Effects of dynamic ventilatory factors on ventilator-induced lung injury in acute respiratory distress syndrome dogs

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BACKGROUND: Mechanical ventilation is a double-edged sword to acute respiratory distress syndrome (ARDS) including lung injury, and systemic inflammatory response high tidal volumes are thought to increase mortality. The objective of this study is to evaluate the effects of dynamic ventilatory factors on ventilator induced lung injury in a dog model of ARDS induced by hydrochloric acid instillation under volume controlled ventilation and to investigate the relationship between the dynamic factors and ventilator-induced lung injuries (VILI) and to explore its potential mechanisms.

METHODS: Thirty-six healthy dogs were randomly divided into a control group and an experimental group. Subjects in the experimental group were then further divided into four groups by different inspiratory stages of flow. Two mL of alveolar fluid was aspirated for detection of IL-8 and TNF-α. Lung tissue specimens were also extracted for total RNA, IL-8 by western blot and observed under an electronic microscope.

RESULTS: IL-8 protein expression was significantly higher in group B than in groups A and D. Although the IL-8 protein expression was decreased in group C compared with group B, the difference was not statistically significant. The TNF-α ray degree of group B was significantly higher than that in the other groups (P<0.01), especially in group C (P>0.05). The alveolar volume of subjects in group B was significantly smaller, and cavity infiltration and cell autolysis were marked with a significant thicker alveolar septa, disorder of interval structures, and blurring of collagenous and elastic fiber structures. A large number of necrotic debris tissue was observed in group B.

CONCLUSION: Mechanical ventilation with a large tidal volume, a high inspiratory flow and a high ventilation frequency can cause significant damage to lung tissue structure. It can significantly increase the expression of TNF-α and IL-8 as well as their mRNA expression. Furthermore, the results of our study showed that small tidal ventilation significantly reduces the release of pro-inflammatory media. This finding suggests that greater deterioration in lung injury during ARDS is associated with high inspiratory flow and high ventilation rate.

KEY WORDS: Acute respiratory distress syndrome; Dynamic factors; Inspiratory flow; Ventilator-induced lung injury

INTRODUCTION

Mechanical ventilation plays an important role in the treatment of acute lung injury and acute respiratory distress syndrome (ALI/ARDS). However, if the ventilator parameters are set incorrectly in the course of treatment, it can cause ventilator-induced lung injury (VILI). A large number of basic experiments have confirmed that the static mechanical ventilation parameters, such as large tidal volume ventilation (VT) and high inspiratory pressure ventilation, which can
cause excessive local or general expansion of alveoli, which are contributive to VILI. ARDSnet has proposed that small tidal volumes and appropriate (PEEP) can reduce the occurrence of VILI. Inspiratory flow is an important determinant of lung mechanical stretch. High inspiratory flow can increase shear stress of alveolar surface and airway and cause deformation of bronchial epithelial cells. High ventilation frequency can increase shear stress, thus resulting in injury to the lung. Therefore, the dynamic factors can produce VILI through mechanical stretch which causes inflammation during mechanical ventilation. But few data are available at present. In this study, aspiration of hydrochloric acid is used to simulate an ARDS model in dogs in an attempt to determine the influence of different ventilatory frequencies and inspiratory flows on additional lung injuries resulted from the treatment of ARDS.

METHODS

Materials and reagents
Thirty-six healthy male mongrel dogs (11–13 kg, average 12.3±0.88 kg, from the Animal Center of School of Medicine Jiaotong University) were used. Goat anti-dog IL-8 antibody (RD Systems, Inc.), goat anti-dog TNF-α antibody (RD Systems, Inc.), mouse SP method II antibody kit, FITC labeled rabbit anti-mouse IgG (Beijing Zhong Shan Biological Reagent Co., Ltd.) were used in addition to DMEM medium, trypsin, HEPES, EGTA and others purchased from Shanghai Biological Engineering Company Limited.

Model establishment
Intravenous injection of 30 mg/kg sodium pentobarbital was used to anesthetize the dogs in preparation for orotracheal intubation. After intubation, the dogs were connected to mechanical ventilators (model PB 760, made in United States). The initial settings were as follows: VT 12 mL/kg, f 16 beats/min, FiO2 1.0, I: E 1:1.5. Under sterile conditions, an arterial line catheter was placed in the right femoral artery to measure arterial blood gas. A central line catheter was placed in the left femoral vein for fluid balance and inhibiting spontaneous breathing with 0.05% succinylcholine, a paralytic agent. When the animals were determined to be stable at PaO2 >400 mmHg, 2.0 mL/kg of hydrochloric acid at pH 1 was slowly introduced into the trachea. 1/3 of the volume was introduced into the dogs in the left lateral decubitus position and then 2/3rd in the right lateral decubitus position. When oxygenation index reached PaO2/FiO2 <200 mmHg, the level of respiratory system compliance reduced by more than 30%, creating the ARDS model.

Animal group
The dogs were randomly divided into 6 groups, with 6 dogs in each group: group N, normal control group; group M, ARDS group; and ventilation group (groups A-D). Group A: small VT, low inspiratory flow, high ventilation frequency; group B: large VT, high inspiratory flow, high ventilation frequency; group C: large VT, high inspiratory flow, low ventilation frequency; group D: large VT, low inspiratory flow, and low ventilation frequency. The factors are shown in Table 1. Lung mechanical parameters were recorded at 0, 1, 2 and 4 hours after the change in ventilatory protocol. The animals were sacrificed after 4 hours of mechanical ventilation, and lung tissues were retained for further examination.

Table 1. Ventilatory protocols and pressure parameters of each group

<table>
<thead>
<tr>
<th>Group</th>
<th>VT (mL/kg)</th>
<th>f (breath/min)</th>
<th>Inspiratory flow (mL/kg/s)</th>
<th>I/E</th>
<th>Inspiratory time (s)</th>
<th>Ppeak (±s, cmH2O)</th>
<th>PMAW (±s, cmH2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>30</td>
<td>6</td>
<td>1:1.0</td>
<td>1.0</td>
<td>9.75±0.31</td>
<td>4.60±0.17</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>30</td>
<td>20</td>
<td>1:1.0</td>
<td>1.0</td>
<td>21.00±0.93&quot;</td>
<td>6.37±0.34&quot;</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>15</td>
<td>17</td>
<td>1:2.3</td>
<td>1.2</td>
<td>19.17±0.91&quot;</td>
<td>5.50±0.43&quot;</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>15</td>
<td>10</td>
<td>1:1.0</td>
<td>2.0</td>
<td>17.67±0.84&quot;</td>
<td>6.33±0.47&quot;</td>
</tr>
</tbody>
</table>

Compared with group A, P<0.05, "P<0.01; compared with group D, P<0.05 versus; Ppeak: peak airway pressure; PMAW: mean airway pressure; 1 cmH2O=0.098 kPa.

Table 2. PCR amplification primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>5-ACTTCCAAGCTGGCTGTGC-3</td>
<td>5-GGCCACTGTCATCAGCTTC-3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5-CCAAGTGACAGCCAGTAGC-3</td>
<td>5-TCTTGATGGCAGAGAGTAGG-3</td>
</tr>
</tbody>
</table>

172 bp
274 bp
The dogs were euthanized quickly by exsanguination (the right femoral artery was opened). Then thoracostomy was performed under sterile condition and the entire right lung was removed immediately. The right lung lobes were divided into several small parts. They were placed into 0.1% DEPC-treated Eppendorf tubes and conventional-disinfection-treated Eppendorf tubes respectively. Then they rapidly placed in liquid nitrogen tanks, and transferred to a –80°C refrigerator for total RNA extraction. Samples of lung tissue were fixed with 4% paraformaldehyde for 24 hours, dehydrated by gradient alcohol, transparentized and embeded in paraffin for HE and IL-8 immunohistochemical staining. Other samples were fixed in 2.5% glutaraldehyde for later electron microscopic examination.

**Lung tissue IL-8, TNF-α protein level, and mRNA determination**

IL-8, TNF-α protein levels were determined by immunohistochemical staining and Western blotting. RT-PCR quantitative analysis of IL-8, TNF-α mRNA relative expression level, experimental PCR primers are shown in Table 2.

**Electron microscope observation**

Fixed lung tissue was treated by the following processes: glutaraldehyde—rinsed with PBS—osmium fixation—rinsed with PBS—progressively dehydrated with acetone—resin (such as EPON812)+acetone penetration—pure resin infiltration—embedding—aggregation—trim—ultrasection—lead citrate and uranyl acetatedye—electron microscope observation of ultrastructure changes of all lung tissue and pictures taken.

**Statistical analysis**

SPSS10.0 statistical software was used for data processing. Data of each group were represented by mean±standard deviation, and the data between the groups were analyzed by one-way ANOVA. \( P<0.05 \) was considered statistically significant.

**RESULTS**

**IL-8 and its mRNA expression**

Chemical staining of immune tissue showed dark brown positive staining of cytoplasm in groups A, B, C, D (Figure 1). The gray value of staining intensity of per unit area was determined using the graphic analysis system. The staining intensity of groups A and D (89.66±3.26, 92.89±4.46) was weaker than that of groups B and C (132.46±9.57, 126.29±5.53) (\( P<0.01 \)). There was no significant difference between groups B

![Figure 1](image-url). Immunocytochemistry for IL-8 in the lung (original magnification ×400). Staining intensity of groups A and D (89.66±3.26, 92.89±4.46) was weaker than that of groups B and C (132.46±9.57, 126.29±5.53) (\( P<0.01 \)), and there was no difference between groups B and C (\( P>0.05 \)).
and C ($P>0.05$). Protein imprint was normal; IL-8 band was found in the other groups except the control group. Analysis using an image analyzer revealed that IL-8 protein expression of group B was higher than that of groups A and D ($P<0.01$), and there was no significant difference between groups A and D ($P>0.05$). IL-8 protein expression of group C was decreased, but there was no significant difference between groups B and C (Figure 2). After PCR and agarose gel electrophoresis, IL-8 was found in the other groups at position 172 bp except the normal group. The IL-8 mRNA content of groups B and C (1.196±0.042, 1.146±0.033) was significantly higher than that of the other groups ($P<0.01$). Statistically, the difference of IL-8 mRNA content change between groups A and D was not significant ($P>0.05$) (Figure 3).

**TNF-α and its mRNA expression**

At 17 KD, specific protein bands can be observed, but no protein bands appeared in the normal group. Compared with β-actin protein band, the gray of group B was higher than that of the other groups ($P<0.01$), but there was no significant difference compared to group C ($P>0.05$) (Figure 2). Statistically, there was no difference in TNF-α protein between groups A and D ($P>0.05$). RT-PCR and gel electrophoresis at 274 bp plane showed specific TNF-α electrophoresis band. Analysis of gray and β-actin bands, the gray degree of group B was significantly higher than that of groups A and D ($P<0.01$) (Figure 3).

**Alveolar fluid IL-8 and TNF-α change**

Protein inspection of the upon alveolar fluid showed a little protein bands in groups B and C. As in Figure 4, IL-8 or TNF-α protein bands didn't exist in the other groups. Possibly, the level of IL-8 in the alveolar fluid in the other groups was too low to be observed.

**Electron microscopy result**

We didn't find any inflammatory cell infiltration in normal alveolar spaces. The alveolar septum was thin with a little of collagenous fibers, and normal type I and type II cells can be seen in group N. In group M, mitochondria in cytoplasm swelling can be seen. Endomembrane of the laminated body disappeared. There was vacuolization, but no inflammatory cell infiltration in normal alveolar spaces. In group A, alveolar spaces appeared to be smaller with inflammatory cell infiltration, thicker alveolar septa and more collagenous and elastic fibers. In group B, alveolar spaces became smaller more obviously, without type I and type II cells but inflammatory cell infiltration. Autocytolysis was seen with thickened alveolar septa, the disorganized inner structures of the alveolar septa, the vague structure of collagenous and elastic fibers, and a large number of necrotic debris. Bleeding occurred in the alveolar septa of group C, and a large number of infiltrated inflammatory cells could be found. The alveolar septa was thicker; while the number of collagenous and elastic fibers increased, type II alveolar epithelial cells were seen. In group D, inflammatory cell infiltration was not found in alveolar spaces. The structures of type I and II cells were complete with a thicker alveolar septa and a little bit increased collagenous and elastic fibers.
DISCUSSION

The mechanisms of VILI have been attributed to stress and strain by lung overstretching at high VT/pressure ventilation. During mechanical ventilation, the lung is subjected to mechanical forces that produce overstretching, compression, and shear stress on bronchial and alveolar structures. Overstretching by high VT may produce lung edema due to physical injury to alveolar-capillary membrane. In this study, we found that large VT, high inspiratory flow and high ventilation frequency increased Ppeak which induced endothelial cells and tissue destruction.

These results suggest that in a normal lung tissue, TNF-α and IL-8 are at a very low level with low expression of their mRNA. The concentration of mRNA cannot be measured by using the aforementioned experimental methods. Based on the same small tidal volume, the difference in inspiratory flow rate and respiratory rate led to differences in TNF-α and IL-8 of lung tissue among groups B, C, and D. TNF-α and IL-8 levels were the highest in lung tissues of group B, and mRNA expression was also the highest. Compared with group B, mRNA expression in group C was markedly decreased but with no other obvious differences. However, the difference between groups C, D, and A was significant. Dogs in group A adopted the publicly recognized small tidal volume model with a permissive hypercapnea protective ventilation method. In group A, the TNF-α, IL-8 protein content and its mRNA expression in the lung tissues increased when compared with the model group, but far lower than groups B and C (P<0.05). Dogs in the group D adopted low inspiratory flow rate and respiratory rate on the basis of large tidal volume, and the results showed that the TNF-α, IL-8 protein content and its mRNA expression in lung tissue decreased. This suggests that high inspiratory flow rate and high respiratory rate can increase the release of inflammatory mediators, induce gene expression, while lower inspiratory flow rate and respiratory rate can prevent the release of inflammatory mediators. Inspiratory flow rate is an important determinant of stress in the lung. High inspiratory flow enhances tensile stress across alveolar surfaces, resulting in greater transmission of kinetic energy to underlying structures despite the same volume change. High inspiratory flow also increases shear stress parallel to the surface of the airways and alveolar walls, distorting lung parenchyma and deforming bronchial epithelial cells with little change in volume.

In this study, small quantities of IL-8 and TNF-α protein bands were found in the alveolar liquid of groups B and C. This may be due to a low sample volume or lung injury. Other studies of lung injuries that result from the use of different ventilation strategies showed that using large tidal volumes can increase the
concentration of TNF-α in BALF. Some studies reported no increase. The reason is that the only occurrence of endotoxin and lung injury will lead to the release of TNF-α.

Electron microscopy results revealed that autolysis and necrosis in group B indicate that high inspiratory flow rate and high respiratory rate result in high shear forces within lung parenchyma. As a result, the production of pulmonary surfactant decreased, the damage to lung epithelial cells increased, and the alveolar surface tension and capillary permeability also increased. This ultimately leads to the formation of pulmonary edema.

This experiment also indicates that large tidal volume ventilation, high inspiratory flow rate and high respiratory rate can lead to cell deformation and necrosis. The study suggests that the strength and magnitude of periodic cell deformation is harmful to lung epithelial cells as static deformation, and may lead to more cell death. In the present study, there were more inflammatory cell infiltration and fibrosis but cell autolysis in group C. This indicates that lowering the frequency of lung ventilation may be protective under large tidal volume. In group A, the swelling of alveolar epithelial cells was not obvious, implying that a small tidal volume ventilation strategy is protective in nature.

Most current studies aim to find that large tidal volume and high inspiratory end pressure can induce VILI. Rich et al. studied VILI using animal models by adopting high tidal volumes and found that at low tidal volumes, the respiratory rate does not affect the expression of inflammatory mediators. In the present study, TNF-α and IL-8 in the high respiratory rate group increased significantly, while the TNF-α and IL-8 expression decreased in the presence of low respiratory rate.

Our experiment confirmed that ventilation injury can promote the aggregation pro-inflammatory cells such as neutrophils and macrophages within the alveolar and its interstitium. Mechanical ventilation can induce neutrophils (representing the main effect cells of lung injury), alveolar epithelial cells (another potential cell releasing inflammatory mediator) to release inflammatory mediators and increase inflammation. Activated neutrophils produce a variety of cytokines, including pro-inflammatory cytokines like TNF-α, IL-1, IL-8 and TGF-β and anti-inflammatory cytokines like TGF-β. Once the hemodynamics of pro-inflammatory cytokines and anti-inflammatory cytokine balance is out of control, expression of inflammatory cytokines increases, resulting in an increase in inflammatory lung injury. IL-8 is the strongest chemotactic factor when ALI/ARDS occurs. With the expression of IL-8 increases, there is an increase in neutrophil recruitment and activation. Under the chemotactic influence of IL-8, not only the neutrophils enter the pulmonary/alveolar endothelium, IL-8 also stimulates cell degranulation within the respiratory system, releasing a large amount of harmful media which results in lung injury. In addition, TNF-α can further activate neutrophils forming a vicious circle.

In conclusion, high tidal volume, high inspiratory flow, and high frequency ventilation strategy can result in serious barotrauma and volume injury. It also promotes inflammatory cell infiltration and activation within the lung tissue. These effects lead to bio-traumatic VILI through a series of injuries. Thus, it is important to reduce inspiratory flow and respiratory rate while using high tidal volumes to prevent any potential lung injury.

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Contributors: Wang RL proposed and wrote the paper. All authors contributed to the design and interpretation of the study and to further drafts.

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