Mechanical Ventilation in Healthy Mice Induces Reversible Pulmonary and Systemic Cytokine Elevation with Preserved Alveolar Integrity

An In Vivo Model Using Clinical Relevant Ventilation Settings

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Background: Mechanical ventilation (MV) may activate the innate immune system, causing the release of cytokines. The resulting proinflammatory state is a risk factor for ventilator-induced lung injury. Cytokine increase results from direct cellular injury but may also result from cyclic stretch alone as demonstrated in vitro: mechanotransduction. To study mechanotransduction in vivo, the authors used an animal MV model with clinically relevant ventilator settings, avoiding alveolar damage.

Methods: Healthy C57BL6 mice (n = 82) were ventilated (tidal volume, 8 ml/kg; positive end-expiratory pressure, 4 cm H2O; fraction of inspired oxygen, 0.4) for 30, 60, 120, and 240 min. Assigned animals were allowed to recover for 2 days after MV. Both pulmonary tissue and plasma interleukin (IL)-1α, IL-1β, tumor necrosis factor α, IL-6, IL-10, and keratinocyte-derived chemokine levels were measured. Histopathologic appearance of lung tissue was analyzed by light microscopy and electron microscopy.

Results: In lung tissue, all measured cytokines and keratinocyte-derived chemokine levels increased progressively with MV duration. Light microscopy showed increased leukocyte influx but no signs of alveolar leakage or albumin deposition. Electron microscopy revealed intact epithelial cell and basement membranes with sporadically minimal signs of partial endothelial detachment. In plasma, increased levels of IL-1α, tumor necrosis factor α, IL-6, and keratinocyte-derived chemokine were measured after MV. In the recovery animals, cytokine levels had normalized and no histologic alterations could be found.

Conclusions: Mechanical ventilation induces reversible cytokine increase and leukocyte influx with preserved tissue integrity. This model offers opportunities to study the pathophysiological mechanisms behind ventilator-induced lung injury and the contribution of MV to the “multiple-hit” concept.

MECHANICAL ventilation (MV) is widely used in general anesthesia and is a lifesaving intervention in critically ill patients. It can, however, induce lung injury in the healthy lung or exacerbate damage in the already injured lung. This has been termed ventilator-induced lung injury (VILI). Clinical studies show that the use of large tidal volumes (V T ≥ 12–15 ml/kg) is associated with a poor prognosis; however, a “lung-protective ventilation strategy” (low tidal volumes [V T < 10–12 ml/kg], optimizing positive end-expiratory pressure [PEEP]) reduces but cannot prevent VILI.

Ventilator-induced lung injury is characterized by the release of inflammatory mediators (especially cytokines), infiltration of leukocytes, alveolar and interstitial edema, alveolar protein deposition, cellular necrosis, and tissue disruption. It is now commonly accepted that increased production of cytokines, especially interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, plays a key role in initiating or perpetuating lung injury.

The clinical relevance of cytokine up-regulation by MV is the resulting proinflammatory state, because this makes the host more vulnerable to a “second hit” (e.g., major surgery). To note, MV itself can be the “second hit” where an already comprised host exists (e.g., MV in the critically ill patient).

Two mechanisms are believed to be responsible for MV-induced cytokine release. The first is direct trauma to the cell with disruption of the membranes, resulting in translocation of cytokines into both the alveolar space and the systemic circulation. This “decompartmentalization” has been demonstrated in vivo. The second has been termed mechanotransduction. In vitro studies show that most pulmonary cells, such as alveolar macrophages, epithelial cells, and endothelial cells, can produce cytokines in response to cyclic stretch. However, the sensing mechanism of these physical forces and the translation into intracellular signals is largely unknown.

In many of the currently available experimental VILI models, injurious MV settings (e.g., V T > 25 ml/kg or peak pressures > 20–40 cm H2O) have been used in healthy animals, or the “multiple-hit” model was used by applying MV in already injured animals. From these study designs, it is not pos-
sible to differentiate whether the observed increase in cytokine levels is the result of decompartmentalization, mechanotransduction, or both.

For a better understanding of the relevant pathophysiologic mechanisms leading to VILI, it is important to study in vivo the effects of ventilation in the healthy lung, using ventilatory protocols analogous to those currently used during general anesthesia and in the intensive care unit patient. We studied the effects of MV in healthy mice, carefully searched for pulmonary cell or tissue disruption, counted leukocyte numbers, and measured cytokine production in lung tissue and plasma.

Materials and Methods

All experiments were approved by the Regional Animal Ethics Committee in Nijmegen, The Netherlands, and performed under the guidelines of the Dutch Council for Animal Care and the National Institutes of Health.

Animals

Experiments were performed in male C57BL6 mice (n = 82; Charles River, Sulzfeld, Germany) aged 10–12 weeks, with weights ranging from 23 to 28 g.

Mechanical Ventilation in Mice

Mice were anesthetized with an intraperitoneal injection of a combination of ketamine, medetomidine, and atropine (KMA): 7.5 µl per gram of body weight of induction KMA mix (consisting of 1.26 ml ketamine, 100 mg/ml; 0.2 ml medetomidine, 1 mg/ml; 1 ml atropine, 0.5 mg/ml; and 5 ml NaCl, 0.9%) was given just before intubation. Animals were orally intubated under direct vision with an endotracheal tube (0.82 mm ID, 1.1 mm OD, length 25 mm). Endotracheal tube position was confirmed by end-tidal carbon dioxide analysis, using mass spectrometry. Subsequently animals were connected to the ventilator (MiniVent®; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany). V₀ was set at 8 ml/kg and frequency was set at 150/min, which is well within the range of measured Vₜ and respiratory rate during spontaneous ventilation in C57BL6 mice. To maintain anesthesia, 5.0 µl per gram of body weight boluses of maintenance KMA mix (consisting of 0.72 ml ketamine, 100 mg/ml; 0.08 ml medetomidine, 1 mg/ml; 0.3 ml atropine, 0.5 mg/ml; and 18.9 ml NaCl, 0.9%) were given, via an intraperitoneally placed catheter, every 30 min. Throughout the experiment, rectal temperature was monitored and maintained between 36°C and 37.5°C using a heating pad.

Study Groups

Animals were divided into seven groups. Group C (n = 9) served as control group: After induction of anesthesia, these mice were killed immediately, without being ventilated. Animals in groups 30 (n = 6), 60 (n = 6), 120 (n = 9), and 240 (n = 9) were ventilated for 30, 60, 120, and 240 min, respectively, and were killed immediately thereafter. In group R (recovery group), animals (n = 6) were exsanguinated and then killed after 2 days of recovery. Anesthesia was discontinued in the group R animals 1 h before extubation. Group D (depleted group) animals (n = 6) were first leukocyte depleted by administering cyclophosphamide as described previously. These animals were then ventilated for 240 min and killed immediately thereafter.

A separate set of experiments (intraarterial blood pressure [IABP] group, n = 15) was conducted to assess whether the chosen anesthetic and ventilation regime resulted in a stable and reproducible cardiorespiratory condition. In these animals, continuous intraarterial carotid blood pressure was measured. Arterial blood gas analysis was performed after 120 min (n = 6) and 240 min (n = 9). The same ventilator settings were used as for the mice in the aforementioned groups. We decided not to include the animals from the IABP group for the cytokine or histopathologic analysis to avoid possible interference with cytokine response resulting from instrumentation induced tissue damage.

In addition, two control experiments were performed. In the first control experiment, animals (n = 12) received the standard ventilation strategy (V₀, 8 ml/kg; PEEP, 4 cm H₂O; FIO₂, 0.4). The lungs were removed after 0 min (n = 4, control), 120 min (n = 4), and 240 min (n = 4) of MV to measure wet/dry ratios. In the second control experiment, animals (n = 4) were ventilated with a V₀ of 16 ml/kg, PEEP of 4 cm H₂O, and FIO₂ of 0.4 for 240 min. The lungs of these mice were used to histopathologically assess the effects of high-V₀ ventilation in our model.

Material Harvesting

After the animals were killed, blood was collected by exsanguination and centrifuged at 14,000 rpm (13,000g) (Eppendorf 5415 C; Nethler-Hinz GmbH, Hamburg, Germany) for 2 min, and plasma was stored at −80°C. Immediately after exsanguination, the heart and lungs were carefully removed en block via midline sternotomy. The right middle lobe was fixed for light microscopy (LM) and electron microscopy (EM), except in animals analyzed for wet/dry ratio. The remaining lung tissue was homogenized for the determination of cytokine concentrations.

Preparation and Analysis of Lung Tissue

For LM, the material was fixed in 4% buffered formalin solution overnight at room temperature, dehydrated,
Table 1. Intraarterial Blood Pressure and Arterial Blood Gas Analysis during Mechanical Ventilation

<table>
<thead>
<tr>
<th>Duration of MV, min</th>
<th>MAP, mmHg</th>
<th>pH</th>
<th>PaO2, mmHg</th>
<th>PaCO2, mmHg</th>
<th>HCO3, mM</th>
<th>BE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>102 (10)</td>
<td></td>
<td>229 (50)</td>
<td>38 (7)</td>
<td>20.4 (1.2)</td>
<td>−4.4 (1.5)</td>
</tr>
<tr>
<td>60</td>
<td>89 (12)</td>
<td>7.36 (0.06)</td>
<td>194 (50)</td>
<td>36 (8)</td>
<td>19.6 (3.1)</td>
<td>−6.3 (2.0)</td>
</tr>
<tr>
<td>120</td>
<td>83 (14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>78 (6)</td>
<td>7.35 (0.07)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>79 (8)</td>
<td>7.35 (0.07)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean (SD).

BE = base excess; MAP = mean arterial pressure; MV = mechanical ventilation; PaCO2 = arterial carbon dioxide tension; PaO2 = arterial oxygen tension.

and embedded in paraplast (Amstelstad, Amsterdam, The Netherlands). Sections of 4-μm thickness were used for further analysis. The enzyme activity of leukocytes was visualized by enzyme histochemistry using chloroacetate esterase staining (Leder). Periodic acid–Schiff staining was performed to analyze for alveolar alveolar presence. Leukocytes were counted manually (20 fields per mouse), and after automated correction for air/tissue ratio, leukocytes per μm² were calculated.

For EM, the material was fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C and washed in the same buffer. The tissue fragments were postfixed in cacodylate-buffered 1% OsO₄ for 120 min, dehydrated, and embedded in Epon 812 (Merck, Darmstadt, Germany). Ultrathin sections were cut on an Ultratome (Leica, Reichert Ultratracs, Vienna, Austria), and contrasted with 4% uranyl acetate for 45 min and subsequently with lead citrate for 4 min at room temperature. Sections were examined in a Jeol 1200 EX2 electron microscope (JEOL, Tokyo, Japan). The evaluating pathologist was blinded to the group and ventilation protocol to which the animal had been assigned.

For wet/dry ratios, both lungs were used; ratios were calculated by measuring lung weight before and after heating for 24 h in a stove at 50°C.

Laboratory Tests

Interleukin-1α and IL-1β were assessed using specific radioimmunoassays, as described previously. Levels of TNF-α, IL-6, IL-10, and keratinocyte-derived chemokine (KC) in lung homogenate and plasma were measured using enzyme-linked immunosorbent assay (for TNF-α, IL-6, and IL-10: CytoSet, BioSource, Camarillo, CA; for KC: ELISA-Kit, R&D Systems, Minneapolis, MN). Lower detection limits were as follows: IL-1α and IL-1β: 40 pg/ml; TNF-α: 32 pg/ml; IL-6: 160 pg/ml; IL-10: 16 pg/ml; and KC: 160 pg/ml. For the assessment of KC in plasma in group 60, insufficient plasma was available; the plasma had to be diluted for analysis, which increased the detection limit to 1,600 pg/ml. To investigate whether lipopolysaccharide contamination was present in our experimental setting, we measured lipopolysaccharide in air, tubing, and the ventilator by Limulus Amebocyte Lysate testing (Cambrex Bio Science, Walkersville, MD; detection limit: 0.06 U/ml).

Statistical Analysis

Data are expressed as mean (SD) when distributed normally (leukocyte counts and wet/dry ratios) and expressed as median (range) otherwise (cytokine concentrations). Statistical analysis was performed with SAS (SAS Institute Inc., Cary, NC) statistical procedures. Because cytokine concentrations are not normally distributed, Kruskal-Wallis procedures were used, with post hoc comparisons of subgroups (Duncan). Data of a particular cytokine concentration variable were ranked, followed by analysis of variance in the General Linear Models procedure using the MEANS procedure with the Duncan option and Bonferroni correction for multiple comparisons. For the analysis of leukocyte counts and wet/dry ratios, analysis of variance was used on non-ranked data with post hoc comparison of group means (Duncan). The level of significance was set at P < 0.05.

Results

Cardiorespiratory Parameters

The animals with an intraarterial canula (IABP group) exhibited stable hemodynamic parameters throughout the experiments. Mean arterial pressure was within normal limits and remained above 65 mmHg in all animals. Blood gas analysis showed normal pH, arterial carbon dioxide tension (PaCO₂), and arterial oxygen tension (PaO₂) levels with a small decrease in base excess (table 1). Two of six animals in group R (recovery group) died directly after extubation. Before extubation, these animals did not differ from surviving subjects in cardiorespiratory parameters. The remaining four animals were stable during the ventilation-free interval, with normal activity and behavior and no respiratory distress or weight loss.

Histologic Examination

Electron microscopy examination of the lung tissue from animals in groups 30, 60, 120, and 240 revealed intact basement membranes and no signs of alveolar...
flooding. Type I pneumocytes sporadically showed signs of minimal membrane disruption and small partial detachment of endothelium (figs. 1A and B). Animals that were allowed to recover (group R) and unventilated animals (group C) showed no signs of membrane disruption or detachment of endothelium (fig. 1C). The four animals in the control experiment that were ventilated with a VT of 16 ml/kg showed significant injury; lungs appeared overinflated (airtrapping) with loss of septal walls and injury of type I pneumocyte (fig. 1D).

Light microscopy examination using periodic acid-Schiff staining showed no intraalveolar albumin. Leder staining revealed a substantially higher number of pulmonary leukocytes after 120 and 240 min of MV (fig. 2). No differences in leukocyte counts were found in the animals that were allowed to recover (group R) compared with the unventilated animals (group C).

Wet/dry ratios showed increased ratios only after 240 min of MV. Data are presented in table 2.

Cytokine Concentration Induced by Mechanical Ventilation

Mechanical ventilation with a VT of 8 ml/kg, PEEP of 4 cm H₂O, and FIO₂ of 0.4 resulted in a significant increase in IL-1α, IL-1β, TNF-α, IL-6, IL-10, and KC in lung tissue homogenate when compared with unventilated animals (group C). These cytokine concentrations increased with the duration of MV, with KC being the first to increase from 30 min of MV onward. IL-1β, TNF-α, IL-6, and IL-10 levels increased after 60 min, whereas IL-1α increased after 240 min compared with group C animals (fig. 3).

In plasma, TNF-α, IL-6, and KC levels were elevated from 120 min onward compared with group C animals, and IL-1α after 240 min of MV. IL-1β and IL-10 levels were not different from those of the group C animals (fig. 4).

When animals were allowed to recover for 2 days (group R), after being ventilated for 240 min, all lung tissue cytokine levels were lower compared with levels found in animals killed immediately after 240 min of ventilation (group 240). IL-1α and KC levels in group R animals were higher compared with group C animals. Plasma levels of TNF-α, IL-6, IL-10, and KC were found to be lower when compared with group 240.

The Effect of Leukocyte Depletion on the Release of Cytokines

In leukocyte-depleted animals (group D), MV resulted in significantly higher levels of lung tissue IL-1α, IL-6, IL-10, and KC compared with group C animals. IL-1β, TNF-α, and KC levels were lower compared with the levels found after 240 min of MV in healthy (non-leukocyte-depleted) animals (group 240). MV resulted in higher plasma levels of KC compared with group C animals. TNF-α, IL-6, and IL-10 levels in plasma were lower compared with the levels found in group 240.

No lipopolysaccharide could be detected in our experimental setting.

Discussion

The current study demonstrates that MV in healthy mice using clinically relevant ventilator settings with low
VT preserves alveolar integrity but induces reversible cytokine increase and leukocyte influx. This rapid increase in cytokine levels and leukocyte influx, however, does not result in persistent inflammation and VILI. Our findings suggest that “noninjurious” or “lung-protective” ventilation does not exist and that even this careful mode of ventilation strategy leads to a reversible inflammatory response. Fortunately, MV in elective, healthy patients rarely leads to clinical significant injury. Apparently, in most circumstances, the lung is able to cope with the MV-induced inflammatory reaction. This is demonstrated in a clinical study by Plotz et al.,36 which showed that 2 h of MV (VT 10 ml/kg) in healthy children, anesthetized for cardiac catheterization, resulted in elevated alveolar IL-6 and TNF-α concentrations without clinical signs of pulmonary dysfunction. In contrast with the current study is essentially different from previous experimental studies. In those studies, either injurious MV settings with large tidal volumes and high peak inspiratory pressures were used.11,12,14,26,27,38 or lungs were preinjured using lipopolysaccharide, hydrochloric acid, or surfactant depletion.13,15,16,28,29 Another major difference is that cytokine levels were measured in lung lavage fluid, whereas we used lung tissue homogenate.11,12,14,38 Lung lavage—by itself potentially injurious because it is often performed using large volumes11,12,38—requires cellular and alveolar leakage for the detection of cytokines. Analysis of lung tissue homogenate allows detection of cytokines before appearance in the alveolar space. This method of material harvesting does not allow the identification of the specific cells responsible for the measured increases in cytokine levels. However, the observed increase in KC after 30 min of MV, before the leukocyte influx, suggests a primary pulmonary origin of this cytokine. This is further supported by the finding of KC elevation in the leukocyte-depleted animals (group D). However, we did not study the possible effects of cyclophosphamide on cytokine synthesis of pulmonary cells.

Table 2. Leukocyte Counts and Wet/Dry Ratios

<table>
<thead>
<tr>
<th>Group</th>
<th>Leukocytes × 10⁶/µm², P Value</th>
<th>Wet/Dry Ratio, P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.1 (1.3), 4.68 (0.014)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>2.4 (1.6), NS</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>2.2 (2.2), NS</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>9.7 (5.0), &lt; 0.05, 4.81 (0.13), NS</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>5.7 (3.1), &lt; 0.05, 5.01 (0.06), &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Recovery</td>
<td>1.2 (0.9), &lt; 0.05*</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean (SD). P values compared with control group (unventilated animals). * P value compared with group 240 (240 min of ventilation). NS = not significant.
In this study, EM analysis revealed that our MV mode almost completely retained histologic integrity, with only sporadically minimal changes in a few samples. Most importantly, basement membranes were not disrupted, signifying alveolar integrity. Although LM has been used for quantitative analysis of leukocytes and can provide some qualitative evidence of lung injury (i.e., alveolar flooding, thickening of alveolar septa), more detailed evaluation of structural changes in the lung requires EM. The effects of large $V_T$ in the animals ventilated with $V_T$ of 16 ml/kg was clearly visible with EM as damage with loss of compartmentalization, consistent with findings in a previous publication. We observed increased wet/dry ratios in animals after 240 min of MV. However, we conclude that this increased wet/dry ratio (without any other signs of possible lung damage as shown by EM and LM examination) is not of clinical significance. This is supported by the finding of the complete recovery of the animals who were allowed to recover after 240 min of ventilation (group R).

Factors affecting cytokine response other than MV were carefully avoided. The possibility of triggering an inflammatory response by invasive procedures, (i.e., insertion of an intraarterial line) was eliminated by performing our experiments in noninvasively monitored
animals, after having documented cardiorespiratory stability in invasively monitored animals (IABP group). A limulus amebocyte lysate test excluded possible aero-
genic lipopolysaccharide contamination during our ex-
periments. Cardiorespiratory parameters and the choice of the anesthetics are known to influence the cytokine profile. In the current study, mean arterial pressure was maintained above 65 mmHg, and blood gas analysis showed normal pH, PaCO\textsubscript{2}, and PaO\textsubscript{2} levels. Only a small decrease in base excess after 120 and 240 min of MV was observed, comparable with other studies.\textsuperscript{11,12} The slight decrease in base excess in the presence of a normal mean arterial pressure unlikely interferes with our ob-
servations. The effect of anesthetics on hemodynamic stability in mice has been studied extensively by Zuur-
bier et al.,\textsuperscript{42} who found KMA mix superior compared with other regimens (e.g., fentanyl–fluanisone–midazol-
alom mix or isoflurane). Some anesthetics, e.g., propo-
fol,\textsuperscript{43} volatile anesthetics,\textsuperscript{44,45} and ketamine,\textsuperscript{46,47} are known to influence cytokine profiles. Ketamine is known to have an inhibitory effect on lipopolysaccha-
ride-induced cytokine production.\textsuperscript{46,48–50} In the current study, all animals received the KMA mix. Ideally, an additional control group of spontaneously breathing an-
imals under KMA anesthesia is needed. However, this will result in hypoventilation with severe respiratory
acidosi and hemodynamic instability. Two mice in group R (recovery group) died immediately after extu-
bation; the cause of death was due to airway problems related to residual effects of the anesthesia. The other mice in group R made uneventful recoveries. By exclu-
sing these confounding factors, we attribute the increase in cytokine levels to MV.

Therefore, even low-V\textsubscript{T} MV induces an inflammatory response and, in a “multiple-hit” situation, might be the additional “proinflammatory hit” resulting in lung injury. Modulation of the inflammatory response may offer strateg-
es to reduce VILI. In this respect, anesthetics may play a role because volatile anesthetics have been shown to exhibit antiinflammatory effects in different organ systems and might be able to modulate the release of cytokines.\textsuperscript{51} The influence of different anesthetics on the inflammatory response in our model needs further inves-
tigation. Recently, Jiang et al.\textsuperscript{52} discovered that Toll-like receptor 4–mediated inflammation by endogenous com-
ounds might also be important in the development of VILI. Further study of the Toll-like receptors and the molecules with which they interact may reveal more insight into the molecular mechanisms of VILI.

Conclusion

The current study shows that in healthy male mice, a short period of "noninjurious" ventilation induces a re-
versable inflammatory reaction, while preserving tissue

integrity. This model offers opportunities to study the pathophysiologic mechanisms of VILI and the contribu-
tion of MV to the “multiple-hit” concept.

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