Mechanical ventilation (MV) is a supportive and life-saving therapy, however, it can cause ventilator-induced lung injury as a common complication. Thus, recruitment manoeuvres (RM) are applied to open the collapsed alveoli to ensure sufficient alveolar surface area for gas exchange. In the light of the fact that positive pressure ventilation is currently the standard treatment for improving pulmonary function, extrathoracic negative pressure is considered as an alternative form of respiratory support. The aim of this study was to estimate the proinflammatory and oxidative response during MV and lung injury as well as the response after RM. All studied parameters were assessed at the following time points: T1-spontaneous breathing, T2- MV, T3- lung injury, T4- RM. During MV (T2) elastase, MPO, ALP release, nitrite and superoxide generation significantly increased, whereas in later measurements a decrease in these values was noted. The MDA plasma concentration significantly (p<0.05) increased at T2, reaching a level of 13.30±0.87 nmol/ml; at other time points the values obtained were similar to the baseline value of 9.94±0.94 nmol/ml, whereas a gradual decrease in SOD activity at time T2-T4 points in comparison with the baseline value was found. During the study both neutrophil activity and oxidative stress indicate exacerbated response after MV and lung injury by bronchoalveolar lavage; however, extrathoracic negative pressure system as the MR ameliorates damaging changes which could further lead to serious lung injury.

**Key words**: neutrophil, mechanical ventilation, lung injury, extrathoracic negative pressure

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**Extrathoracic negative pressure ameliorates lung injury during mechanical ventilation in experimental pigs**

J. Wessely-Szponder¹, T. Szponder², A. Fijalkowska-Nestorowicz³, R. Bobowiec¹, A. Sobczyńska²

¹ Department of Pathophysiology, Chair of Preclinical Veterinary Sciences, Faculty of Veterinary Medicine, University of Life Sciences, 20-033 Lublin, Poland, ² Department and Clinic of Animal Surgery, Faculty of Veterinary Medicine, University of Life Sciences, 20-612 Lublin, Poland, ³ Department of Anaesthesiological and Intensive Care Nursing, Medical University, 20-093 Lublin, Poland

**Abstract**

Mechanical ventilation (MV) is a supportive and life-saving therapy, however, it can cause ventilator-induced lung injury as a common complication. Thus, recruitment manoeuvres (RM) are applied to open the collapsed alveoli to ensure sufficient alveolar surface area for gas exchange. In the light of the fact that positive pressure ventilation is currently the standard treatment for improving pulmonary function, extrathoracic negative pressure is considered as an alternative form of respiratory support. The aim of this study was to estimate the proinflammatory and oxidative response during MV and lung injury as well as the response after RM. All studied parameters were assessed at the following time points: T1-spontaneous breathing, T2- MV, T3- lung injury, T4- RM. During MV (T2) elastase, MPO, ALP release, nitrite and superoxide generation significantly increased, whereas in later measurements a decrease in these values was noted. The MDA plasma concentration significantly (p<0.05) increased at T2, reaching a level of 13.30±0.87 nmol/ml; at other time points the values obtained were similar to the baseline value of 9.94±0.94 nmol/ml, whereas a gradual decrease in SOD activity at time T2-T4 points in comparison with the baseline value was found. During the study both neutrophil activity and oxidative stress indicate exacerbated response after MV and lung injury by bronchoalveolar lavage; however, extrathoracic negative pressure system as the MR ameliorates damaging changes which could further lead to serious lung injury.

**Key words**: neutrophil, mechanical ventilation, lung injury, extrathoracic negative pressure
Introduction

Mechanical ventilation (MV) despite its beneficial role in life-threatening conditions, is potentially injurious and has been involved in some side effects, including ventilator induced lung injury (VILI). VILI in the form of damage to lung tissue is caused by pro-inflammatory responses, increased oxidative stress and secondary infections, which can be followed by sepsis or systemic inflammatory response syndrome leading to increased mortality (Schilling et al. 2013, Karadottir et al. 2015). Wolthuis et al. (2009) in their study conducted on two commonly used mice strains stated that VILI occurs in healthy lungs and not just in animals with a pre-existing lung injury. In animals with intact circulating neutrophils, conventional mechanical ventilation increases alveolar-endothelial barrier permeability, impairs oxygenation, and alters lung morphology. It is estimated that conventional MV (positive pressure ventilation) in humans leads to the activation of neutrophils and the release of cytotoxic products, and these products may lead to further damage to lung tissues. The mechanisms by which MV induces neutrophil activation may include direct lung stress and cytotoxic mediator release (Zhang et al. 2002).

It has been shown in previous studies that neutrophils are a major component of cellular host defence but they may also cause tissue damage in many lung disorders including acute respiratory distress syndrome (ARDS) (Zhang et al. 2002). Mechanical ventilation activates neutrophils which release certain enzymes, elastase is one of these, it cleaves elastin, interstitial collagens, and basement membrane proteins leading to lung tissue injury (Kaynar et al. 2008). Its release is correlated with a degree of systemic inflammatory response and multiple organ failure (Zhang et al. 2002). Myeloperoxidase (MPO) released by activated neutrophils exhibits powerful pro-oxidative and pro-inflammatory properties. The content of this enzyme in neutrophils serves both as an index of their degranulation and their activation (Loria et al. 2008). Alkaline phosphatase (ALP), in turn, is a specific marker of damage to pneumocytes type I and a marker of the proliferation of pneumocytes type II in the course of lung tissue injury (Liu et al. 2017).

In the light of the fact that, during MV, hyperoxia can cause oxidative stress with systemic injuries, assessment of the oxidant/antioxidant balance is of great importance (Filho et al. 2012, Pires et al. 2012, Sun et al. 2015). The response to injurious oxidative stress is the induction of cytoprotective antioxidant enzymes, which is critical in the cellular detoxification of reactive oxygen species (ROS) and in the maintenance of cellular redox homeostasis (Sun et al. 2015). Superoxide dismutase (SOD) is a key antioxidant enzyme neutralizing the superoxide anion. However, several studies have indicated that during oxidative stress, an increase in serum malondialdehyde (MDA) and a decrease in plasma SOD activity, indicating an imbalance between the pro-oxidant and anti-oxidant states in the organism, leads to an imbalance in systemic redox status (Chakraborty et al. 2007, Pires et al. 2012).

Ventilator induced lung injury is a common complication in patients receiving MV. Therefore, the application of recruitment manoeuvres (RM) is advocated for restoring a normal functional residual capacity by re-aerating atelectasis and areas of airway closure, thereby increasing the alveolar surface for gas exchange (Tusman et al. 2014). Many approaches to lung recruitment have been proposed to improve pulmonary function; however, the optimal method is still under debate. Positive pressure ventilation is currently the standard treatment, whereas extrathoracic negative pressure is an alternative form of respiratory support.

The aim of our study was to estimate if MV and lung injury caused by bronchoalveolar lavage evoke a pro-inflammatory and oxidative response and the extent and reversibility of these changes during RM with whole-body external negative pressure. For this purpose we detected the neutrophil secretary response, the generation of free radicals and antioxidants, as well as time-related changes of these parameters during the study. It was conducted for the purpose of assessing the safety of the model used with respect to neutrophil reactivity as well as oxidative and antioxidant response.

Materials and Methods

All protocols were in accordance with the ARRIVE guidelines and were approved by the Ethics Committee (no. 43/2014). Five Landrace (pbz) pigs weighing 52.3±5kg were included in the study. Firstly, intramuscular premedication with xylazine 2 mg/kg anaesthesia was induced using propofol 3 mg/kg and maintained with a continuous intravenous infusion of propofol 10-12 mg/kg/h as well as fentanyl 4-6 μg/kg/h and a single dose of pancuronium bromide 0.1-0.2 mg/kg. The animals were then placed in the supine position, tracheotomized and intubated (9 mm ID) and mechanical ventilation (Bennett 840, Minneapolis, MN, USA) was introduced in a volume-controlled mode with a tidal volume of 8-10 ml/kg, an inspiratory: expiratory ratio of 1:2 and a fraction of inspired oxygen of 1.0. A respiratory rate was applied to maintain the arterial carbon dioxide partial pressure within the range of 4.7-6.0 kPa. A continuous infusion of lactated Ringer’s solution (8-10 ml kg/h) was administered throughout the study. The body temperature of the
animals was maintained at 37°C with a heating lamp. After surgical preparation and initial measurements, the acute lung injury was produced by repeated bronchoalveolar lavage using 0.9% sodium chloride (1.5-1.8 L, 37°C) until the arterial oxygen partial pressure remained stable below 13.3 kPa. Thereafter, the animals were secured in a whole-body sized chamber and a continuous extrathoracic negative pressure was created using a vacuum pump. The negative pressure protocol consisted of exposure to 16 cm H2O decompressions, while the lung ventilation mode was held unchanged.

Arterial blood samples were drawn at the following time points: baseline in spontaneously breathing pigs (T1 during 30 min of anaesthesia, spontaneous breathing, SB), during mechanical ventilation (T2 at about 2 hrs, MV), after bronchoalveolar lavage with NaCl (T3-lung injury-about 30 min, LI), and after recruitment manoeuvres (about 30 min, T4-RM). Arterial gases were analysed using a Gem Premier 3500 blood gas analyser (Instrument Lab., Berdford, MA, USA). Measurements of pH, arterial partial pressure of carbon dioxide (PaCO2), arterial partial pressure of oxygen (PaO2), bicarbonate (HCO3), and base excess (BE) were made.

After completing the study protocol, all animals were euthanized with an intravenous injection of high-dose potassium chloride during deep anaesthesia until asystole was observed on the electrocardiogram.

Blood (5 ml) for further assays was collected from the marginal auricular vein into a syringe using EDTA as an anticoagulant and a complete blood cell count analysis was performed using a Vet EXIGO analyser (Boule Medical AB). For cell culture, neutrophils were isolated from whole blood using the method described previously (Wessely-Szponder and Szponder, 2010). Briefly, the red blood cells were lysed by the addition of 0.83% ammonium chloride and the blood was then centrifuged at 700 x g for 15 min at 4°C. The purified cells (75-80% pure PMNs, more than 90% viable) were re-suspended in a modified phosphate saline buffer (PBS) and were adjusted to a final concentration of 1 x 10⁶ cells/ml.

The degranulation of neutrophils was assayed on the basis of elastase, MPO, and ALP release and related to maximal enzyme content obtained after treatment of cells with 0.5% Triton X-100 (Sigma). The assay of elastase activity was performed on the basis of cleavage of azocasein (Sigma) as a substrate at 25°C for 10 min, thereafter absorbance was assessed at 490 nm using BioTek EL800 (BioTek Poland). MPO release was determined by measuring the absorbance at 490 nm after 10 min of incubation of the sample with an equal volume of o-phenylendiamine (OPD-Sigma). ALP activity was estimated after 10 min incubation at 25°C with an equal volume of 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP-Sigma) and absorbance then was measured at 405 nm. Superoxide anion production was measured using the method described previously. Briefly, neutrophils were incubated with 0.1% nitroblue tetrazolium (NBT-Sigma) solution at room temperature for 10 minutes and absorbance was then read at 545 nm. The nitric oxide (NO) level was determined as described previously (Wessely-Szponder and Szponder, 2010). The obtained values were expressed as a concentration of nitrite, as a stable product of NO, which accumulates in the medium.

The plasma concentration of MDA was measured using a spectrophotometric method based on its reaction with thiobarbituric acid (Wessely-Szponder et al. 2015). Superoxide dismutase (SOD) anti-oxidant enzyme activity was measured by assessing epinephrine degradation and adenochrome formation. SOD activity in plasma was assessed spectrophotometrically by measuring the absorbance at a wavelength of 480 nm. The data were expressed as SOD units per ml of plasma (Filho et al. 2012).

Each experiment was repeated using cells isolated from five pigs (n=5) and all measurements were performed in duplicate. Data are expressed as the mean ± standard deviation for continuous variables. The significance was identified by one-way ANOVA using Dell™ Statistica™ 13.1 (StatSoft Poland). Differences were considered statistically significant when p<0.05 compared to the baseline.

Results

Heart rate, systolic blood pressure and body temperature were maintained at relatively constant levels during anaesthetic protocols. Initially, the arterial O2 and CO2 partial pressure were in the physiological range. There were no significant differences in blood pH, BE, bicarbonate and PaCO2 during the whole experiment (T1-T4), but the PaO2 increased significantly after MV. Moreover, we documented significant hypoxemia after lung lavage and good recovery after RM. The results of arterial blood gas measurements are presented in Table 1.

We estimated that in the 2nd measurement (T2) elastase release significantly increased from 49.26±1.2% to 54.66±4.48% of maximal release (p<0.05). In further measurements of elastase, activity slightly decreased. MPO release was highest during MV (T2), then decreased, but was maintained at a level significantly higher than the baseline measurement (p<0.05). Alkaline phosphatase increased significantly during MV and then decreased and reached a value of...
18.84±2.58% which was significantly lower as compared with T1 and T2 (p<0.05) (Fig.1).

Nitrite generation increased significantly during MV (T2) and then gradually decreased at later time points (Fig. 2). A similar effect was observed regarding superoxide generation; however, results obtained at T3 and T4 were comparable with the baseline (Fig. 3).

The MDA plasma concentration increased significantly (p<0.05) at T2 reaching a level of 13.30±0.87 nmol/ml; at other time points the values obtained were similar to the baseline value of 9.94±0.94 nmol/ml. Our study revealed a gradual decrease of SOD activity at time points T2-T4 in comparison with the baseline value (Table 2).

**Table 1. Arterial blood gas analysis.** *p<0.05 vs. T1.

<table>
<thead>
<tr>
<th></th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.38±0.014</td>
<td>7.4±0.13</td>
<td>7.34±0.084</td>
<td>7.42±0.077</td>
</tr>
<tr>
<td>PaCO₂ (mmHg)</td>
<td>46.5±13.00</td>
<td>45±1.4</td>
<td>53±1.41</td>
<td>50.00±9.89</td>
</tr>
<tr>
<td>O₂ (mmHg)</td>
<td>56.00±2.83</td>
<td>395.5±193*</td>
<td>45±16.9*</td>
<td>514±21.21*</td>
</tr>
<tr>
<td>HCO₃ (mmol/l)</td>
<td>27.65±8.83</td>
<td>28.36±7.23</td>
<td>28.8±4.8</td>
<td>31.75±0.06</td>
</tr>
<tr>
<td>BE</td>
<td>-2.5</td>
<td>-3.55</td>
<td>-3.05</td>
<td>-6.75</td>
</tr>
</tbody>
</table>

**Fig. 1.** Enzyme release by porcine neutrophils isolated during spontaneous breathing (SB, T1), mechanical ventilation (MV, T2), lung injury (LI, T3), and recruiting manoeuvres (RM, T4), * p<0.05 in comparison with baseline value (T1). Data are expressed as means ±SE and are representative of two independent experiments, with five pigs (n=5).

Discussion

The present study shows that MV, lung injury and RM are accompanied by changes in neutrophil secretory response, oxidant/antioxidant balance and arterial blood gas parameters. It is known that MV increases neutrophil recruitment and MPO activity in lung homogenates in animals and humans. Moreover, the potential role of neutrophils as major effector cells in the generation of VILI has been clearly demonstrated by several experimental studies (Zhang et al. 2002, Kaynar et al. 2008, Wolthuis et al. 2009). Thus, the first aim of our study was to assess the influence of MV on the secretory response of circulating neutrophils. We found that MV causes a transiently increased inflammatory response from circulating neutrophils. We found that MV causes a transiently increased inflammatory response from circulating neutrophils on the basis of an increase in elastase, MPO and ALP release. According to Pires et al. (2012), MV for 30 min triggered an inflammatory response in mouse lung, as estimated on the basis of TNFα release and a four-fold increase of MPO activity in lung homogenates. This kind of response was previously described by Turunen et al. (2006) as a rapid activation of circulating phagocytes after MV in preterm infants and by Zhang et al. (2002) in healthy volunteers.
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In our study increased elastase release during MV was found. Kaynar et al. (2008) reported pronounced neutrophil elastase activity during MV in mice and underlined the beneficial role of elastase in the improvement of the permeability of neutrophils by epithelium to cross the transvascular barrier. Conversely, inhibi-
tion of this enzyme leads to the accumulation of neutrophils in pulmonary vasculature where these cells exert an acute injurious effect on the lung tissues. ALP, in turn, after the initial increase at MV, decreased at later time points to levels below the baseline value. In general, since a decrease of all of these values at later time points was observed we concluded that increased activity was mild and transient and presumably does not cause severe lung injury.

Greater amounts of nitrites as products of NO generation by neutrophils as well as higher superoxide generation were assessed during MV, and MPO release also increased at this time point, as the pro-oxidative and pro-inflammatory marker described by other authors (Pires et al. 2012). Some reports suggest that oxygen and related species (oxidants) may contribute to a number of lung disorders. The lung exists in a high-oxygen environment and together with its large surface area and blood supply, is susceptible to injury mediated by these oxidants (Rai and Phadke 2006). It has been shown that MV is a modulator of oxidative stress (Pires et al. 2012), which is observed 180 min. after the onset of MV (de la Osa, 2014). During oxidative stress the association between lipid peroxidation in the lungs and MV has been reported previously. In our study the MV generated a higher plasma concentration of MDA, whereas in the study of Pires et al. (2012), an increased concentration of MDA was found in lung tissues.

In the course of the study after lung injury with repeated bronchoalveolar lavage a decrease in neutrophil secretory activity in comparison with values obtained at T2 was noted. According to Hoth et al. (2009) increased neutrophil response after lung injury was observed 3 h after injury with maximal enzyme release 24 h after injury. These authors, on the basis of immunoblotting and immunohistochemical assays, reported increased elastase activity in the lung tissue together with an influx of neutrophils into BAL, 24 h after injury.

During MV we noted increased ROS generation leading to oxidative stress determined on the basis of an increased MDA plasma level. Then, during the course of the study, bronchoalveolar lavage and an increased level of neutrophil reactivity and oxidative response were detected until RM. As noted by Matute-Bello et al. (2008) in the case of the combination of MV with a lung injury caused by bronchoalveolar lavage it is difficult to determine the extent to which the lung injury is caused by bronchoalveolar lavage, by the MV, or both. In our experiment lung lavage was preceded by MV; thus, we estimated that major changes in neutrophil activity and oxidative antioxidant balance were generated by MV.

A gradual decrease in plasma SOD activity over consecutive time points of our study was observed. The potential of oxidants to damage pulmonary tissue depends on antioxidant defense mechanisms that act against oxidative stress (Rai and Phadke 2006). Pires et al. (2012) reported a significant decrease in SOD activity during MV. The reduction of SOD activity might be in part due to the loss of enzyme specific activity caused by oxidants and would also be associated with superoxide overproduction. When evaluating the correlation between oxidative stress and injurious MV, Filho et al. (2012) estimated an increase in MDA and a decrease in SOD. Increased MDA was involved in endothelial dysfunction and overexpression of ROS. A decrease in SOD, in turn, could be explained by the reduced host self-defense mechanisms against free radicals.

The RM was accompanied by a decrease in ALP and SOD activity, and MPO release remained high, whereas MDA concentration, elastase release, NO and superoxide generation were at a level similar to the baseline value. According to de la Osa et al. (2014) RM did not cause oxidative stress in newborn piglets. There are contradictory data concerning RM influence on inflammatory mediators. As mentioned in some studies, RM increased the expression of inflammatory mediators (Tusman et al. 2014). In contrast, experimental and clinical data showed that RM did not affect, or even decreased, the inflammatory response. Moreover, RM did not induce significant pro-inflammatory responses in healthy pigs undergoing one lung ventilation during thoracic anaesthesia (Schilling et al. 2013). Different modes of MV strategy strongly influence the intensity and distribution of inflammation caused by neutrophils in the lungs. Protective ventilation and alveolar recruitment improved gas exchange and reduced inflammatory cell activity, especially in dependent lung regions. The main cause of reduced inflammatory response was decreased neutrophil metabolic activity (phosphorylation rate), without changes in local cell counts (de Prost et al. 2013).

In our study as expected, significant hypoxemia was observed after lung lavage with prompt recovery following lung recruitment. Similar results were obtained by other authors in experiments using a rabbit model of acute lung injury caused by bronchoalveolar lavage (Rotta et al. 2001, Piacentini et al. 2004). According to Matute-Bello et al. (2008), bronchoalveolar lavage leads to hypoxemia which is rapidly reversible by RM. The lung lavage by itself has little consequence in terms of permeability changes or inflammation. Epithelial damage occurs only when the lung lavage is followed by an injurious ventilatory strategy. In our study the observed alterations were mild and reversible. Therefore, the beneficial effects of RM have been demonstrated in an animal model of alveolar collapse induced by surfactant depletion.
Conclusion

This study described both anaesthetic procedures developed as a model during the experiment and reactions of the patient. The results obtained at evaluated time points, namely during spontaneous breathing, mechanical ventilation, lung injury and recruitment with external negative pressure, reflect the actual situations and problems encountered in clinical practice. We revealed that both the pro-inflammatory neutrophil response and oxidative stress in this porcine model of lung injury were ameliorated by RM performed as a protective strategy to restore the optimal conditions in the lungs and for the prevention of serious lung injury. The obtained results may be extrapolated to human medicine and veterinary anaesthesiology.

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References


