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Original Article

TLR4 contributes to mechanical ventilation induced lung injury in the rabbits

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Abstract: Background: TLR4 contributes to acute lung injury occurrence and development. However, the role of TLR4 in ventilator-associated lung injury remains unclear. This study aimed to detect TLR4 expression in the lung tissue subjected to mechanical ventilation and explore the relationship between TLR4 and VILI. Methods: 30 male health New Zealand rabbits were randomized into control, low tidal volume group and high tidal volume group. The control group received tracheal intubation but no mechanical ventilation while low tidal volume group and high tidal volume group received mechanical ventilation at 6 ml/kg and 20 ml/kg, respectively. PaO2, MDA content and SOD activity were measured. The expression of TLR4 and tumor necrosis factor α (TNF-α) in lung tissue was detected by immunohistochemical analysis. Results: The damages of lung tissue were severe in high tidal volume group, and PaO2, MDA content and SOD activity were significantly lower in high tidal volume group compared to control group and low tidal volume group ($P<0.05$). Furthermore, both TLR4 and TNF-α expression levels were significantly higher in high tidal volume group than in low tidal volume group and control group ($P<0.05$). Conclusions: During high tidal volume ventilation TLR4 expression in lung tissue is significantly increased, accompanied by pathological and biochemical changes in lung tissues. TLR4 may play an important role in the initiation and subsequent maintenance of VILI inflammation, and is a promising target for the prevention and treatment of VILI.

Keywords: Ventilator-induced lung injury, Toll receptor 4, tumor necrosis factor, mechanical ventilation

Introduction

As an important means in the treatment of various causes of respiratory failure, mechanical ventilation has been widely used in the clinical. However, improper use of mechanical ventilation will lead to ventilator-induced lung injury (VILI) [1]. Toll-like receptors (TLRs) are cell transmembrane pathogen pattern recognition receptors that recognize molecular patterns associated with microbial pathogens [2]. Different members of TLRs recognize different pathogens, for example, TLR4 is responsible for the recognition of the endotoxins of Gram-negative bacteria and forms the first line of defense against the pathogens by playing an important role in the innate immunity [3].

Recent studies show that TLR4 is involved in inflammation and contributes to acute lung injury occurrence and development [4]. However, the role of TLR4 in ventilator-associated lung injury remains unclear. Therefore, this study aimed to detect the expression of TLR4 in the lung tissue subjected to mechanical ventilation and explore the relationship between TLR4 and VILI.

Materials and methods

Animals and groups

Thirty healthy male adult New Zealand white rabbits (weight 2.0-2.5 kg) were provided by the Animal Experimental Center of Hubei Medical College, and randomly divided into control group, low tidal volume group and high tidal volume group (n=10). Control group: rabbits underwent intraperitoneal anesthesia using 10% chloral hydrate (5 ml/kg body weight), and underwent tracheotomy, intubation and the right carotid artery after successful anesthesia, without mechanical ventilation. Low tidal volume group and high tidal volume group: rabbits un-

Table 1. PaO2 in each group

Group	No.	PaO2 before experiment (mmHg)	PaO2 after experiment (mmHg)
Control	10	122.5±7.4	119.7±6.3
Low tidal volume	10	124.1±8.9	98.3±7.6*
High tidal volume	10	118.6±6.5	67.2±5.9*,#

*P<0.05 compared to control group, #P<0.05 compared to low tidal volume group.

derwent intraperitoneal anesthesia using 10% chloral hydrate (5 ml/kg body weight), and underwent tracheotomy, intubation and the right carotid artery after successful anesthesia, then underwent mechanical ventilation using the animal ventilator with the tidal volume of was 6 ml/kg body weight and 20 ml/kg body weight in low tidal volume group and high tidal volume group, respectively. The mechanical ventilation frequency was 50 beats/min, inspiratory to expiratory ratio was 1:3, FiO2 was 21%, and ventilation lasted 24 h. At the end of ventilation, all rabbits were killed and right lower lobe specimens were taken for pathological and biochemical analysis. Animal experiments were approved by animal ethics committee at the Zhongnan Hospital, Wuhan University.

Measurement of partial pressure of oxygen in arterial blood

Artery blood was taken from the rabbits before and after mechanical ventilation, and partial pressure of oxygen in arterial blood (PaO2) was immediately measured with a blood gas analyzer (i-SATA, USA).

Detection of oxidative stress

MDA content in the lung lysates was detected by using thiobarbituric acid (TBA) kit (Jiancheng Bioengineering Institute, Nanjing, China) and SOD activity in the lysates was detected by using Xanthine oxidase assay kit (Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocols.

Immunohistochemical analysis

Lung tissues were fixed in paraformaldehyde and paraffin embedded, and then cut into 4 μm thick serial sections. The sections were washed with phosphate buffered saline (PBS) three times, 10 min each time, and then blocked by incubation with 2% goat serum in PBS containing 0.3% Triton X-100 (PBS-X) for 1 h at room temperature. Next the sections were incubated with anti-rabbit TLR4 or anti-TNF-α primary antibody (Boster, Wuhan, China) at 4°C overnight, and then incubated with secondary antibody and DAB chromogen. The section were counterstained with Hematoxylin and eosin and observed under optical microscope. The staining was analyzed by Image-Pro 6.0 software.

Statistical analysis

Data were represented as x ± s and analyzed by SPSS13.0 software. The differences between groups were compared by using analysis of variance. P<0.05 was considered significant difference.

Results

PaO2 of lung tissues in each group

As shown in **Table 1**, before mechanical ventilation, no significant difference in PaO2 was observed among the rabbits in each group. However, after mechanical ventilation PaO2 decreased in both low tidal volume group and high tidal volume groups (P<0.05 vs. control group), and PaO2 decreased further in high tidal volume group (P<0.05 vs. low tidal volume group).

Histopathology of lung tissues in each group

In the lung tissues in control group, alveolar structure and bronchial epithelium were intact, no inflammatory cell infiltration was observed in interstitial lung (**Figure 1A**). In the lung tissues in low tidal volume group, only a small amount of inflammatory cell infiltration was observed, and there was no pulmonary interstitial edema (**Figure 1B**). In the lung tissues in high tidal volume group, diffuse pulmonary interstitial edema and inflammatory cell infiltration were observed, alveolar septal was thickened, the rupture of alveoli and alveolar hemorrhage were also observed (**Figure 1C**).

Oxidative stress in lung tissues in each group

As shown in **Table 2**, MDA content was significantly higher in low tidal volume group and high

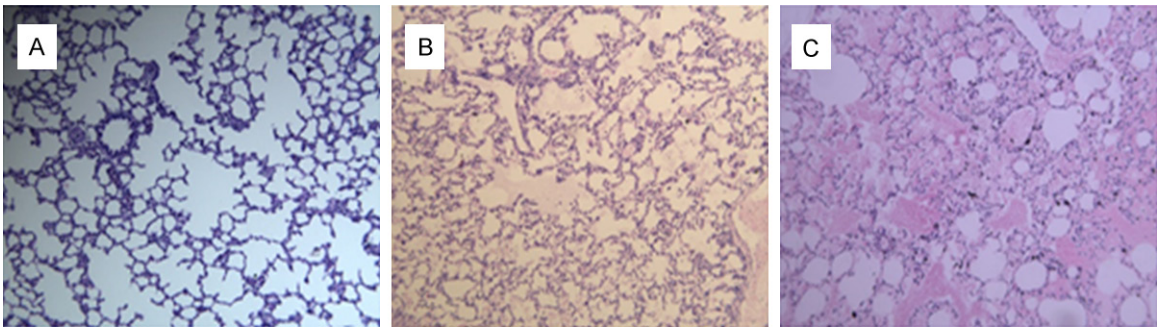

Figure 1. Histopathology of lung tissues in each group. A. Control group. B. Low tidal volume group. C. High tidal volume group. Shown were representative images of HE staining. Magnification fold: ×200.

Table 2. Oxidative stress in lung tissues in each group

Group	No.	MDA value (nmol/mg)	SOD activity (U/mg)
Control	10	5.24±0.81	108.96±10.72
Low tidal volume	10	7.20±0.97*	90.02±6.26*
High tidal volume	10	9.66±1.16*,#	70.46±9.25*,#

*P<0.05 compared to control group, #P<0.05 compared to low tidal volume group.

tidal volume group than in control group, and MDA content was significantly higher in high tidal volume group than in low tidal volume group. In contrast, SOD activity was significantly lower in low tidal volume group and high tidal volume group than in control group, and SOD activity was significantly lower in high tidal volume group than in low tidal volume group (P<0.01).

TLR4 and TNF-α expression in lung tissues in each group

Immunohistochemical analysis of TLR4 in lung tissues showed that TLR4 staining was very weak in control group, strong in low tidal volume group, and very strong in high tidal volume group (**Figure 2**). Similarly, immunohistochemical analysis of TNF-α in lung tissues showed that TNF-α staining was very weak in control group, strong in low tidal volume group, and very strong in high tidal volume group (**Figure 3**). Quantitative analysis of staining showed that both TLR4 and TNF-α expression levels were significantly higher in low tidal volume group and high tidal volume group than in control group. Furthermore, both TLR4 and TNF-α expression levels were significantly higher in high tidal volume group than in low tidal volume group control (**Table 3**). In addition, TLR4 expression level was positively correlated with TNF-α expression level, with a correlation coefficient of 0.947.

Discussion

VILI is a serious complication of mechanical ventilation, and numerous studies have shown that inflammatory cells and cytokines play an important role in the pathogenesis of VILI. A variety of inflammatory cytokines such as IL-6 and IL-8 are released after alveolar epithelium is mechanically pulled, leading to the recruitment of large numbers of neutrophils and macrophages in the lung and the resulting damages to alveolar epithelial and endothelial cells [5-7]. In this study we showed that in high tidal volume group, TNF-α expression in lung tissue was significantly increased. In addition, we observed diffused pulmonary edema and inflammatory cell infiltration in this group. These data indicate that mechanical ventilation induces inflammatory response in the lung.

Toll-like receptors are important molecular pattern recognition receptors of the innate immune cells and recognize molecular structure of bacteria surface to activate the innate immune system to produce pro-inflammatory cytokines [8]. At present toll-like receptors have been found to be distributed in various organs, and play important role independently or jointly in recognizing different pathogens. LPS is an important structure recognized by TLR4 and it induces the activation of TLR4 signaling pathway. In acute lung injury caused by bacterial infection, TLR4 initiates early inflammation and contributes to the occurrence and development of acute lung

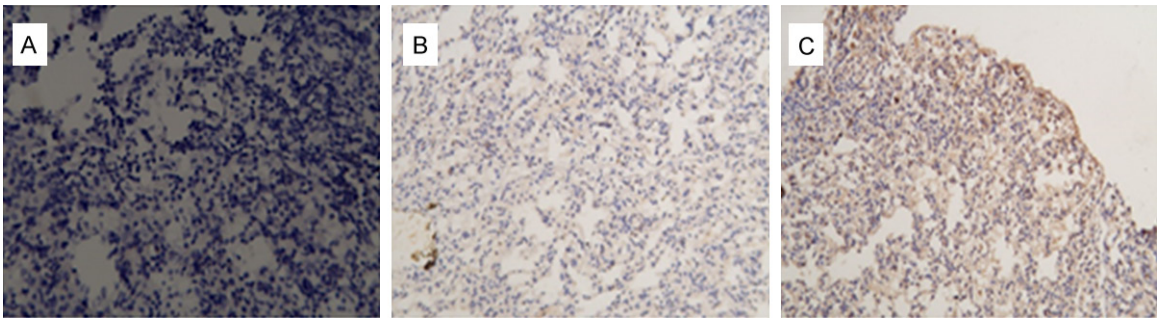

Figure 2. Immunohistochemical staining of TLR4 in lung tissues in each group. A. Control group. B. Low tidal volume group. C. High tidal volume group. Shown were representative images of TLR4 staining. Magnification fold: ×200.

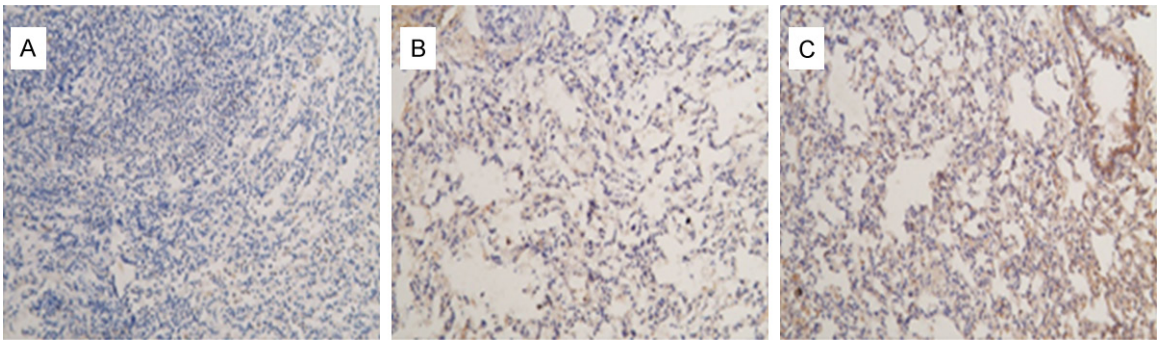

Figure 3. Immunohistochemical staining of TNF-α in lung tissues in each group. A. Control group. B. Low tidal volume group. C. High tidal volume group. Shown were representative images of TNF-α staining. Magnification fold: ×200.

Table 3. TLR4 and TNF-α expression in lung tissues in each group

Group	No.	Relative TLR4 level	Relative TNF-α level
Control	10	10.22±3.94	17.1±1.38
Low tidal volume	10	53.99±7.70*	71.2±24.7*
High tidal volume	10	151.43±20.56*,#	251.6±27.1*,#

*P<0.05 compared to control group, #P<0.05 compared to low tidal volume group.

injury [9]. In addition, a recent study showed that the inhibition of TLR4 ameliorated VILI [10]. Therefore, we hypothesized that TLR4 may play an important role in mediating VILI.

In this study we showed that TLR4 expression in lung tissues of high tidal volume group was significantly higher (P<0.05) than that of control group and low tidal volume group, and there was a positive correlation between TLR4 and TNF-α expression. The mechanism may be that the binding of TLR4 and ligand promotes the activation of NF-κB, which then activates the transcription of inflammatory cytokines such as TNF-α and IL-6. These inflammatory cytokines may further promote the release of TLR4, thus forming a vicious cycle for progressive inflammation [11]. Furthermore, we found that MDA content increased significantly while SOD activity decreased significantly in high tidal volume group, which indicates excessive oxidative stress during VILI. It was reported that active oxygen radicals can activate TLR4 mediated inflammatory signaling pathways [12]. Therefore, we speculate that oxidative stress induced by hyperoxide and peroxide in VI can lead to the activation of TLR4 mediated inflammation, thereby aggravating lung injury.

In summary, we found that during high tidal volume ventilation TLR4 expression in lung tissue was significantly increased, accompanied by pathological and biochemical changes in lung tissues. TLR4 may play an important role in the initiation and subsequent maintenance of VILI inflammation, and is a promising target for the prevention and treatment of VILI.

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Disclosure of conflict of interest

None.

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