Lung protective mechanical ventilation
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Citation for published version (APA):
Chapter 12

A Physiological Model of Ventilator–Induced Lung Injury in Mice

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Submitted for publication
Abstract

**Background:** In most models of ventilator–induced lung injury (VILI) unphysiological large tidal volumes ($V_t$) are being used. Too large $V_t$ potentially lead to hemodynamic instability and as such may hamper extrapolation of study results to the clinical setting. In addition, animals frequently develop metabolic acidosis which may influence endpoints of VILI.

**Methods:** We set up a clinical relevant model of VILI using more physiological $V_t$ in 2 mice strains, C57Bl/6 and BALB/c mice. Baseline parameters were obtained from spontaneous breathing mice and used as a reference. Mechanically ventilated mice were randomized into low $V_t$ (~7.5 ml/kg) or high $V_t$ (~15 ml/kg). In addition, normal saline or sodium bicarbonate to correct for hypovolemia were compared.

**Results:** With saline infusion animals developed severe metabolic acidosis. Infusion of sodium bicarbonate completely compensated for the metabolic acidosis. With high $V_t$, VILI developed in both mice strain.

**Conclusion:** To facilitate translation of findings in animal studies on mechanical ventilation into the clinical setting, we advocate the use of more physiological $V_t$ in models of VILI. Although endpoints of VILI were not importantly influenced by the type of fluid administered, we recommend to use sodium bicarbonate instead of normal saline as fluid to prevent metabolic acidosis.
Introduction

Mechanical ventilation (MV) is mandatory during general anesthesia and is an indispensable tool in the treatment of patients with respiratory failure. However, MV can induce lung injury in healthy lungs or aggravate pre-existing lung injury, a phenomenon known as ventilator–induced lung injury (VILI) in animals, or ventilator–associated lung injury (VALI) in humans [1-3]. Overdistension and shear stress forces generated with MV have been implicated as the cause of VILI [2,4-7]. Clinical studies in patients with acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) demonstrate that lung–protective MV with the use of lower tidal volumes (V_t) reduces mortality and attenuates inflammation [1,8-11].

An incremental approach to the management of respiratory failure requires adequate animal models to test various treatment strategies. However, existing animal models have considerable imperfections. In most animal models of VILI, very high V_t and inspiratory pressures have been used [12-16]. Too high V_t may compromise systemic circulation, eventually leading to shock. In some models of VILI a fall in arterial blood pressure is even part of the protocol [15,16]. It is difficult to extrapolate results from such studies into human clinical practice, as clinicians have widely adopted protective ventilation strategies in patients with ALI. Another problem may be that infusion of saline solution to correct for low arterial blood pressures leads to metabolic acidosis in models of VILI [17,18]. Metabolic acidosis may influence several endpoints of VILI.

The aim of this investigation was to set up a model of VILI, which closely reflects the clinical setting. In 2 commonly used mice strains 2 clinically relevant V_t-settings were compared. We closely monitored whether the chosen MV–strategies influenced hemodynamics. In addition, we compared intraperitoneal administration of either normal saline or sodium bicarbonate to correct for hypovolemia.

Materials and Methods

The study was approved by the Animal Care And Use Committee of the Academic Medical Center. Animal procedures were carried out in compliance with Institutional Standards for Human Care and Use of Laboratory Animals.

Animals

Experiments were performed in male C57Bl/6 mice (n = 66) and BALB/c mice (n = 66, Charles River, Someren, the Netherlands), aged 8 – 10 weeks, with weights ranging from 19 to 25 gram. Two groups of control animals served either as non–ventilated controls for blood gas analysis at baseline (n = 6 for each strain) or as non–ventilated controls after 5 hours (n = 6 for each strain). The other animals were all mechanically ventilated with 2
different MV–strategies (see below) and 2 different fluid support strategies (see below). Thus, 6 groups of animals of each strain were formed.

**Instrumentation and anesthesia**

Throughout the experiments rectal temperature was maintained between 36.5 – 37.5°C using a warming path. Anesthesia was achieved with intraperitoneal injection of a mix of ketamine (Eurovet Animal Health B.V., Bladel, the Netherlands), medetomidine (Pfizer Animal Health B.V., Capelle a/d IJssel, the Netherlands), and atropine (Pharmachemie, Haarlem, the Netherlands) (KMA). Induction anesthesia consisted of 126 mg/kg ketamine, 0.2 mg/kg medetomidine and 0.5 mg/kg atropine. Maintenance anesthesia consisted of 36 mg/kg ketamine, 0.04 mg/kg medetomidine and 0.075 mg/kg atropine. Maintenance–mix was administered via an intraperitoneal catheter (PE 10 tubing, BD, Breda, the Netherlands) every hour.

**Mechanical ventilation strategies**

A Y–tube connector, 1.0 mm outer diameter and 0.6 mm inner diameter (VBM Medizintechnik GmbH, Sulz am Neckar, Germany) was surgically inserted into the trachea under general anesthesia. Mice were placed in a supine position and connected to a human ventilator (Servo 900 C, Siemens, Sweden). Simultaneously, 6 mice were pressure controlled ventilated with either an inspiratory pressure of 10 cmH₂O (resulting in V₅ ≈ 7.5 ml/kg; low V₅, LV₅) or an inspiratory pressure of 18 cmH₂O (resulting in V₅ ≈ 15 ml/kg; high V₅, HV₅). In C57BL/6 mice, respiratory rate was set at 120 breaths/min and 70 breaths/min with LV₅ and HV₅, respectively; in BALB/c mice, respiratory rate was set at 100 breaths/min and 70 breaths/min with LV₅ and HV₅, respectively. These respiratory settings resulted in normal PaCO₂-values after 5 h of MV. Positive end expiratory pressure (PEEP) was set at 2 cmH₂O with both MV–strategies. The fraction of inspired oxygen was kept at 0.5. The inspiration to expiration ratio was kept at 1:1 throughout the experiment. A sigh (sustained inflation with 30 cmH₂O) for 5 breaths was performed every 30 minutes.

**Fluid support strategies**

Mice received an intraperitoneal bolus of 1 ml normal saline 1 hour before start of MV, followed by 0.2 ml normal saline or 0.2 ml sodium bicarbonate administered via the intraperitoneal catheter every 30 minutes.

**Hemodynamic and ventilatory monitoring**

Systolic blood pressure and heart rate were non–invasively monitored using a murine tail–cuff system (ADInstruments, Spenbach, Germany). Blood pressure and pulse were measured directly after start of MV, after 2.5 and 5 hour of MV. The data were recorded on a data acquisition system (PowerLab/4SP, ADInstruments). Systolic blood pressure and heart rate were averaged from three consecutive measurements.
VT was checked hourly with a plethysmograph system. A minimum of 5 consecutive breaths was selected for analysis of the digitized VT-signals.

**Study groups**

Non-ventilated control mice for blood gas analysis at baseline (for both strains n = 6): animals were handled one week before the experiment to decrease stress activation. After induction of anesthesia with isoflurane arterial blood was taken from the left ventricle by heart puncture within 30 seconds.

Non-ventilated control mice after 5 hours (for both strains n = 6): mice received half the dose of induction anesthesia, were spontaneously breathing and sacrificed after 5 hours.

LVT—mice receiving either normal saline (n = 12) or sodium bicarbonate (n = 12) and HVTL—mice receiving either saline (n = 12) or sodium bicarbonate (n = 12) were mechanically ventilated for 5 hours and then sacrificed. Half of these mice were sacrificed and blood was drawn from the vena cava inferior into a sterile syringe, transferred to EDTA–coated tubes and immediately placed on ice. Blood samples of 2 mice were pooled together. Subsequently, bronchoalveolar lavage fluid (BALF) was obtained from the right lung; the left lung was used to measure wet/dry ratios. The other half of these mice were used for blood gas analysis. Blood was sampled from the carotid artery. The lungs of these mice were used for homogenate (right lung) and histopathology (left lung).

For wet/dry ratios the left lung was weighed and subsequently dried for three days in an oven at 65°C. The right lung was removed and snap frozen in liquid nitrogen. These frozen specimens were suspended in 4 volumes of sterile isotonic saline and subsequently lysed in 1 volume of lysis buffer (150 mM NaCl, 15 mM Tris [tris(hydroxymethyl)aminomethane], 1 mM MgCl₂H₂O, 1mM CaCl₂, 1% Triton X-100, 100 μg/ml pepstatin A, leupeptin and aprotinin, pH 7.4) and incubated at 4°C for 30 min. Homogenates were spun at 3400 rpm at 4°C for 15 minutes after which the supernatants were stored at –20°C until assayed.

BALF was obtained by instilling three times 0.5 ml aliquots of saline by a 22-gauge Abbocath–T catheter (Abbott, Sligo, Ireland) into the trachea. Approximately, 1.0 ml of BALF was retrieved per mouse and cell counts were determined using a hemacytometer (Beckman Coulter, Fullerton, CA). Subsequently, differential counts were done on citospin preparations stained with a modified Giemsa stain, Diff–Quick (Dade Behring AG, Düdingen, Switzerland). Supernatant was stored at -80°C for total protein level, thrombin–antithrombin complexes (TATc) and plasminogen activator inhibitor (PAI)–1 measurements.

For histopathology lungs were fixed in 4% formalin and embedded in paraffin. 4μm sections were stained with hematoxylin–eosin (H&E), and analyzed by a pathologist who
was blinded for group identity. To score lung injury we used a modified VILI histology scoring system as previously described [12].

Assays

Total protein levels in BALF were determined using a Bradford Protein Assay Kit (OZ Biosciences, Marseille, France) according to manufacturers’ instructions with bovine serum albumin as standard. Cytokine levels in lung homogenates were measured by enzyme–linked immunosorbent assay (ELISA) according to the manufacturer’s instructions. Tumor necrosis factor (TNF)–α, interleukin (IL)–6, macrophage inflammatory protein (MIP)–2 and keratinocyte–derived chemokine (KC) assays were all obtained from R&D Systems (Abingdon, UK). TATc levels in BALF were measured with a mouse specific ELISA as previously described [19]. Levels of PAI–1 were measured by means of a commercially available ELISA (Kordia, Leiden, the Netherlands).

Statistical analysis

All data in the results are expressed as means ± SD or median ± interquartile range, where appropriate. To detect differences between groups, the Dunnett method and Dunn method were used, in conjunction with one–way analysis of variance and Kruskall–Wallis tests. A p-value of < 0.05 was considered significantly. All statistical analyses were carried out using SPSS 12.0.2 (SPSS, Chicago, IL).

Results

Hemodynamic and ventilatory monitoring

All mechanically ventilated animals survived 5 hours of MV after which they were sacrificed. Control animals survived anesthesia and were also sacrificed after 5 hours. Hemodynamic monitoring demonstrated stable conditions throughout the experiment (figure 1): systolic blood pressure and heart rate remained stable in all animals for the complete duration of MV, with no differences noted between the two MV–strategies. Also, no hemodynamic differences were noted between animals that received normal saline and animals infused with sodium bicarbonate.

While blood gas analysis from LV–mice and HV–mice using normal saline revealed metabolic acidosis after 5 hours of MV, with the use of sodium bicarbonate blood gas analysis after 5 hours of MV was normal to near normal (table 1 and 2). Arterial oxygen tension in C57Bl/6 mice was significantly higher in HV–mice as compared to LV–mice (p < .001). No differences regarding oxygenation were found between MV–groups in BALB/c mice.
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Figure 1 C57BI6 mice (A and C) and BALB/c mice (B and D): Hemodynamic parameters during 5 hours of mechanical ventilation: Systolic blood pressure and heart rate (beats/min) was measured at three time points (t = 0, 2.5 and 5 hours after start of mechanical ventilation) in mice ventilated with low tidal volumes (VT) and high VT, and with two different fluid therapies (normal saline; NaCl and sodium bicarbonate; NaBic). Data represent means of 12 mice.

Table 1 Arterial blood gas analysis in C57BI6 mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low VT NaCl</th>
<th>Low VT NaHCO₃</th>
<th>High VT NaCl</th>
<th>High VT NaHCO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.34 (0.05)</td>
<td>7.42 (0.05)</td>
<td>7.11 (0.07)</td>
<td>7.37 (0.08)</td>
<td></td>
</tr>
<tr>
<td>PaCO₂ (mmHg)</td>
<td>39.3</td>
<td>35.3 to 39.5</td>
<td>41.2 to 51.3</td>
<td>40.8 to 44.3</td>
<td></td>
</tr>
<tr>
<td>PaO₂ (mmHg)</td>
<td>193 (36)</td>
<td>168 (48)</td>
<td>173 (51)</td>
<td>161 (50)</td>
<td></td>
</tr>
<tr>
<td>HCO₃ (mmol/l)</td>
<td>21.1</td>
<td>23.6 to 25.9</td>
<td>14.4 to 24.1</td>
<td>25.2 to 14.7</td>
<td></td>
</tr>
<tr>
<td>BE</td>
<td>-3.9</td>
<td>-12.3</td>
<td>0.15</td>
<td>-15.9</td>
<td>-0.7</td>
</tr>
</tbody>
</table>

Data are mean (SD) or median [IQR]; Control, spontaneously breathing mice; Low VT, mice ventilated for 5 h with a VT of 7.5 ml/kg; High VT, mice ventilated for 5 h with a VT of 15 ml/kg. n = 6 per group. * p < .001 vs. control mice.
Table 2 Arterial blood gas analysis in BALB/c mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low Vt</th>
<th>High Vt</th>
<th>NaCl</th>
<th>NaHCO₃</th>
<th>NaCl</th>
<th>NaHCO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.42 (0.04)</td>
<td>7.17 (0.07)*</td>
<td>7.41 (0.07)</td>
<td>7.23 (0.06)*</td>
<td>7.49 (0.02)</td>
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<tr>
<td>PaCO₂ (mmHg)</td>
<td>34.4</td>
<td>50.1</td>
<td>45.0</td>
<td>33.7</td>
<td>31.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCO₃ (mmol/l)</td>
<td>21.4</td>
<td>16.6</td>
<td>28.0</td>
<td>14.6</td>
<td>24.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BE</td>
<td>-1.3</td>
<td>117</td>
<td>4.1</td>
<td>-12.8</td>
<td>2.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are mean (SD) or median [IQR]; Control, spontaneously breathing mice; Low Vt, mice ventilated for 5 h with a Vt of 7.5 ml/kg; High Vt, mice ventilated for 5 h with a Vt of 15 ml/kg. n = 6 per group. * p < .05; † p < .001 vs. control mice.

Lung injury with different tidal volumes settings

In C57Bl/6 mice lung wet/dry ratios were significantly higher with both MV–strategies compared with controls. Wet/dry ratios in HV–mice did not differ from LV–mice. For BALB/c mice only higher lung wet/dry ratios were found in HV–mice (figure 2A), and differences between LV–mice and HV–mice were statistically significance (p < .05). In accordance, total BALF–protein levels were significantly higher in HV–mice as compared to control in both mice strains (figure 2B).

![Figure 2](image-url)
In BALB/c mice total BALF–protein levels were statistically different between LV,–mice and HV,–mice (p < .05). The numbers of neutrophils in BALF were significantly higher in HV,–mice as compared to control mice in both mouse strains (figure 2C). Neutrophil count in BALF from HV,–mice did not differ from LV,–mice. The histopathological changes were minor. For both mice strain the pulmonary histopathology score was higher in both MV–groups as compared to controls.

Figure 3 C57BL/6 mice and BALB/c mice: Pulmonary levels of interleukin (IL)–6 (A), tumor necrosis factor (TNF)–α (B), macrophage inflammatory protein (MIP)–2 (C) and keratinocyte–derived chemokine (KC) (D) in lung tissue homogenate after 5 hours of mechanical ventilation in control (C) mice, mice ventilated with low tidal volumes (LV,.) and high V, (HV,.), and with two different fluid therapies (normal saline; NaCl and sodium bicarbonate; NaBic). Data represent median and interquartile range of six mice. *p < .05; †p < .01; ‡p < .001 vs. control.
Mechanically ventilated mice in the HV₁-group of both mice strain demonstrated higher pulmonary levels of TNF-α, IL-6, MIP-2 and KC as compared to control mice (figure 3).

Pulmonary MIP-2 levels were higher in LV₁-mice as compared to control in both mice strain. IL-6 levels were also higher in LV₁-mice, but only in BALB/c mice. For IL-6 there was a statistically significant difference between LV₁- and HV₁-mice (p < .01 for both mice strain). Systemic levels of IL-6 and KC were elevated in the both ventilation groups, with higher levels in the HV₁-group (Figure 4). Plasma levels of TNF-α and MIP-2 were below the detection limit of the assay.

TATc–levels in BALF were significantly higher in HV₁-mice in both mice strains as compared to control (figure 5A). For BALB/c mice levels of TATc were significantly higher in the HV₁–group as compared to the LV₁–group (p < .05). Levels of PAI–1 were not significantly different after MV in C57Bl/6 mice (figure 5B). BALB/c mice did show increased PAI–1 levels after MV with HV₁–MV as compared to control and higher levels of PAI–1 were found in HV₁-mice as compared to LV₁-mice (p < .01).

Lung injury with different fluid support strategies
The different fluid support strategies showed no difference in endpoint measurements, except for pulmonary MIP-2 levels. MIP-2 levels were significantly higher in C57Bl/6 mice that received sodium bicarbonate as compared to control (figure 3C). For mice that received saline no significant increase in MIP-2 was observed after MV for 5 hours.
Discussion

We developed and tested a model of VILI in 2 commonly used mice strains using clinically relevant $V_t$ and preventing hypovolemia with fluid support. By using a clinically relevant $V_t$ and fluid support we prevented shock. By using sodium bicarbonate instead of normal saline metabolic acidosis was prevented. We developed a model which enhances translation of results into clinical practice and/or future studies.

To our best knowledge this is the first study that compares more physiological $V_t$ then previously used in a model of VILI. VILI was clearly present with the use of HV after 5 hours of MV. Interestingly, with LV $V_t$ VILI also developed, although to a lesser extent. This finding is in accordance with a previous report, where low $V_t$ (8 ml/kg) for 4 hours resulted in a reversible inflammatory reaction, while preserving tissue integrity [20]. Thus, even the use of LV is to be considered injurious when ventilating mice. Of note, however, this finding is not supported by human data. Indeed, patients ventilated with LV ($6 \text{ ml/kg}$) for 5 hours did not show upregulation of pulmonary inflammatory mediators as opposed to patients ventilated with HV ($12 \text{ ml/kg}$) [21].

Figure 5 C57Bl/6 mice and BALB/c mice: Thrombin–antithrombin complexes (TATc) levels (A) and plasminogen activator inhibitor (PAI)–1 levels (B) in bronchoalveolar lavage fluid after 5 hours of mechanical ventilation in control (C) mice, mice ventilated with low tidal volumes (LV) and high $V_t$ (HV), and with two different fluid therapies (normal saline; NaCl and sodium bicarbonate; NaBic). Data represent median and interquartile range of six mice. *$p < .05$ vs. control; †$p < .001$ vs. control.
Unrecognized differences in MV between mice and the human setting may be responsible for this difference. Our model has several limitations. First, $V_T$ in HV_{1}–mice are still quite large (~15 ml/kg). Although there is underuse of lung–protective ventilation with the use of LV in ALI/ARDS–patients [22] and patients at risk for ALI/ARDS [23], in the clinical arena $V_T$ have declined gradually over the past 10 years [24,25]. Indeed, in our institution $V_T$ declined from 9–10 to 7–8 ml/kg predicted bodyweight [26]. However, $V_T$ of as large as 15 ml/kg are reported to be used [27,28]. Therefore our comparison may still reveals relevant information on lung injury caused by MV. Second, LVT ventilation can promote development of atelectasis. Allen et al. recently demonstrated that periodic recruitment with relatively frequent deep inflations during ventilation with LV can improve oxygenation, ventilation and lung mechanical function with no evidence of lung injury by 2 h in mechanically ventilated mice [29]. In our model we used deep inflation every 30 minutes to prevent atelectasis. It may be speculated this is not frequent enough and some pulmonary inflammation seen in LVT mice may be caused by atelectotrauma.

Although it is well known that acid–base parameters are reliable indicators of the general condition of the animal, these parameters are not or only partly assessed in previous murine models of MV [12,30–32]. Acid–base balance in spontaneously breathing mice are mainly under isoflurane–anesthesia [17] and reported values on pH are rather acidic [33]. It has been suggested that mice have a considerably lower alveolar and arterial PCO$_2$ than other mammals (PaCO$_2$ ranging from 33–41 mmHg). However, instrumentation of animals cannot be completely excluded as causative [34]. Here we show normal values for pH and PaCO$_2$ in C57Bl/6 mice and BALB/c mice after brief anesthesia. Our animals developed metabolic acidosis when normal saline was used. Metabolic acidosis in mice can be induced by isoflurane anesthesia and/or saline administration [17,18]. In the present study we only found subtle differences in endpoints of VILI between the two fluid therapies. Nevertheless, we favor the use of sodium bicarbonate instead of normal saline as fluid support therapy to prevent metabolic acidosis, since severe acidosis may influence unmeasured endpoints of VILI. In daily clinical practice correction of severe acidemia (pH < 7.20) can be achieved by administration of sodium bicarbonate [35].

In our study, in C57Bl/6 mice the systolic blood pressure and heart rate remained stable during the complete duration of anesthesia. By contrast, BALB/c mice had higher systolic blood pressures and higher heart rates after induction of anesthesia as compared to later timepoints. This could be due to the fact that in BALB/c mice nervus vagus activity is increased to a lesser extent by medetomidine as in C57Bl/6 mice [18]. Systolic blood pressures in our study are comparable with values from another mouse ventilation model [12].

We found higher systemic levels of KC and IL-6 as compared to control mice and levels were higher in HV_{1}–mice. This finding is in accordance with data from human studies.
Indeed, in patients with ALI/ARDS a lung-protective MV-strategy using LVf and sufficient PEEP-levels resulted in significantly lower systemic inflammatory mediators as compared to ALI/ARDS patients ventilated with a more conventional MV-strategy, using HVf [9].

The term mechanical ventilation-induced coagulation has recently been introduced [36]. Several studies suggest pulmonary coagulopathy to be a feature of VILI too. Indeed, we have shown that MV using large Vf resulted in increased alveolar thrombin generation [37]. It is likely that the alveolar epithelium can initiate intra-alveolar coagulation by expressing active tissue factor [38]. Recently, we also showed MV with large Vf to attenuate fibrinolysis in rats, in part via upregulation of PAI–1 [39,40]. These results are in line with results from the present study.

In conclusion, we set up a more physiological murine model of VILI in two commonly used mice strains by using physiological Vf and sodium bicarbonate infusion to correct for hypotension and prevent acidosis. Lung injury was found with both Vf used in our experiments. Importantly, with larger Vf more injury was found. We recommend the use of more physiological Vf in future studies of VILI. Although most endpoints of VILI were not influenced by the type of fluid administration, we recommend to use sodium bicarbonate instead of normal saline as fluid support therapy to prevent metabolic acidosis in models of VILI.
References

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