Effects of mechanical ventilation of isolated mouse lungs on surfactant and inflammatory cytokines

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ABSTRACT: Mechanical ventilation of the lung is an essential but potentially harmful therapeutic intervention for patients with acute respiratory distress syndrome. The objective of the current study was to establish and characterize an isolated mouse lung model to study the harmful effects of mechanical ventilation.

Lungs were isolated from BalbC mice and randomized to either a non-ventilated group, a conventionally ventilated group (tidal volume 7 mL·kg⁻¹, 4 cm positive expiratory pressure (PEEP)) or an injuriously ventilated group (20 mL·kg⁻¹, 0 cm PEEP). Lungs were subsequently analysed for lung compliance, morphology, surfactant composition and inflammatory cytokines.

Injurious ventilation resulted in significant lung dysfunction, which was associated with a significant increase in pulmonary surfactant, and surfactant small aggregates compared to the other two groups. Injurious ventilation also led to a significantly increased concentration of interleukin-6 and tumour necrosis factor-α in the lavage.

It was concluded that the injurious effects of mechanical ventilation can effectively be studied in isolated mouse lungs, which offers the potential of studying genetically altered animals. It was also concluded that in this model, the lung injury is, in part, mediated by the surfactant system and the release of inflammatory mediators.


Recent clinical studies have indicated that the mechanical ventilation strategy utilized in patients with acute lung injury (ALI) and Acute Respiratory Distress Syndrome (ARDS) may have significant impact on patient outcome [1, 2]. A recent multi-centre, NIH-sponsored clinical trial demonstrated that ventilation with a tidal volume of 6 mL·kg⁻¹ significantly reduced mortality in patients with ARDS compared to a tidal volume of 12 mL·kg⁻¹ [3]. However, not all studies utilizing low tidal volume ventilation have demonstrated significant reductions in mortality [4, 5]. This apparent contradiction among clinical studies may be related to a number of factors that may have been different among studies, such as disease severity, specific ventilatory strategies and the specific patient populations.

One of the factors complicating design and interpretation of clinical studies is the fact that there is still an incomplete understanding of the mechanisms by which mechanical ventilation affects the lung. As such, clinical trials usually focus on blood gases and/or other physiological parameters to monitor their ventilatory strategies. With a more detailed knowledge on how injurious ventilation affects the lung, it may be possible to develop biochemical approaches to indicate whether a specific ventilation strategy is injurious to the lung, prior to gross physiological abnormalities.

A number of animal models have been developed to examine the mechanisms of ventilator-induced lung injury [6-9]. The authors previously studied a non-perfused ex vivo rat lung model to examine mechanical and inflammatory factors causing lung injury [9, 10], and to identify inflammatory cytokines associated with injurious ventilatory strategies. One insightful approach to identifying important basic mechanisms is to use transgenic and knock out animals in which specific genes are altered or deleted. Since the most common transgenic animal is the mouse, the objective of the present study was to develop an isolated mouse lung model to investigate injurious mechanical ventilation strategies. Similar to previous studies, the authors chose the isolated lung model to avoid systemic responses to the different ventilation strategies and therefore isolate lung-specific responses to mechanical ventilation. Furthermore, since there is contradicting data regarding the effects of mechanical ventilation on pulmonary surfactant [11, 14], the authors chose to examine how injurious ventilation affected the pulmonary surfactant system in mice along with lung morphology, compliance and the concentrations of inflammatory cytokines in the lung.

Methods

Animal preparation

Lungs were harvested from normal male BalbC mice (22-28 g). The mice were anaesthetized using an intraperitoneal injection of pentobarbital sodium...
(70 mg·kg⁻¹). The mice were then sacrificed by cutting the descending aorta, an endotracheal tube was then inserted and the lungs were rapidly excised via a midline sternotomy. The excised lungs were randomized into one of three groups: 1) a control group which was not ventilated; 2) a conventional mechanical ventilation group (CMV), in which the lungs were placed in a 37°C humidified chamber and ventilated. The first five inflations consisted of 20 µL each, followed by inflations of 50 µL volumes until a pressure of 25 cmH₂O was reached. Subsequently lungs were deflated in a similar stepwise fashion while pressures were recorded.

**Assessment of lung injury**

Five lungs from each group were analysed for lung compliance using static pressure-volume curves. These curves were constructed by connecting the lungs to a syringe-pump and pressure-transducer. Following two inflations to a pressure of 25 cmH₂O, the lungs were inflated with air in a stepwise fashion with the pressure being recorded following each inflation. The mean airway pressures were monitored and recorded at 30 min intervals. The animal procedures were approved by the animal-use subcommittee of the University of Western Ontario.

**Morphological analysis**

Following the measurement of the pressure-volume curves, the lungs were inflated to a pressure of 15 cmH₂O and then fixed at this pressure by submerging the inflated lung in 10% neutral buffered formalin. The lungs from each animal were sectioned sagittally and embedded in tissu for histological evaluation. Sections were cut at 5 µm thickness and stained with haematoxylin and eosin stain. The lungs were examined and the degree of lung injury was scored as: normal, mild, moderate or severe, taking into consideration several different features of early lung injury such as atelectasis, overdistension, congestion haemorrhage and oedema. The distribution of injury within the lung was assessed as either diffuse (uniform involvement) or patchy (non-uniform involvement). All assessments were carried out by a pathologist blind with respect to the experimental groups.

**Pulmonary surfactant analysis**

For analysis of the surfactant system, the isolated lungs from each of the three groups were lavaged using three washes of 1 mL 0.15M NaCl. Each wash consisted of instilling the saline, withdrawing it and re-instilling and withdrawing it twice more. The recovered lavages were combined and the total volume was recorded. The lavage was then spun at 150 × g for 10 min to remove cellular debris. The 150 × g supernatant was utilized for measurement of total surfactant, surfactant protein D, and phosphatidylcholine species determination. In addition, a 1-mL aliquot of the 150 × g supernatant was used to separate the large surfactant aggregates (LA) from the small surfactant aggregates (SA) via centrifugation at 40,000 × g for 15 min [15]. The pellet of the 40,000 × g spin (the LA fraction) was resuspended in 300 µL 0.15M NaCl and frozen at -20°C until further use. The SA-fraction was also stored at -20°C.

Phospholipid-phosphorus measurements were used to determine the amount of surfactant and its subfractions. Aliquots of the different samples were extracted as described by Blas and Dyer [16]. The phospholipid-containing chloroform-phase was then dried and utilized for determination of phosphorus using a modification of the method of Duck-Chong [17].

Electrospray ionization mass spectrometry was carried out in the positive ion mode on a triple quadrupole instrument (model api 365, Sciex, Concord, ON, Canada). This method allows the detection and quantification of several of the molecular species of phosphatidylcholine present in the 150 × g supernatant [18]. For this analysis, approximately 10 µg phospholipid was extracted using the method of Blas and Dyer [16]. The chloroform extract was dried and the lipids were resuspended in 900 µL chloroform-methanol (1:8). Immediately prior to analysis 100 µL 0.01 M NaOH was added to the sample, and the sample was injected into the mass spectrometer at a liquid flow rate of 10 µL·min⁻¹. The peak intensities on the mass spectrum were used to calculate the relative amounts of the major phosphatidylcholine species. These included PC, palmitoyl-phosphatidylethanol PC, dipalmitoyl-phosphatidyl PC (DP-PC), palmitoyl-oleoyl PC and palmitoyl-oleoyl PC.

Surfactant protein D (SP-D) was analysed by sandwich enzyme-linked immunosorbent assay (ELISA) using a polyclonal rabbit anti-rat SP-D antibody. Rabbit anti-rat SP-D polyclonal antibodies were diluted (1:400) in 0.1 M NaHCO₃-buffer (pH 9.6) and coated overnight at 4°C on a polystyrene 96 well microtitre plate (maxisorb, NUNC). The microtitre plates were blocked with 2% BSA in washing solution (50 mM Tris-HCL, 150 mM NaCl, 0.05% Tween 20, pH 7.4, 200 µL·well⁻¹) for 60 min at room temperature followed by six washes. Samples and standards, consisting of pooled normal mouse lavage, were diluted (1:2, 1:4 and 1:8) in washing solution containing 0.1% BSA. The diluted samples and standards were applied to the microtitre plate (50 µL·well⁻¹) and incubated for 1 h at room temperature. Plates were washed and 50 µL·well⁻¹ biotin-conjugated anti-rat SP-D was applied to the plate for 1 h. After washing, the plates were incubated with horseradish peroxidase conjugated streptavidin (0.1 mg·mL⁻¹, Sigma Chemical Co. St Louis, MO, USA), diluted in washing solution containing 0.1% BSA (50 µL·well⁻¹, room temperature, 1 h). Subsequently, the plates were washed and concentration of SP-D was determined by measuring the bound horseradish peroxidase using 150 µL·well⁻¹
tetramethylbenzidine reagent (100 mg·mL⁻¹ in 1 mM H₂O₂ in 0.1 M citric acid buffer, pH 4.0). The reaction was stopped by adding 50 μL 2 M H₂SO₄ and absorption was measured at 450 nm. SP-D levels were determined relative to the pooled mouse lavage standards.

**Cytokine analysis**

A separate cohort of animals (n=7/group) was used to measure the concentrations of the pro-inflammatory cytokines tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and the anti-inflammatory cytokine, interleukin-10 (IL-10). For these analyses lungs were lavaged with two 1-mL aliquots of 0.15 M NaCl. The lavage was then centrifuged at 200 x g for 10 min at 4°C and the supernatant was aliquoted into 350 μL aliquots that were immediately frozen in liquid nitrogen and then stored at -70°C. The concentrations of these cytokines in the lavages were measured by opti-eta kits from Pharmingen, using the instructions provided by the supplier.

**Statistics**

All data are expressed as mean±SEM. Statistical analysis was performed using the SPSS-statistical software package for Windows, version 9.0.0 (SPSS Inc, Chicago, IL, USA). Differences among ventilation strategies were examined by analysis of variance with a Tukey post hoc analysis. Probability values lower than 0.05 were considered statistically significant.

**Results**

A total of 69 mice were used in this study. Due to surgical complications resulting in air leaks, six lungs were eliminated from the study, before being randomized to any of the three groups. The resulting isolated lungs were randomized to one of the three experimental groups outlined in the methods. Lungs randomized to one of the two ventilated groups were placed in the humidified chamber and ventilated for 2 h. At the start of ventilation, lungs in the CMV group had an average peak airway pressure of 14.9±0.8 cmH₂O which increased significantly during the ventilation period to 18.7±0.8 cmH₂O. The lungs ventilated with the injurious strategy (IMV) had an initial peak airway pressure of 26.3±1.0 cmH₂O which significantly increased to 31.7±0.7 cmH₂O (p<0.05) during the 2 h of ventilation. Comparisons of the subsets of lungs used for either surfactant analysis, morphological assessment or cytokine measurements revealed that within each ventilation group there were no significant differences in peak airway pressures.

Figure 1 shows the pressure-volume curves from the lungs of the three experimental groups (n=5/group). On the inflation limb of the curve, the control group had significantly higher volumes than the other two groups starting at a pressure of 14 cmH₂O and continuing until the highest pressure (25 cmH₂O). Comparison between the two ventilated groups revealed that the CMV group had significantly higher volume only at a pressure of 25 cmH₂O. During deflation, the control group remained significantly more inflated than the other two groups until a pressure of 9 cmH₂O for the CMV group and 5 cmH₂O for the IMV group. The CMV group had significantly higher volumes than the IMV groups from 25–8 cmH₂O.

Representative light micrographs of the lungs from the three different groups are shown in figure 2. Lungs were fixed after inflation to 15 cmH₂O and lungs from the control group showed no evidence of lung injury (fig. 2). Lungs from the CMV and IMV groups showed similar histological features of injury with moderate atelectasis, overdistension and congestion and mild haemorrhage in patchy distribution (fig 2b and c). There was no evidence of inflammatory infiltrates in any of the lungs examined.

The amounts of total surfactant as well as large (LA) and small (SA) surfactant aggregates are shown in figure 3. In the control group the total amount of surfactant was 6.0±0.8 mg phospholipid·kg⁻¹ bodyweight which consisted of 52±4% LA. The CMV group had amounts of surfactant and surfactant subfractions that were not significantly different than the control. Significantly more surfactant was obtained from the lavage of the lungs in the IMV group (p<0.05 versus control and CMV). Analysis of the surfactant subfractions revealed that increase in total surfactant was due to a significant increase in the SA fraction compared to the control and CMV group. This also resulted in a significantly lower percentage of LA present in the lavage of the IMV group compared to the two other groups (34±2% versus 52±4% and 50±4% for control and CMV respectively (p<0.05)).

Extracted aliquots of the total surfactant was analysed for the relative amount of dipalmitoylphosphatidylcholine (DPPC) by ion-spray mass spectrometry. A total of six specific phospholipid peaks were analysed and their relative amounts are shown in
normal controls. The results are shown in table 1. Compared to the control group, both ventilatory groups had consistently lower concentrations of SP-D. However, due to the variable levels of SP-D in the control group this difference was not statistically significant.

A separate group of lungs were used to analyse TNF-α and IL-6 concentrations in the lung lavage (fig. 5). The results revealed that the IMV group had significantly higher TNF-α concentrations compared to the other groups (fig. 5a). The concentrations of IL-6 in the IMV and CMV groups were significantly higher than the control group. There was no significant difference between the CMV and IMV groups. The concentrations of the anti-inflammatory cytokine IL-10 was also measured in the lavage fluid of the three experimental groups. These concentrations were not significantly different among the three groups (226±56 pg/mL,

130±30 pg/mL and 257±64 pg/mL for control, CMV and IMV respectively).

**Discussion**

Mechanical ventilation remains a necessary therapeutic intervention for patients with ALI and ARDS [19]. A significant role for mechanical ventilation in the
progression of lung injury, and possibly in the progression of ARDS into multi-organ failure, has been suggested [20]. Mechanical ventilation may affect the lung through the release of inflammatory cytokines and through alterations of the pulmonary surfactant system [9, 11, 12, 14, 21–23]. In the current study an isolated mouse lung model was utilized to study the effects of mechanical ventilation on these two aspects of lung homeostasis. The isolated lung model was chosen specifically to address the lung-specific effects of ventilation without the influence of other systemic factors. Previously, a similar model was utilized using isolated rat lungs [9, 10] and it was decided to adapt this model to mice, in order to, in future studies, be able to make use of genetically modified animals.

The authors were specifically interested in how injurious ventilation affected the surfactant system and the inflammatory cytokines in these isolated mouse lungs. There is abundant evidence in the literature that high tidal volume ventilation without sufficient end-expiratory pressure is harmful to the lung [6, 8–10]. However, there is significant species variability regarding the specific levels of tidal volume that are needed to produce lung injury.

Preliminary experiments were performed in which isolated mouse lungs were ventilated with a variety of tidal volumes. A tidal volume of 30 mL·kg⁻¹ and 40 mL·kg⁻¹, which has been used in isolated rat lungs,

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<th>Table 1. – Relative amounts of surfactant protein D (SP-D) in two ventilation groups</th>
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Data represent mean±SEM percent of control. CMV: conventional mechanical ventilation; IMV: injurious mechanical ventilation.

![Fig. 4. – Relative amounts of phosphatidycholine species. Five phospholipid species were determined lyso-phosphatidylycholine (Lyso-), dipalmitoyl-PC (16:0-16:0) palmitoyl-palmitoleoyl PC (16:0-16:1) palmitoyl-myristoyl PC (16:0-14:0) and palmitoyl-oleoyl PC (16:0-18:1) by ion-spray mass spectrometry. (I): control, (II): conventional mechanical ventilation (CMV), (III): injurious mechanical ventilation (IMV). *: p<0.05 versus CMV.](image1)  

![Fig. 5. – Concentrations of a) tumour necrosis factor (TNF)α and b) interleukin (IL)-6 in the lung lavage obtained from the three experimental groups control, conventional mechanical ventilation (CMV) and injurious mechanical ventilation (IMV). Cyto-kine concentrations were determined by ELISA. *: p<0.05 versus CMV and control.](image2)

also caused significant lung injury in these mouse lungs. However, in approximately 60% of the lungs these strategies resulted in air leaks. It was therefore decided to use a tidal volume of 20 mL·kg⁻¹, 0 cmH₂O PEEP as the injurious strategy. This injurious strategy was compared with a more conventional ventilation strategy; a tidal volume of 7 mL·kg⁻¹, 4 cmH₂O PEEP. These two groups were ventilated for 2 h at 37°C and were compared to a nonventilated control group.

The results showed that 2 h of injurious ventilation of isolated mouse lungs led to a decreased compliance consistent with lung injury. Light microscopy confirmed this lung injury, although significant morphological evidence of lung injury was also observed in the CMV group. However, compared to the CMV group, the lung injury in the IMV group was associated with increases in inflammatory cytokines and also significant alterations of the surfactant system. This suggests that these two effects contribute to the development of lung dysfunction in this model.

Alterations of surfactant have been reported in studies of patients with ARDS [24–26], animal models of ARDS [15, 27–29], and in response to ventilation in rabbits and rats [11–14]. This study is the first to evaluate the effects of ventilation on pulmonary surfactant in mouse lungs. Pulmonary surfactant consists of phospholipids and surfactant associated proteins.
and can be separated into two subfractions, the LA and SA. The LA represent the active form of surfactant whereas the SA are not surface active [30]. The decreased percentage of LA in the injured group is consistent with a number of observations in both ARDS models and in ventilated rabbits [11, 12, 15, 29]. Considering the evidence that stretching causes surfactant secretion [31, 32], it is reasonable to assume that LA secretion occurs during high tidal volume ventilation and is responsible for the increased total amount of surfactant. During high tidal volume ventilation, these LA will be converted to the inactive SA form resulting in an observed increase in SA and, as a consequence, the decreased percentage of LA. Interestingly, the percentage of large aggregates in the injured group was due to an increased amount of SA obtained from these lungs; the actual amount of LA was similar in the injured group when compared to the other two groups. Although it is well established that SA do not reduce surface tension, it is not known if an increased amount of SA in the lungs can interfere with the function of the LA. This possibility requires further investigation.

As mentioned, the surfactant results are consistent with previous observations. However, in the previous study using isolated rat lungs; it was found that injurious ventilation (40 mL·kg⁻¹ · 0 cmH₂O PEEP) actually increased the percentage of LA [14]. The reason for this difference is not known. Interestingly, surfactant analysis in rat models of lung injury, such as systemic sepsis, ozone-exposure and radiation pneumonitis have also demonstrated an increased percentage of LA [27, 33–36]. Although in other rat studies decreases in LA have been observed [13, 37], it appears that the metabolism of surfactant aggregates in rats is different than that of other species, including humans. Therefore, studies to examine the effects of ventilation on surfactant aggregates in the mouse, appear to be more reflective than those observed in other species.

An obvious disadvantage of the use of mice to study the surfactant system is the low amount of material that is recovered from these small lungs. Classical chromatographic methodologies to study surfactant composition would require a large number of pooled lung lavages to obtain reliable data [38]. A relatively new approach for compositional analysis of surfactant was recently reported by Postle et al. [18], namely ion spray mass spectrometry. This technique offers a sensitive and accurate alternative to chromatographic methods and can be performed on individual mouse samples. Using this technique the authors determined the amount of the main surface tension reducing component of surfactant namely dipalmitoylphosphatidylcholine (DPPC) relative to the other main phosphatidylcholine species in surfactant. A significantly lower percentage of DPPC was found in the IMV group compared to the CMV group; Previous studies have focused mainly on the effects of mechanical ventilation on total surfactant, surfactant subfractions and surfactant activity. The compositional data provided here demonstrate that mechanical ventilation can also significantly affect the actual phospholipid-composition of surfactant. These compositional changes could occur through increases in phospholipase-A2 activity in the airspace, which would result in an increased lyso-PC, or through alterations in the biosynthetic pathways of surfactant.

In addition to the alterations of pulmonary surfactant, ventilation induced increases in the concentration of the two pro-inflammatory cytokines, TNF-α and IL-6 in the lung lavages were also detected. In contrast, the anti-inflammatory cytokine, IL-10, did not appear to be affected by mechanical ventilation. In general, the role of the inflammatory cytokines in ventilation induced lung injury and potentially in the development of multi-organ failure is not clear [20]. However, this study confirms the previous observations in isolated rat lungs, that the mechanically ventilated lung can be a significant source of inflammatory mediators [9, 39]. The impact of these mediators on lung compliance and/or on surfactant alterations requires further study. The current mouse model offers an advantage over the previously used models for such studies since it offers the feasibility of using transgenic animals deficient in a specific mediator. These types of experiments may help elucidate whether inflammatory cytokines are the result of ventilation induced lung injury or if they are active components contributing to the lung injury.

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