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The roles of NF-kB in the development of lung injury after one-lung ventilation

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Abstract. – OBJECTIVE: To explore the roles of NF-kB in the development of lung injury after one-lung ventilation.

MATERIALS AND METHODS: Eighteen Sprague-Dawley (SD) rats were randomly divided into 3 groups including control group, one-lung ventilation (OL) group and NF-kB inhibitor pyrrolidine dithiocarbamate (PDTC) group, with 6 rats in each group. Rats in OL and PDTC groups were used to establish one-lung ventilation model, and rats in PDTC group were subjected to intravenous injection of NF-κB specific inhibitor PDTC at 30 min before model construction. One-lung ventilation was performed for 3 h, and arterial blood gas analyzer was used for blood gas analysis. The hemodynamics and respiratory mechanics parameters were detected. The respiratory index (RI) and oxygenation index (OI) were calculated. The pathological changes of lung tissue were observed by HE staining. The levels of TNF-α, IL-1β, IL-6, and IL-8 in lung tissue were detected by ELISA. The expression levels of p65, p-p65, p-IκBα and IκBα and the activity of NF-kB in lung tissue were detected by Western Blot.

RESULTS: Compared with OL group, HR, RI and W/D were significantly reduced and MAP and OI were significantly increased in PDTC group ($p<0.05$). Compared with OL group, alveolar fluid exudation, pulmonary interstitial thickening and inflammatory cell infiltration were significantly improved in PDTC group. The levels of TNF-α, IL-1β, IL-6 and IL-8 in PDTC group were significantly lower than in OL group ($p<0.05$). The ratios of p-p65/p65 and p-IκBα/IκBα and the activity of NF-kB in OL group were significantly reduced than in PDTC group ($p<0.05$).

CONCLUSIONS: NF-kB can promote lung injury after one-lung ventilation, and the inhibition of NF-kB may be a new way for the treatment of this disease.

Key Words:
One-lung ventilation, Lung injury, NF-kB, PDTC, Inflammatory response.

Introduction

One-lung ventilation (OLV) has been widely used in a variety of thoracic procedures, including lung, aortic, mediastinal and esophageal surgery[1]. Although one-lung ventilation is not a mandatory step for those surgical operations, it can always improve the access to operation field, which in turn shorten the operation time[2]. With the continuous increased expertise of anesthesiologists on the operations of double-lumen tubes, one-lung ventilation now has been used in most of the thoracic operations with lung involved[3]. Lung injury is one of the main causes of death after thoracic surgery. Recent investigations[4] have shown that high tidal volumes and over hydration of one-lung ventilation are closely correlated with the development of postoperative lung injury. Lung perfusion during one-lung ventilation can inevitably cause transpulmonary shunting, impaired or even hypoxemia[5], which in turn leads to the progression of lung injury. Therefore, how to reduce or avoid lung injury after one-lung ventilation has become a hot research field for the application of this technique. The development of lung injury after one-lung ventilation is a complex procedure, and inflammation plays an essential role in this process[6]. NF-kB pathway has been proved to play pivotal role in the development of various diseases including lung diseases[7,8]. It's well known that NF-kB can aggregate inflammatory response by regulating the expression of a variety of inflammatory factors[9,10]. In view of the pathogenesis of lung injury and the functionality of NF-kB pathway, it will be reasonable to propose that NF-kB pathway may participate in the progression of lung injury caused by one-lung ventilation, and the inhibition of NF-kB pathway may inhibit the development of postoperative lung injury.

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In this study, one-lung ventilation-induced lung injury was established, and NF-kB specific inhibitor PDTC was used to treat the rat model to investigate the roles of NF-kB in the development of lung injury after one-lung ventilation. We found that NF-kB pathway was activated after lung injury and NF-kB pathway inhibition significantly inhibited the development of lung injury from various aspects.

Materials and Methods

Animals

Specific-pathogen-free (SPF) grade Sprague-Dawley (SD) rats (200-220 g) were purchased from Institute of Zoology, Chinese Academy of Science. Rats were raised in SPF animal room at room temperature of 22-24°C and relative humidity of 50-60% for one week. Rats were allowed to access food and water freely. The present study was approved by the Animal Ethics Committee of our institute.

Animal Grouping and Treatment

Eighteen SD rats were randomly divided into control group, one-lung ventilation (OL) group and NF-kB inhibitor pyrrolidine dithiocarbamate (PDTC) group, with 6 rats in each group. Rats in OL group were fixed in supine position after anaesthesia through the intraperitoneal injection of pentobarbital (Jiangsu Hengrui Medicine Co., Ltd., Jiangsu, China) at a dose of 50 mg/kg. Right femoral vein was separated to establish venous channel and right femoral artery was separated to monitor arterial pressure. The main trachea was separated and cut to insert 3.5 mm endotracheal tube (Portex, Keene, NH, USA). After intravenous administration of pancuronium (Jiangsu Nhwa Pharmaceutical Co., Ltd, Jiangsu, China) at a dose of 2 mg/kg/h[11], right-lung mechanical ventilation was performed for 3 h. Ventilation parameters were: inhalation oxygen concentration (FiO2), 100%; RR, 60 times/min; tidal volume (VT), 10 ml/kg; suction ratio, 1:2. Intravenous infusion of 0.5% sodium pentobarbital for maintenance anesthesia, which was performed at a rate of 4 ml/kg/h[12] using micro-pump. Thirty minutes before model construction, rats in PDTC group were subjected to intravenous injection of NF-κB specific inhibitor PDTC (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 50 mg/kg. Rats in control group were treated with the same method described above except OLV.

Measurement of Hemodynamics and Respiratory Mechanics Parameters

The following variables were recorded at T0 and T1: heart rate (HR), mean arterial pressure (MAP), respiratory frequency (RR) and tidal volume (VT). The minute ventilation was calculated (MV) according to the formula: MV=VT×RR.

Determination of Respiratory Index (RI) and Oxygenation Index (OI)

Arterial blood samples (0.5 ml) were collected before (T0) and after mechanical ventilation (T1). Arterial blood gas analyzer was used for blood gas analysis to calculate RI and OI. RI=$P(A\text{-}a)O_2$/PaO_2, $P(A\text{-}a)O_2$ is the alveolar-arterial O_2 tension difference, and PaO_2 is partial pressure of oxygen. $P(A\text{-}a)O_2 = FiO_2 \times (PB\text{-}PaH_2O\text{-}PaCO_2)/R\text{-}PaO_2$, where PB is atmospheric pressure, PaH_2O is saturation pressure of water vapor, and R is ventilator. $OI = PaO_2/FIO_2$.

Determination of Wet/Dry (W/D) Ratio of Lung Tissue

After anesthesia through intraperitoneal injection of pentobarbital at a dose of 50 mg/kg, rats were sacrificed by cervical dislocation. Lung tissue was collected from right middle lobe and blood was removed using filter paper to measure the wet weight (W). Lung tissue was then baked in 80ºC incubator for 72 h to measure dry weight (D). W/D ratio = [W(mg)-D(mg)]/D (mg).

Histopathological Examination

The lung upper right lobe tissue was fixed in 4% neutral formaldehyde solution for 24 h; then, the tissue was dehydrated through a series of increasing alcohol concentrations. After paraffin-embedding, the tissue was cut into 5 μm slices. The tissue was transferred to the glass slides and hematoxylin and eosin (HE) staining was performed by using a kit (Beyotime, Jiangsu, China). Slices were sealed with neutral resin, and histopathological changes were observed under an optical microscopy (Olympus BX51, Olympus, Tokyo, Japan). The lung injury was scored[13] for edema, neutrophil infiltration, hemorrhage, bronchiole epithelial desquamation, and hyaline membrane formation. A score scaled at 0 to 4 represents the severity: 0 for no or very minor, 1 for modest and limited, 2 for intermediate, 3 for widespread or prominent, and 4 for widespread and most prominent. The cumulative total score was the lung injury score.

ELISA to Detect the Levels of TNF-α, IL-1β, IL-6, and IL-8 in Lung Tissue

After ventilation, right lung tissue was collected and ground, followed by centrifugation to collect supernatant. Levels of TNF-α (RTA00), IL-1β (RLB00) and IL-6 (R6000B) were detected by ELISA kits (R&D Co., Ltd., Minneapolis, MN, USA). Levels of IL-8 (ABIN4970046) were detected by ELISA kit (Abbexa Co., Ltd., Cambridge, UK). All operations were performed in strict accordance with the instructions of the kits.

Western Blot to Detect the Level of NF-kB Related Proteins and the Activity of NF-kB in Lung Tissue

The total protein was extracted from lung tissue which collected from right anterior lobe by using traditional method and protein concentration was measured by BCATM Protein Assay Kit (Pierce Biotechnology, Waltham, MA, USA). Then, 50 μg of protein were subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, followed by transmembrane under 20 V for 1 h to polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk, membranes were incubated with primary antibodies including rabbit anti-rat p-P65 antibody (1:2000, AF-3393, Affinity Biosciences, OH. USA), rabbit anti-rat P65 antibody (1:2000, 06-418, Calbiochem, Darmstadt, Germany), rabbit anti-rat IκBα antibody (1:1500, Assay-R12-2938, Los Angeles, CA, USA), rabbit anti-rat p-IκBα antibody (1:500, OM197719, OmnimAbs, Alhambra, CA, USA) and rabbit anti-rat nuclear factor (NF)-κB (1:500, MAB3026, Calbiochem, Darmstadt, Germany) overnight at 4ºC. After washing with TBST for 3 times, 10 min for each time, membranes were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (1:10000, AP187P, Calbiochem, Darmstadt, Germany) at room temperature for 1 h. After washing with TBST for 3 times, 10 min for each time, color development was performed with ECL. With β-actin as endogenous control, Image J 2.1 software was used to calculate the relative expression levels of each protein.

Statistical Analysis

All data were presented as mean ± standard deviation (SD). Data were analyzed with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Statistical comparisons between two groups were analyzed by *t*-test. Multiple comparisons were evaluated by repeated measures analysis of variance (ANOVA) followed by the LSD *t*-test or Kruskal-Wallis test. $p<0.05$ was considered to be statistically significant.

Results

Comparison of Hemodynamic and Respiratory Mechanics Parameters

As shown in Figure 1, there were no significant differences in HR, MAP, VT, and MV between groups at T0 ($p>0.05$). At T1, compared with Control group, MAP was significantly decreased in OL and PDTC groups ($p<0.05$), while HR was significantly increased in OL and PDTC groups ($p<0.05$). Compared with OL group, MAP was increased in PDTC group ($p<0.05$), while HR was decreased in PDTC group ($p<0.05$). There were no significant differences in VT and MV between groups at T1 ($p>0.05$).

Comparison of RI and OI Between Groups

As shown in Figure 2, no significant differences in PaO_2, $PaCO_2$, RI, and OI were found between groups at T0 ($p>0.05$). At T1, compared with Control group, $PaCO_2$, and RI were significantly increased in OL and PDTC groups ($p<0.05$); compared with OL group, $PaCO_2$, and RI were significantly reduced in PDTC group ($p<0.05$). Compared with Control group, PaO_2 and OI were significantly reduced in SL and PDTC groups ($p<0.05$). Compared with OL group, PaO_2 and OI were significantly increased in PDTC group ($p<0.05$).

Comparison of W/D Between Groups

As shown in Figure 3, compared with control group, W/D was significantly increased in OL group ($p<0.05$), while there were no significant differences in W/D between PDTC group and control group ($p>0.05$). Compared with OL group, W/D was significantly decreased in PDTC group ($p<0.05$).

Comparison of Lung Pathological Examination Results Between Groups

In control group, alveolar wall was thin, the endothelial cells of the lung tissue were intact, and no inflammatory cell infiltration was observed (Figure 4). In OL group: alveolar wall was thickened and edema was observed; alveolar structure was disordered and alveolar edema fluid exudation was found; serious inflammatory cell infiltration

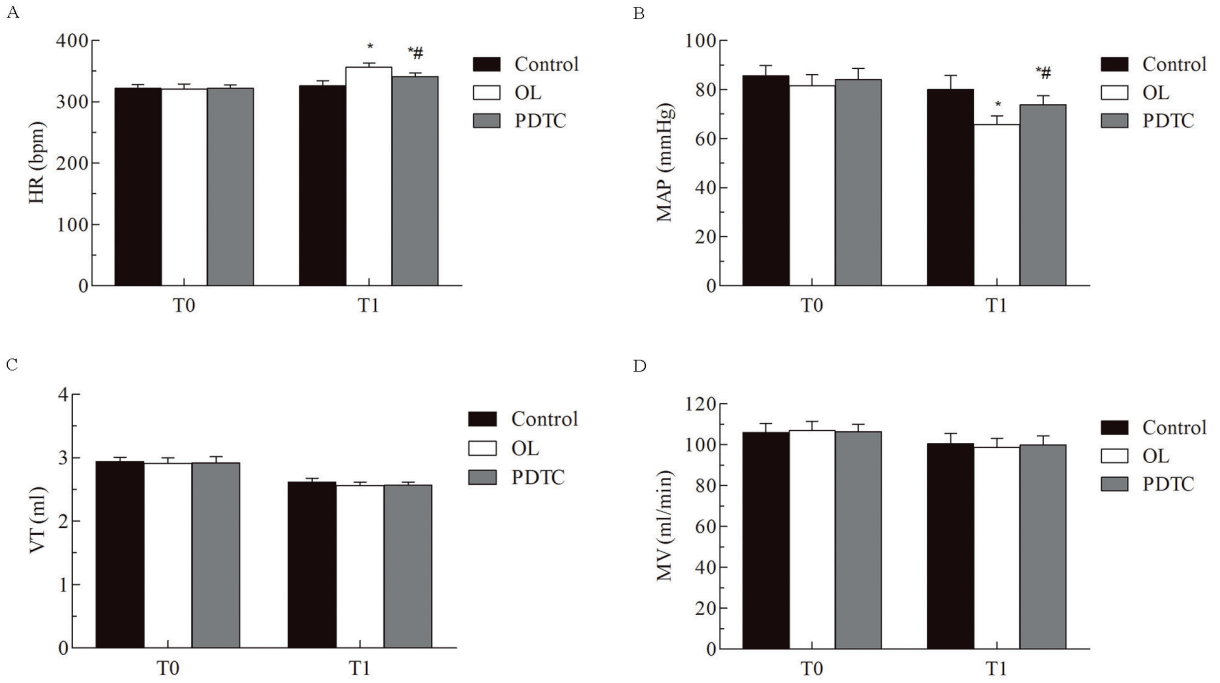

Figure 1. Comparison of hemodynamics and respiratory mechanics parameters between groups. ***(A)*** HR at different time points in each group. ***(B)*** MAP at different time points in each group. ***(C)*** VT at different time points in each group. ***(D)*** MV at different time points in each group. $^{*}p<0.05$, compared with control group; $^{\#}p<0.05$, compared with OL group.

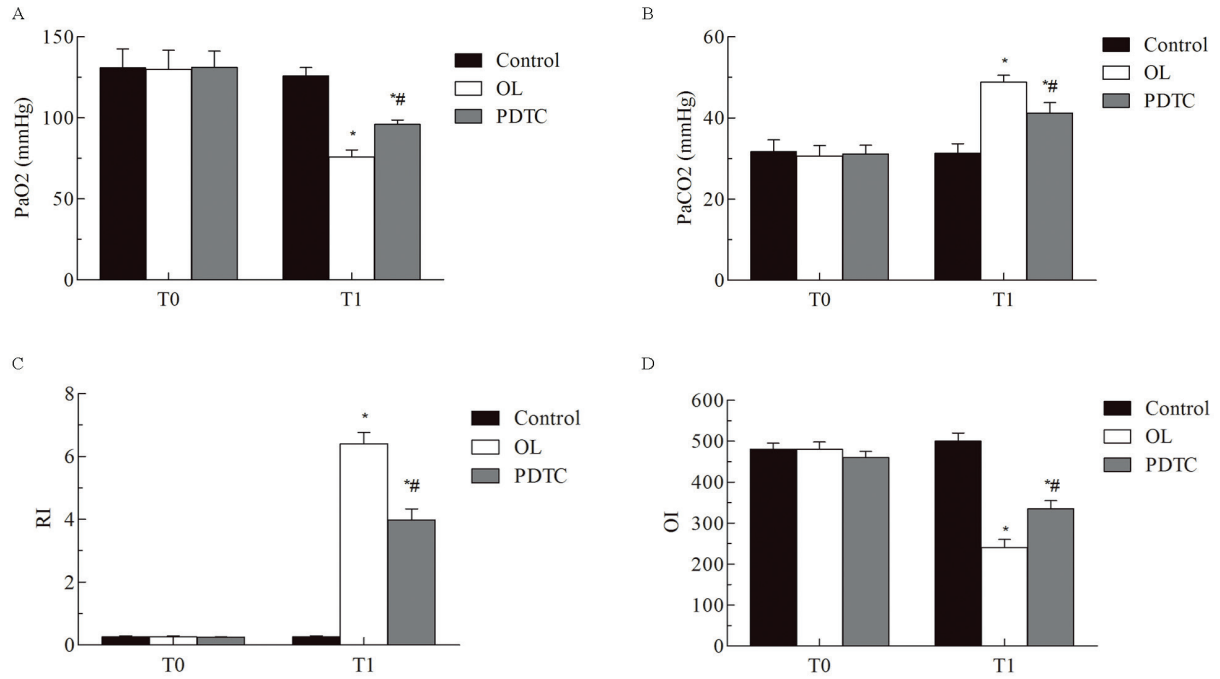

Figure 2. RI and OI at different time points in each group. ***(A)*** PaO_2 at different time points in each group. ***(B)*** $PaCO_2$ at different time points in each group. ***(C)*** RI at different time points in each group. ***(D)*** OI at different time points in each group. $^{*}p<0.05$, compared with control group; $^{\#}p<0.05$, compared with OL group.

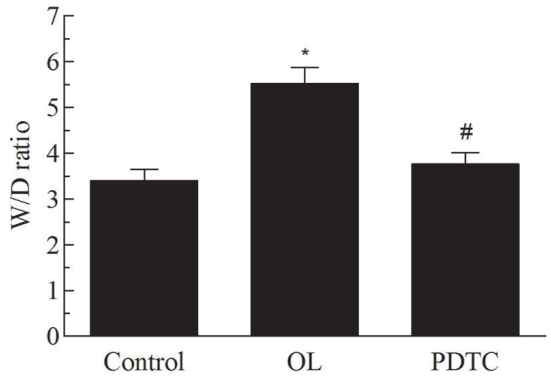

Figure 3. Comparison of W/D between groups. $^{*}p<0.05$, compared with control group; $^{\#}p<0.05$, compared with OL group.

was observed. Compared with OL group, alveolar fluid exudation, pulmonary interstitial thickening and inflammatory cell infiltration were significantly improved in PDTC group. Compared with control group, the score was significantly increased in OL and PDTC groups ($p<0.05$). Compared with OL group, the score was significantly decreased in PDTC group ($p<0.05$).

Comparison Levels of TNF-α, IL-1β, IL-6, and IL-8 Between Groups

The levels of TNF-α, IL-1β, IL-6, and IL-8 were detected by ELISA (Figure 5). Compared with control group, levels of TNF-α, IL-1β, IL-6, and IL-8 were significantly increased in OL group ($p<0.05$), while there were no significant diffe-

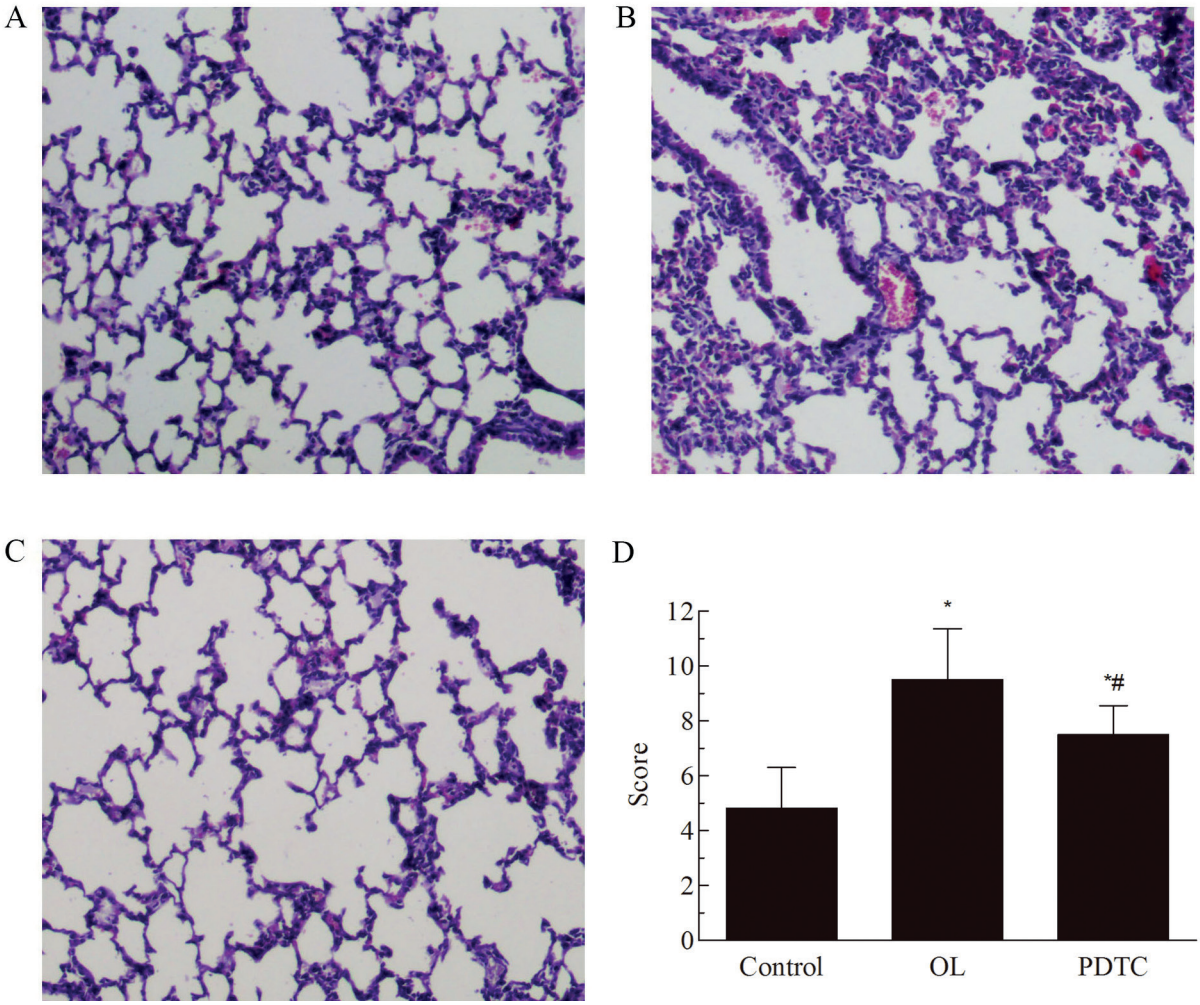

Figure 4. Comparison of lung pathological examination results between groups (×100). ***(A)*** Control group. ***(B)*** OL group. ***(C)*** PDTC group. ***(D)*** Lung tissue injury score. $^{*}p<0.05$, compared with control group; $^{\#}p<0.05$, compared with OL group.

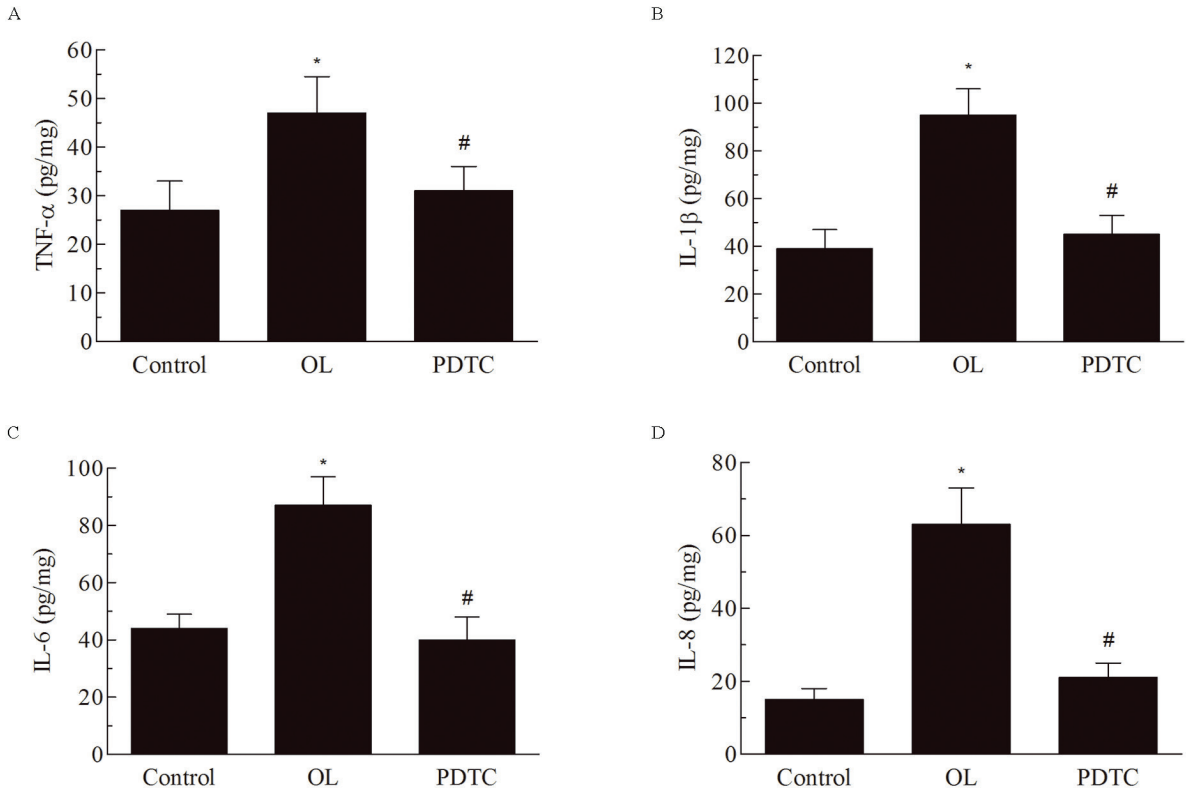

Figure 5. Comparison of levels of TNF-α, IL-1β, IL-6, and IL-8 between groups. ***(A)*** Comparison of levels of TNF-α in each group. ***(B)*** Comparison of levels of IL-1β in each group. ***(C)*** Comparison of levels of IL-6 in each group. ***(D)*** Comparison of levels of IL-8 in each group. $^{*}p<0.05$, compared with control group; $^{\#}p<0.05$, compared with OL group.

rences between PDTC group and control group. Compared with OL group, levels of TNF-α, IL-1β, IL-6, and IL-8 were significantly reduced in PDTC group ($p<0.05$).

Comparison of Expression Levels of NF-kB Pathway-Related Proteins and The Activity of NF-kB Between Groups

Expression levels of p-p65, p65, p-IκBα, and IκBα proteins and the activity of NF-kB were detected by Western blot, and the ratios of p-p65/p65 and p-IκBα/IκBα were calculated. As shown in Figure 6, compared with control group, ratios of p-p65/p65 and p-IκBα/IκBα were significantly increased in OL group ($p<0.05$), while there were no significant differences between PDTC group and control group ($p>0.05$). Compared with OL group, ratios of p-p65/p65 and p-IκBα/IκBα were significantly decreased in PDTC group ($p<0.05$). Compared with control group, protein expression of NF-kB was significantly increased in OL group ($p<0.05$). Compared with OL group, protein expression of NF-kB was decreased in PDTC group ($p<0.05$).

Discussion

The development of lung injury after one-lung ventilation is a complex process involving various factors. Smith et al[14] have shown that the release of excessive inflammatory cytokines caused by surgical manipulation, lung collapse, reperfusion and re-expansion contributed significantly to the development of lung injury after one-lung ventilation. TNF-α, IL-1β, IL-6, and IL-8 are four pro-inflammatory factors that achieve their biological functions by promoting inflammatory responses[15-17]. In this study, level of TNF-α, IL-1β, IL-6, and IL-8 were significantly increased in rats treated with one-lung ventilation, indicating the existence of inflammatory response in those rats. The results showed that hemodynamic parameters changed significantly after one-lung ventilation 3 h. HR of the rats with one-lung ventilation increased significantly and MAP decreased significantly. There were no significant differences in VT and MV. In addition, lower PaO_2 and higher $PaCO_2$ were observed in rats with one-lung ventilation. Respira-

tory index (RI) is a simple and practical indicator of lung ventilation and oxygen exchange function, while oxygenation index (OI) is usually used to evaluate the required intensity of ventilator for the maintenance of oxygenation. In general, higher RI and lower OI indicate better outcomes. In this study, lower RI and higher OI were observed in rats with one-lung ventilation, suggesting that the risk of death was increased by one-lung ventilation. Wet/dry ratio of lung tissue is a reliable index for detection of lung edema. In our study, the W/D ratio in OL group was significantly higher than that in the control group. In addition, pathological structure of lung tissue was also seriously affected after one-lung ventilation. All these data suggest that one-lung ventilation for 3 h can seriously affect lung function, which is consistent with previous studies that one-lung ventilation for 1 h can significantly increase the occurrence of lung injury[1]. NF-kB pathway plays pivotal roles in various biological processes including the development of lung injury induced by different factors. In the study of endotoxin-induced acute lung injury, Everhart et[18] reported that the sustained activation of NF-kB pathway was closely correlated with the degree of lung injury. As a transcription factor, NF-kB can regulate the expression of various genes involved in cell growth, immunity and inflammation[19], and many of those genes have been proved to be involved in the development of acute lung injury[20]. In our study, ratios of p-p65/p65 and p-IκBα/IκBα were significantly increased in rats treated with one-lung ventilation, indicating that NF-kB pathway was activated by one-lung ventilation. Researches have proved that the inhibition of NF-kB pathway can significantly improve the conditions of lung injury. Recently Wang et al[21] reported that apigenin treatment could significantly improve the conditions of acute lung injury by decreasing production of pro-inflammatory cytokines through the inactivation of NF-kB pathway. In the study of intestinal ischemia-reperfusion injury,

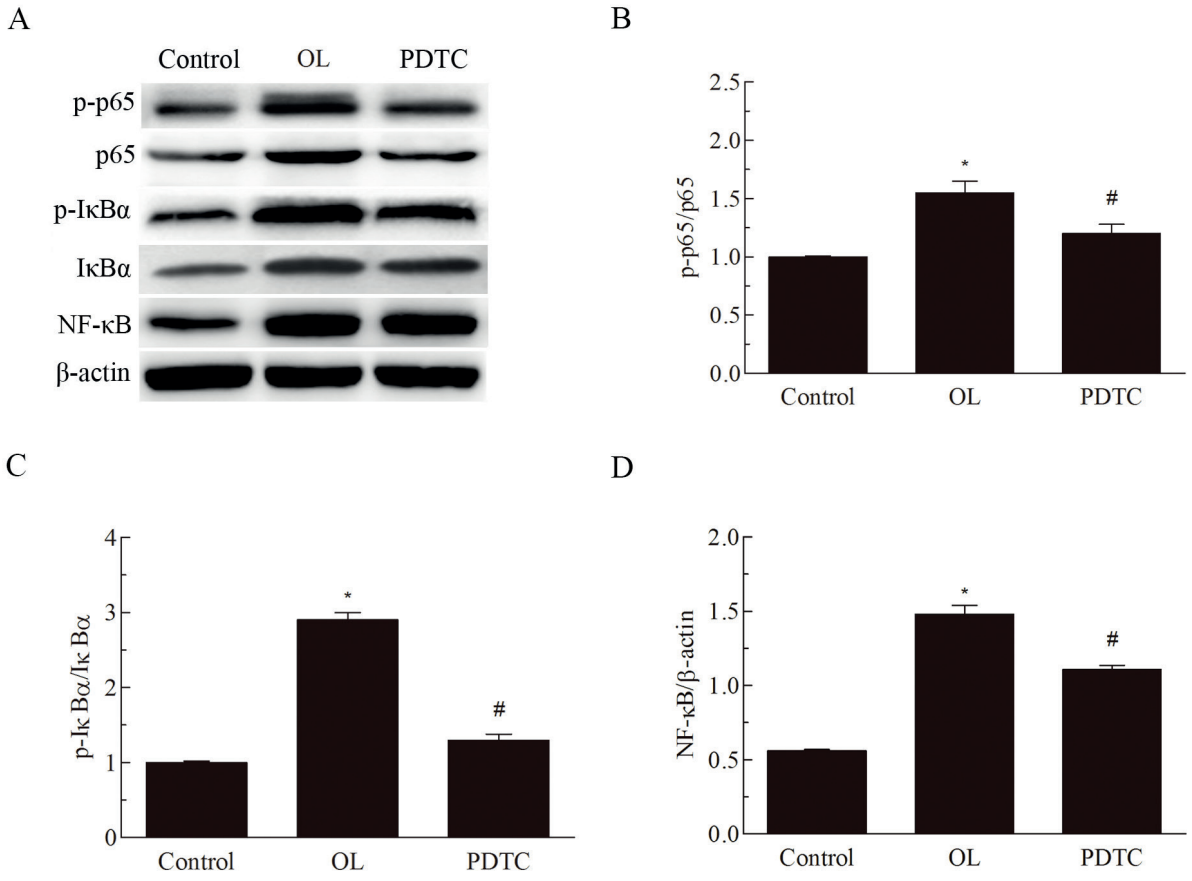

Figure 6. Comparison of expression levels of NF-kB and related proteins between groups. ***(A)*** Western blot results. ***(B)*** Comparison of ratios of p-p65/p65 between groups. ***(C)*** Comparison of ratios of p-IκBα/IκBα between groups. ***(D)*** The activity of NF-kB. $^{*}p<0.05$, compared with control group; $^{\#}p<0.05$, compared with OL group.

Fan et al[22] observed that curcumin treatment could significantly inhibit the formation of lung lesion, and this inhibitory function of curcumin was very likely to be achieved by inhibiting NF-kB pathway to reduce inflammatory response. In another study, Guo et al[23] reported that the downregulation of angiopoietin-like protein 4 can protect acute lung injury caused by lipopolysaccharide through the regulation of the signal transduction of SIRT1/NF-κB pathway. All those researches suggest that the inhibition of NF-kB signaling transduction pathway may also be a promising therapy for the treatment of lung injury caused by one-lung ventilation. As a specific inhibitor of NF-kB, pyrrolidine dithiocarbamate, or PDTC, has been proved to be effective in the treatment of various human diseases. A recent study found that PDTC could attenuate surgery-induced cognitive dysfunction and neuroinflammation possibly by inhibiting the activation of NF-kB[24]. In another study, Ivan et al[25] referred that PDTC treatment could be a promising approach to reduce UVB-induced skin oxidative stress and inflammation. In addition, the inhibition of NF-kB with PDTC has also been proved to be effective in alleviating acute lung injury induced by lipopolysaccharide[26]. By detecting the activity of NF-kB, we proved that it is feasible to use PDTC to inhibit NF-kB. In this study, PDTC was found to be able to significantly reduce the increased levels of pro-inflammatory factors TNF-α, IL-1β, IL-6, and IL-8 caused by lung injury after one-lung ventilation. In addition, PDTC also significantly improved the pulmonary function parameters as well as the pathological structure of lung tissue. All those results suggest that NF-kB is involved in the development of lung injury after one-lung ventilation, and the inhibition of NF-kB can significantly inhibit the progression of lung injury.

Conclusions

We have demostrated that the activation of NF-kB pathway is involved in the development of lung injury after one-lung ventilation possibly by increasing the levels of pro-inflammatory factors, and the inhibition of NF-kB pathway with PDTC could significantly inhibit the progression of postoperative lung injury. Our study provided a novel approach for the treatment of lung injury caused by one-lung ventilation. However, the conclusions of this work still need to be confirmed by clinical studies.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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