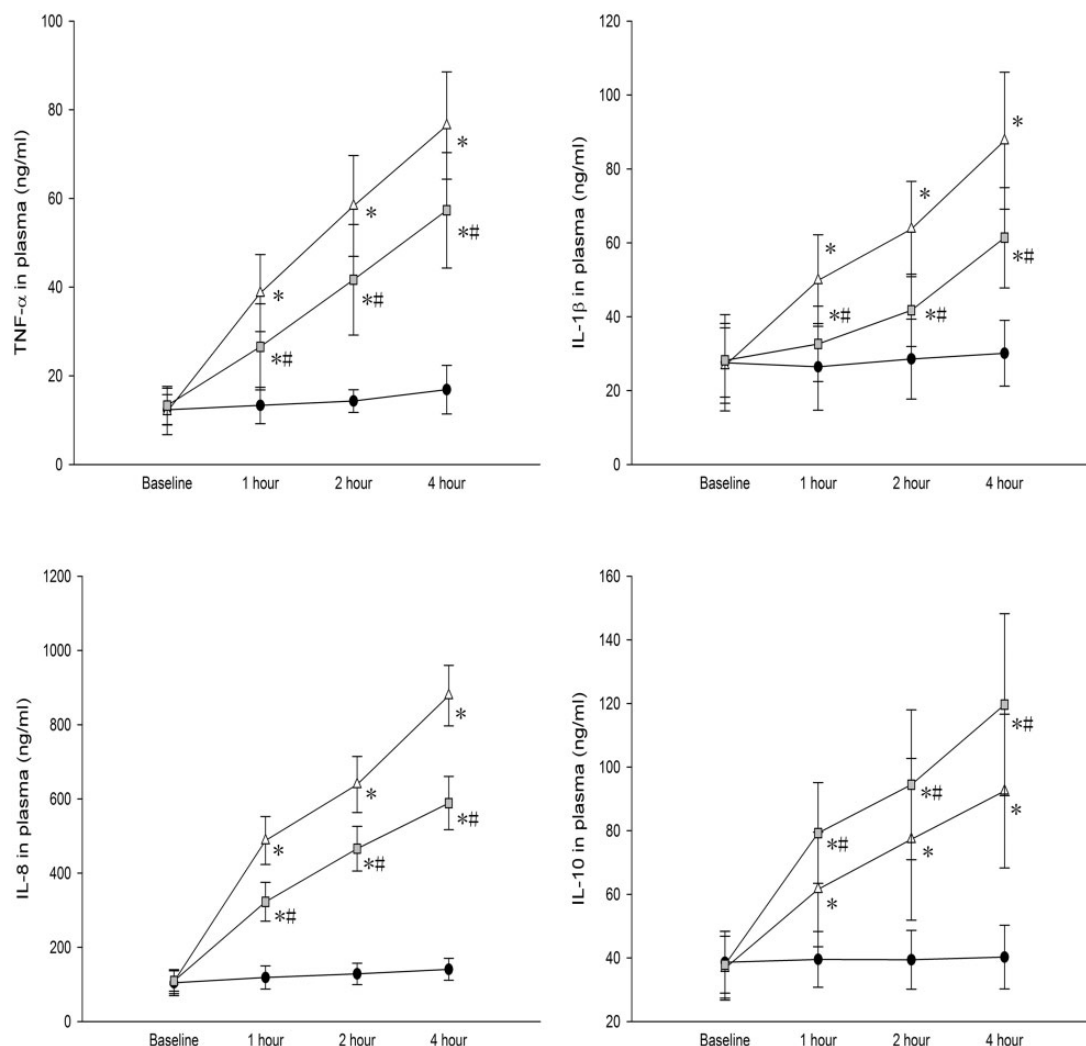


Figure 5 TNF- α , IL-1 β , IL-8, and IL-10 levels in serum of the different groups are shown. * $P < 0.05$, compared with the sham group; # $P < 0.05$, compared with the control group. (●, Group S; △, Group C; □, Group B)

significantly greater, while dynamic compliance and PaO₂/FiO₂ ratio were significantly lower within 4 h of ventilation. Peak pressure was significantly lower, while dynamic compliance and PaO₂/FiO₂ ratio were significantly higher in the budesonide group, compared to the control group. After seven days, PaO₂/FiO₂ ratio in both control and budesonide groups were significantly lower than the sham group. PaO₂/FiO₂ ratio in the budesonide group was higher than the control group after seven days (347.52 ± 56.74 vs. 203.31 ± 39.27 vs. 240.75 ± 26.36 ; $P < 0.001$, $P < 0.001$, $P = 0.045$) (Figure 1).

Budesonide improved alveolocapillary permeability

W/D weight ratio and total protein in BALF significantly increased in both control and budesonide groups, compared to the sham group, but were significantly lower in the budesonide group than in the control group; (2.23 ± 0.64 vs. 5.56 ± 1.81 vs. 3.33 ± 1.22 ; $P < 0.001$, $P = 0.04$, $P = 0.01$),

(95.87 ± 19.52 vs. 369.83 ± 70.09 vs. 212.78 ± 39.87 ; $P < 0.001$, $P < 0.001$, $P < 0.001$) (Figure 2).

Budesonide inhibited inflammation

White cell count and percentage of neutrophils in BALF were higher in both control and budesonide groups than in the sham group, but were significantly lower in the budesonide group compared to the control group; (104.63 ± 49.92 vs. 473.56 ± 158.36 vs. 288.43 ± 68.61 ; $P < 0.001$, $P < 0.001$, $P < 0.001$), (3.54 ± 1.14 vs. 9.48 ± 3.39 vs. 5.62 ± 2.28 ; $P < 0.001$, $P = 0.03$, $P = 0.01$) (Figure 3). In addition, neutrophil elastase concentrations were significantly higher in both control and budesonide groups, compared to the sham group, but were lower in the budesonide group than in the control group (56.23 ± 19.45 vs. 298.78 ± 89.56 vs. 187.82 ± 54.93 ; $P < 0.001$, $P < 0.001$, $P < 0.001$) (Figure 3).

TNF- α , IL-1 β , IL-8, and IL-10 levels were significantly higher in BALF and peripheral blood of both control and

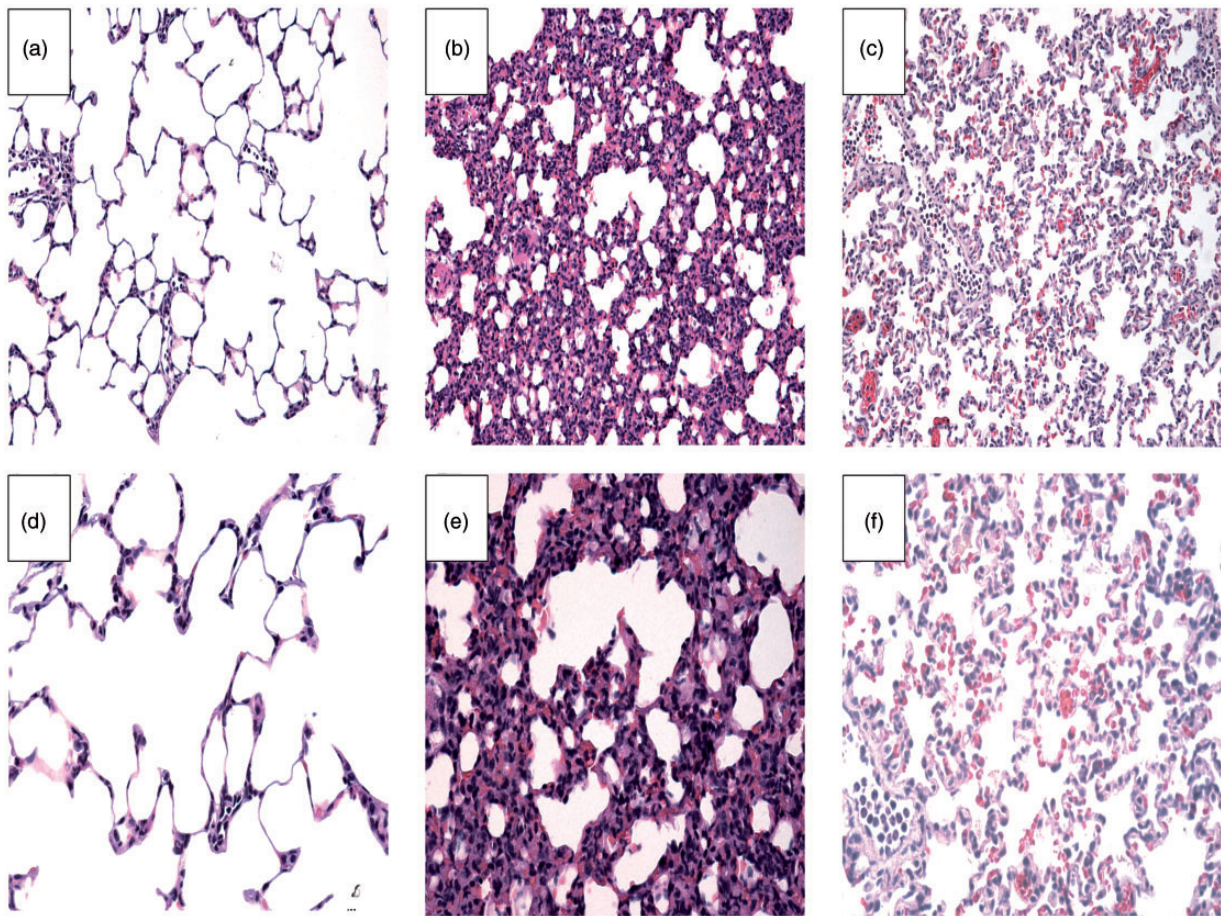


Figure 6 Histopathological analysis of lung tissues among different groups is shown. (A, D) sham group, (B, E) control group, and (C, F) budesonide group. A–C: $\times 200$; D–F: $\times 400$. Lungs in the saline group showed thickened alveolar wall, edema and less alveolar space, and obvious inflammatory cell infiltration. Budesonide significantly decreased endotoxin-induced histopathological injury. (A color version of this figure is available in the online journal.)

budesonide groups than in the sham group. Compared to the control group, TNF- α , IL-1 β , and IL-8 levels in BALF were significantly lower; but IL-10 levels were significantly higher in the budesonide group (Figures 4 and 5).

Budesonide attenuated lung injury

We observed typical ARDS pathologic aspects using light microscopy in the saline group such as severe edema, alveolar wall thickening, hyaline membrane formation, hemorrhage, and neutrophil infiltration in lung parenchyma. These lung tissue damages were notably reduced in the budesonide group (Figure 6). Lung injury scores in the control, saline and budesonide groups were 1 (range: 1 to 2), 3 (range: 2 to 4) and 2 (range: 2–3), respectively. Lung injury was significantly reduced in the budesonide group compared to the saline group ($P < 0.05$).

Budesonide improved rabbit survival with endotoxin-induced ARDS

To investigate the long-term therapeutic effect of budesonide on ARDS induced by intravenous injection of endotoxin, we analyzed the survival of rabbits with endotoxin-induced ARDS. Survival rate of rabbits treated with

budesonide was 30%, compared to 0% for rabbits that received saline ($P < 0.001$) (Figure 7).

Discussion

In this study, we found that budesonide not only significantly reduced short-term lung injury including improved ventilation function, increased PaO₂/FiO₂ and reduced inflammation, but also improved the seven-day outcome such as decrease of alveolocapillary permeability and edema, histological injury, and survival in endotoxin-induced lung injury.

Endotoxin is derived from the cell wall of gram-negative bacteria and has been implicated as a major factor in the pathogenesis of septic shock caused by gram-negative bacteria.²⁰ Endotoxin is known to induce infiltration of inflammatory cells and overproduction of cytokines, severe hypoxemia, decrease of compliance, accumulation and activation of neutrophil, increase of endothelial and epithelial permeability, and parenchymal injury.²¹ In experimental animals, endotoxin challenge causes pathophysiologic changes that are similar to those observed in human patients with septic shock.^{5,6} Endotoxin-induced ARDS could result in severe inflammation response, and lead to

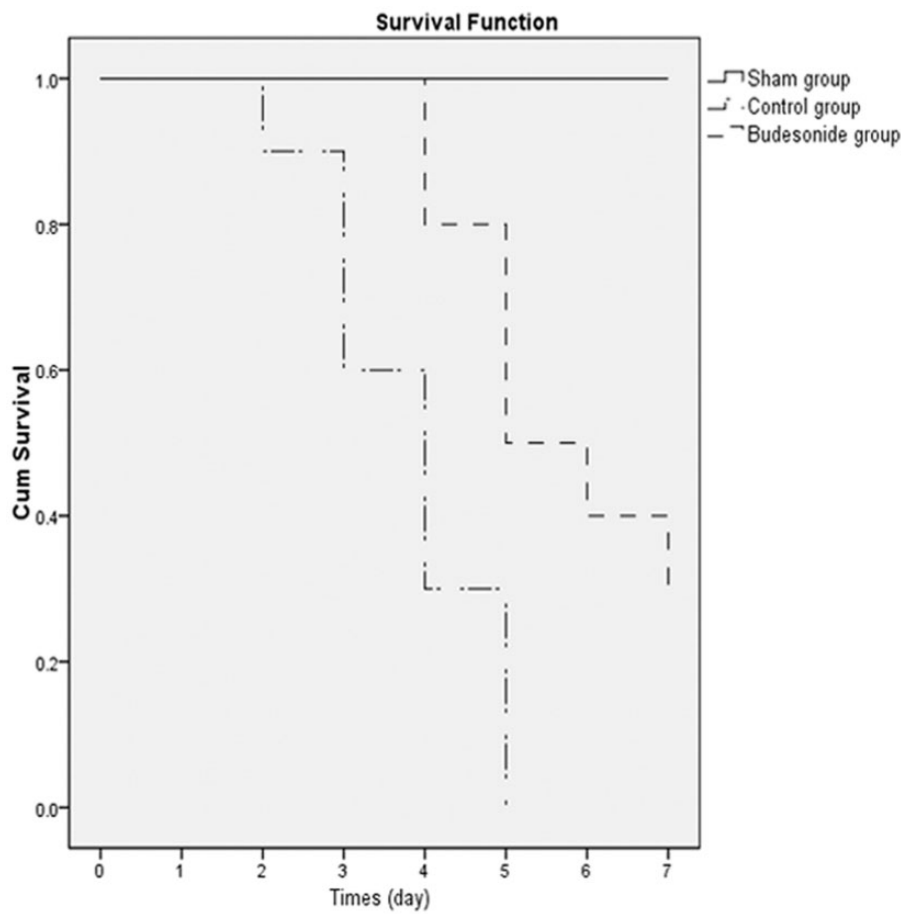


Figure 7 Effects of budesonide on rabbit survival with endotoxin stimulation are shown. Survival was observed for seven days after the endotoxin challenge. Results were expressed as percent of survival ($n = 10$). Survival rate was estimated by the Kaplan-Meier method. Budesonide significantly delayed endotoxin-induced rabbit death

lung edema and injury.^{5,6} Effective control of inflammation could be the best treatment for ARDS. However, there are no effective drugs that could reduce endotoxin-induced lung injuries. Although glucocorticoids have been used for treating ARDS via inhibition of NF- κ B and inflammation,¹¹⁻¹⁴ systemic immunosuppression¹⁵ of glucocorticoids may allow infection to spread. Compared with other glucocorticoids, inhaled budesonide not only provides more effective local anti-inflammation, but also less systemic immunosuppression. More importantly, no evidence associated between budesonide, increased frequency or severity of infectious diseases,^{22,23} and safety of long-term budesonide applications has been accepted. In this study, we investigated the effects of budesonide on both short-term and long-term outcomes of ARDS to evaluate whether budesonide could be applied in patients with ARDS.

We found that inhaled budesonide significantly reduced peak pressure, and increased compliance and $\text{PaO}_2/\text{FiO}_2$ ratio, indicating that airway responsiveness was inhibited by budesonide. Indeed, budesonide reduces airway responsiveness in asthma patients,²⁴ and it also improves airway pressure, compliance, and $\text{PaO}_2/\text{FiO}_2$ ratio in chlorine gas- or meconium aspiration-induced ARDS models.^{16,17} In addition, improved respiratory function may be associated

with alveolocapillary permeability improvement. In this study, budesonide significantly reduced protein contents in BALF and lung edema. The protective effects of budesonide on alveolocapillary permeability might be due to local anti-inflammatory action.²⁵

In endotoxin-induced ARDS, neutrophil elastase plays an important role in mediating neutrophil extravasation, tissue damage, and endothelial cell injury.²⁶ Elastase inhibition could provide protection against lung injury.²⁷ In this study, severe lung tissue injury increased neutrophil infiltration, and increased neutrophil elastase levels were observed in BALF after endotoxin injection. Compared to the control group, budesonide dramatically ameliorated hyaline membrane formation and hemorrhage areas, and significantly reduced lung injury scores. In addition, budesonide also significantly decreased white blood cell count, as well as the percentage of neutrophils and neutrophil elastase in BALF. Collectively, these data indicate that budesonide significantly inhibited the infiltration and activation of neutrophils.

A complex network of inflammatory cytokines, chemokines, and anti-inflammatory cytokines plays a major role in mediating, amplifying, and perpetuating lung injury during ARDS. During ARDS, TNF- α and IL-1 β are key

cytokines involved in inflammatory processes of ARDS.^{2,28} IL-1 β stimulates the production of a variety of chemotactic cytokines and directly causes injury to endothelial and epithelial cells.²⁹ TNF- α is not only an important pro-inflammatory cytokine, but also promotes apoptosis in ARDS.^{29,30} Furthermore, IL-8 is the most important chemokine of neutrophils, and promotes adherence, activation, and release of lysosomal enzymes of neutrophils during ARDS. In this study, budesonide significantly inhibited TNF- α , IL-1 β , and IL-8 production in BALF; decreased inflammation; and reduced endotoxin-induced lung injury, which was consistent with previous reports associated with budesonide applications for ARDS or lung inflammation after lung ventilation.^{16-18,25} Budesonide also inhibits T cell-mediated epithelial inflammation, as well as inflammatory neutrophil recruitment and alveolar macrophage infiltration.^{24,31,32} In addition to inhibiting pro-inflammatory factors, budesonide amelioration on ARDS also includes the promotion of budesonide on anti-inflammatory factors.²⁵ IL-10 can mediate anti-inflammatory responses through suppressing TNF- α and other pro-inflammatory cytokines.³³ Budesonide increases IL-10 levels, but decreases pro-inflammatory factors in serum.³⁴ Together, these data indicate that budesonide modulated immune cell response by adjusting pro-inflammatory cytokines and anti-inflammatory cytokines, and extended the immunoregulatory role of budesonide in different injury models.

Budesonide could reduce inflammation-induced lung injury.¹⁶⁻¹⁹ However, Liu et al. have shown that a high dosage of dexamethasone was ineffective in reducing phosgene-induced lung injury.³⁵ This may be due to the fact that phosgene-induced lung injury is non-inflammatory and cardiogenic, and the effect of corticosteroids in lung injury depends on its anti-inflammatory property. Moreover, among various lung injury factors including endotoxin, meconium, bleomycin, and chlorine, endotoxin is mostly used to model the consequences of bacterial sepsis, and it could mimic human ARDS caused by sepsis with high reproducibility.³⁶ ARDS were caused by meconium, bleomycin, and chlorine in only few patients, compared with endotoxin. In addition, the physiopathology of ARDS that was caused by these factors could not mimic human ARDS.³⁶

In conclusion, in this study, we demonstrated that budesonide could improve respiratory function, ameliorate lung injury and epithelial permeability, decrease edema, and inhibit local inflammation in an endotoxin-induced ARDS rabbit model. Therefore, budesonide inhalation is a potential approach for ARDS therapy in clinical practice. Results of this study may provide a new clinical therapeutic strategy for ARDS caused by lung infection.

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ETHICS APPROVAL

This study was approved by the Experimental Animal Ethics Committee of Harbin Medical University. In caring for the animals, investigators adhered to the Care and Use of Laboratory Animals prepared by the Experimental Animal Ethics Committee of Harbin Medical University.

Authors' contributions: WG and N-YJ carried out the immunoassays. WG and N-YJ participated in the design of the study, performed the statistical analyses, and drafted the manuscript. All authors read and approved the final manuscript.

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