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Healthy Lung Tissue Response to Mechanical Ventilation in an Experimental Porcine Model

JIRI FREMUTH[1], JIRI KOBR[1], KATERINA PIZINGEROVA[1], LUMIR SASEK[1], PETR JEHLICKA[1], JANA ZAMBORYOVA[1], ONDREJ HESS[2], JANA VRZALOVA[3], JAROSLAV RACEK[4] and ZBYNEK TONAR[5,6]

[1]Pediatric intensive care unit (PICU), Department of Pediatrics, Departments of [4]Clinical Biochemistry and Hematology, and [5]Histology and Embryology, [2]Šikl Institute of Pathological Anatomy, [3]Central Laboratory of Immunoanalysis, and [6]Biomedical Centre, Faculty of Medicine, Charles University of Prague in Pilsen, Pilsen, Czech Republic

Abstract. *Background: The aim of this comparative study was to assess the impact of two different settings of tidal volume (Vt) on the function and morphology of the mechanically ventilated lungs during a 12-h period. Materials and Methods: A total of 32 animals were randomly divided into two groups. Group A included piglets ventilated with a Vt of 6 ml/kg and group B piglets ventilated with a Vt of 10 ml/kg. Lung functions and pulmonary mechanics were evaluated after 1 and 12 h of mechanical ventilation. Morphological changes of the lung tissue were evaluated at the end of the study. Results: Twelve hours of lower Vt ventilation was associated with the development of respiratory acidosis but minimal histological changes. Higher Vt led to pronounced histological changes in terms of proliferation and apoptosis and a decrease of dynamic compliance, with a trend towards lower oxygenation during the study. Conclusion: Mechanical ventilation with a Vt of 6 ml/kg induces minimal histological lung parenchymal changes in terms of proliferation and apoptosis. Positive pressure mechanical ventilation with Vt of 10 ml/kg does not protect lung tissue and induces substantial proliferative and apoptotic changes within the lung parenchyma. Positive pressure mechanical ventilation with Vt of 10 ml/kg does not guarantee protection of healthy pulmonary tissue in the absence of a priming pulmonary insult.*

Mechanical ventilation is an irreplaceable tool in the treatment of critically-ill patients but, as has been shown, ventilation at a high tidal volume (Vt) can result in lung damage (1-3). Ventilation of patients with acute respiratory distress syndrome with a high Vt is even associated with increased mortality (4, 5). Mechanical ventilation practices have changed over the past four decades, with Vt decreasing significantly, especially for patients with acute lung injury (6).

Clinical trials have documented that mechanical ventilation of healthy lungs with high conventional Vt (12 ml/kg) contributes to the development of lung injury in comparison with patients ventilated at lower Vt, and this has been associated with sustained cytokine production as measured in blood and tracheal aspirate (7-9). The goal of prospective experimental and clinical studies should be to evaluate optimal ventilator management strategies for patients without lung injury or respiratory distress syndrome. Most experimental models of ventilatory-induced lung injury use very high Vt that are considerably higher than those used in the clinical management of patients.

The duration of mechanical ventilation is relatively short and may be too short to model clinical conditions. Such an approach prevents conclusions being drawn on the deleterious effects of mechanical ventilation in the absence of pre-existing lung injury. It is important to emphasize that so-called lower Vt in fact are normal for mammals. A growing body of evidence supports the use of protective strategies of mechanical ventilation in an effort to reduce the regional end-inspiratory stretch, thereby avoiding alveolar mechanical damage, as well as a regional inflammatory response (10).

We chose a mechanical ventilation strategy that closely reflects the human setting by using clinically relevant Vt, preventing shock and gross lung morphological changes, and comparing a lower Vt (6 ml/kg) with a higher Vt (10 ml/kg) with respect to several endpoints. After twelve hours of mechanical ventilation lung function was assessed using standard ventilation indices and a quantitative histochemical analysis of lung tissue was performed.

Correspondence to: Jiri Fremuth, MD, Ph.D., Department of Pediatrics, PICU, Faculty of Medicine, Charles University, alej Svobody 80, 304 60 Plzen-Lochotin, Pilsen, Czech Republic. E-mail: fremuthj@gmail.com

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Aims of the study. To develop a clinically relevant model of mechanical ventilation using two different Vt, evaluate the influence of mechanical ventilation on lung functions (oxygenation and ventilation) and pulmonary mechanics, and to assess the degree of lung tissue trauma development, *i.e.* proliferative and apoptotic changes.

Materials and Methods

This experimental study was approved by the Institutional Ethics Committee (PRVOUK P36) and carried out at an accredited experimental site of the Faculty of Medicine in Pilsen, Czech Republic.

Animal preparation and randomisation. A total of 32 white piglets, average weight 24.4 kg (range=19.0-28.7 kg) were included in the study. All animals were pre-medicated with atropine (0.07 mg/kg) and azaperon (5.0 mg/kg) intramuscularly. A bolus of intravenous thiopental (2.0 mg/kg) was used for induction of anaesthesia and tracheal intubation. Continuous intravenous infusion of fentanyl (3.0 μg/kg/h) and azaperon (2.0 mg/kg/h) was used for analgosedation. Pancuronium bromide (0.1 mg/kg/h) was used intravenously in all animals to induce neuromuscular block.

A central venous catheter (5F) was inserted percutaneously into the internal jugular vein and an arterial line (22G) was surgically inserted into the femoral artery. Urinary drainage was accomplished by surgical cystostomy. An infusion of Ringer's solution was given at a rate of 2.5 ml/kg/h intravenously. Electrocardiography (ECG), pulse oximetry (SpO_2) and invasive blood pressure were continuously monitored.

The functions of extra-pulmonary organs were deliberately not influenced pharmacologically and no other medication was given throughout the study.

The animals were randomly divided into two groups at the time of intubation according to the Vt used. Group A included animals ventilated with a Vt of 6 ml/kg. Group B included animals ventilated with a Vt of 10 ml/kg. All piglets were ventilated in the supine position, using Siemens Servo 900C (Siemens-Elema, Sweden) and volume-controlled ventilation mode (VCV). Uniform ventilator settings were used in both groups: positive end expiratory pressure (PEEP) 6 cmH_2O, fraxion of inspired oxygen (FiO_2) 0.4, inspiratory-to-expiratory time ratio (I:E) ratio 1:2, 30 breaths/min.

Protocol of the study. After performing all invasive procedures, a one-hour recovery interval followed. Clinical examination, ventilator and circulatory parameters were recorded and blood samples were taken at the end of the recovery interval (time 1). The clinical assessment was accomplished and monitored parameters were then recorded at one-hour intervals. After 12 hours of mechanical ventilation, a second set of blood samples was obtained (time 12). Echocardiographic examination was performed at time 1 and time 12. All animals were euthanased by administering cardioplegic solution (10% Thomas sol. 3 ml/kg intravenously). Thoracotomy was performed and both lungs were extirpated. One sample from each West zone (I-III) was obtained.

The quality of ventilation was evaluated by arterial blood gases (ABG) analysis and calculation of the following indices was performed (Microsoft Excel 2010, Microsoft, Redmond, USA): alveolar-arterial oxygen difference ($AaDO_2$), oxygenation index (OI), ventilation index (VI), hypoxemic ratio (PaO_2/FiO_2 ratio), dynamic compliance (C_{dyn}), airway resistance (R_{aw}).

Histological processing. Tissue blocks measuring approximately 1×1.5×2 cm were excised from the lungs and fixed with 4% buffered formalin solution. The tissue samples represented all three vertical zones according to West *et al*., based upon the relationship between the pressure in the alveoli, in the arteries, and the veins (11). The tissue blocks were embedded in paraffin. While preserving the cutting plane perpendicular to the lung surface, a series of 5-μm-thick histological sections was processed. The sections were stained with haematoxylin-eosin to examine the overall morphology and exclude preparation artifacts. For immunohistochemical detection of proliferation we used the Ki-67 antigen (monoclonal mouse anti-human, clone MIB-1; Dako, Glostrup, Denmark). Ki-67 is a nuclear protein preferentially expressed during active phases of the cell cycle (G_1, S, G_2, and M phases), but absent in resting cells (G_0-phase). To detect apoptosis, we investigated activated caspase 3 (polyclonal rabbit anti-human SignalStain Asp175 detection kit; Cell Signaling Technology, Danvers, MA, USA). Activated caspase 3 is a critical executioner of apoptosis responsible for the proteolysis of many key nuclear and plasmatic proteins. While Ki-67 positivity was found in the nuclei only, caspase 3 positivity was found in nuclei as well as in the cytoplasm. Ten micrographs per tissue sample and immunohistochemical method were taken at ×40 magnification in a systematic, uniform yet random manner. They represented the whole area of the lung tissue, *i.e.* every part of the tissue had the same probability of being sampled. Starting with a randomly selected micrograph, at least 1,000 nucleated cell profiles were counted per tissue sample; the staining method using an unbiased counting frame. Epithelial *vs*. connective tissue cells were not discriminated. Quantitative immunohistochemical analysis consisted of calculating the proliferative and apoptotic indices. We calculated the proliferation index as the ratio between the nuclear Ki-67-positive cells and the total number of cells counted (12). We recorded the apoptotic index as the ratio between nuclear caspase 3-positive cells (13) and the total number of cells counted. For quantification, we used the Cell Counter and Grid Overlay plugins of ImageJ software (National Institutes of Health, Bethesda, MD, USA; Schneider *et al*., 2012) (14).

Statistics. The data are presented as medians and first and third quartiles. The data were processed with Statistica Base 9 (StatSoft, Inc., Tulsa, OK, USA). Wilcoxon paired test was used to assess differences in ventilatory parameters between the time intervals within groups. The Kruskal–Wallis test was used for comparisons of these parameters between groups. A value of $p<0.05$ was considered statistically significant. The Mann-Whitney U-test was used to assess differences in proliferation and apoptotic indices between groups. For this purpose, the data from all tissue samples and West zones of all individuals were pooled. A value of $p<0.001$ was considered statistically significant.

Results

Macroscopic appearance. The macroscopic appearance of the lung tissue was normal in all animals from group A (Figure 1). On the contrary, the lungs of animals from group B showed signs of focal hyperinflation and condensation with local hyperaemia. The trachea and bronchial tree were

Table I. *Parameters of lung mechanics and ventilatory indices in group A (Vt=6 ml/kg) and group B (Vt=10 ml/kg). The Table compares data at 1 hour (time 1) and 12 hours (time 12) in both groups. Data are presented as medians and first and third quartiles. p<0.05 was considered statistically significant.*

Parameter	Group A (n=11)		*p*-Value	Group B (n=21)		*p*-Value
	Time 1	Time 12		Time 1	Time 12	
pH	7.29 (7.2; 7.31)	7.22 (7.16; 7.33)	NS	7.36 (7.33; 7.42)	7,39 (7.28; 7.47)	NS
pO_2 (kPa)	17.9 (13.7; 40.3)	14.1 (11.9; 25.5)	NS	15.5 (10.7; 32)	14,4 (10.5; 28.5)	<0.05
$PaCO_2$ (kPa)	8.5 (7; 10)	10.85 (9.2; 13.2)	<0.05	6.8 (5.9; 8.9)	6,7 (6; 8.1)	NS
$AaDO_2$ (kPa)	7.58 (–0.83; 15.25)	10.51 (5.75; 14.25)	NS	10.57 (–0.30; 16.45)	13.40 (–2.75; 19.88)	<0.05
OI	2.65 (1.29; 3.92)	3.26 (2.08; 4.22)	NS	2.59 (1.95; 4.47)	3,86 (2.46; 5.63)	<0.05
PaO_2/FiO_2 ratio (mmHg)	336.07 (208.00; 605.31)	291.64 (225.30; 424.32)	NS	332.59 (229.59; 600.00)	262.85 (197.00; 535.09)	<0.05
VI	27.24 (22.18; 37.68)	39.56 (23.43; 57.60)	<0.05	27.63 (21.45; 32.44)	27.95 (21.38; 54.59)	NS
C_{dyn} (ml/cmH_2O/kg)	0.87 (0.79; 0.95)	0.705 (0.59; 0.85)	NS	0.77 (0.67; 1.04)	0.73 (0.56; 0.83)	<0.05
R_{awe} (cmH_2O/l/s)	0.76 (0.57; 0.84)	0.87 (0.65; 0.91)	NS	0.89 (0.81; 1.15)	0.91 (0.86; 1.27)	NS
PIP (cmH_2O)	13 (12.3; 14.2)	14.1 (12.9; 16.9)	NS	19.8 (17; 21.9)	21 (19.5; 23)	NS
P_{aw} (cmH_2O)	8.3 (7; 8.9)	9.1 (7.7; 10)	<0.05	9.2 (8.3; 10.2)	9.9 (9.2; 11)	<0.05
Vt (ml/kg)	6.1 (6; 6.6)	6.1 (6; 6.4)	NS	10 (9.8; 10.9)	10 (9.7; 10.2)	<0.05
VE (l/min)	6 (5; 6.2)	4.8 (4; 6.8)	NS	7.3 (6.6; 8.8)	7.4 (6.2; 9.2)	NS

$AaDO_2$: Alveolar-arterial oxygen difference; OI: oxygenation index, VI: ventilation index; PaO_2/FiO_2 ratio: hypoxemic ratio; Cdyn: dynamic lung compliance; Rawe: dynamic airway resistance; NS: not significant. Comparisons of time differences between groups A and B were not significant.

macroscopically normal. Signs of lung injury were more pronounced in the dorsal, dependent parts of the lungs and were bilaterally symmetrical (Figure 2).

Ventilatory indices and lung mechanics. The level of oxaemia was stable throughout the study in group A, which was characterised by unchanged values of PaO_2 and OI. The exchange of oxygen on alveolar capillary membranes (PaO_2/FiO_2 ratio, $AaDO_2$) did not change significantly during the experiment in group A. Significant elevations of CO_2 led to an increase in VI and development of respiratory acidosis in this experimental group. Ventilatory mechanics (C_{dyn}, R_{awe}) did not change significantly during the study in group A (Table I).

Twelve hours of higher Vt ventilation was associated with the development of deterioration in alveolar capillary membrane oxygen exchange, which led to significant changes in $AaDO_2$, OI and PaO_2/FiO_2 ratio. The higher Vt guaranteed stable levels of capnia and VI. Mechanics of ventilation were affected, resulting in significant decreases in C_{dyn}.

Histological changes, proliferation and apoptosis. Overall morphology was assessed using haematoxylin-eosin staining. Mechanical ventilation with the higher Vt was associated with apparent cellular inflammatory infiltration, which was pronounced around terminal bronchioli and in the interstitial and alveolar spaces (Figure 3). In comparison, the inflammatory changes in group A were attenuated and limited to peribronchiolar and interstitial spaces (Figure 3). These changes were observed in all three West zones.

Ki-67 immunohistochemical staining showed a higher degree of proliferation in group B (Figure 3) in comparison with group A (Figure 3). The maximal proliferative changes were apparent in the perivascular, interstitial and peribronchiolar spaces. The rate of proliferation as assessed by proliferation index was statistically higher in Group B compared to Group A (Figure 4).

Immunohistochemical staining of activated caspase 3 revealed more positive cells in issue from group B compared to group A (Figure 3). The rate of apoptosis assessed by the apoptotic index was also significantly higher in group B compared to group A (Figure 4).

Discussion

We developed a clinically-relevant experimental model of mechanical ventilation of healthy lungs using two different Vt employed in everyday clinical practice: a Vt of 6 ml/kg, generally used in protective ventilation strategy and a Vt of 10 ml/kg, which corresponds to the upper limit of Vt used in routine clinical practice (15-18).

The ventilatory settings were deliberately not changed during the study. Our study was based on the hypothesis that each form of positive pressure mechanical ventilation induces biological stress, the intensity of which depends on the Vt. We are not aware of another similarly designed experimental study in pigs, using these two levels of Vt and exposing animals to mechanical ventilation for 12 h.

Low Vt ventilation was associated with development of respiratory acidosis, which further progressed during the

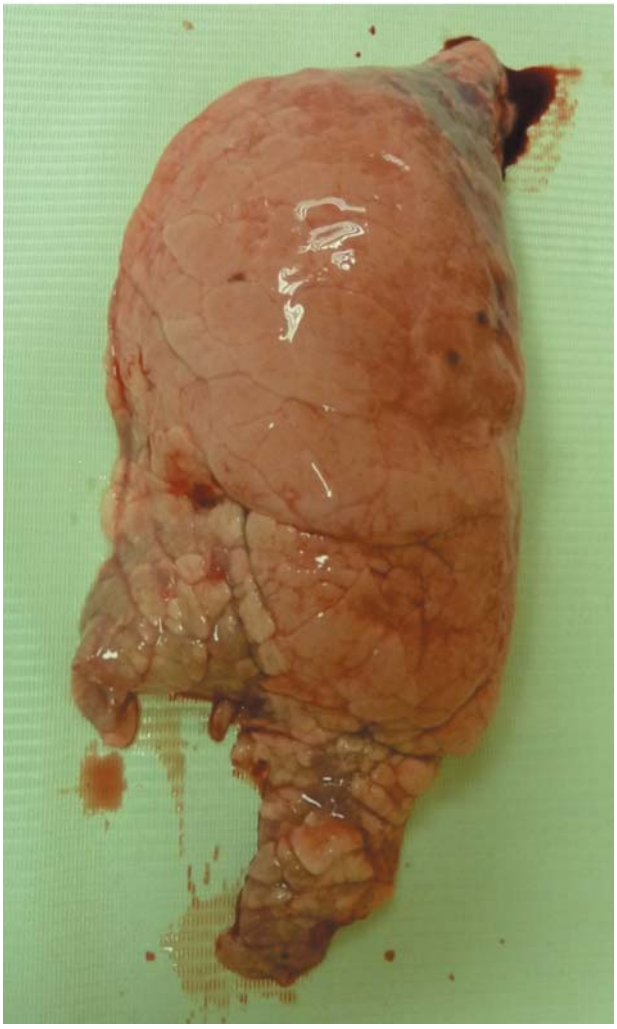

Figure 1. *A macroscopic image of the left lung from an animal of group A (Vt 6 ml/kg). Normal appearance.*

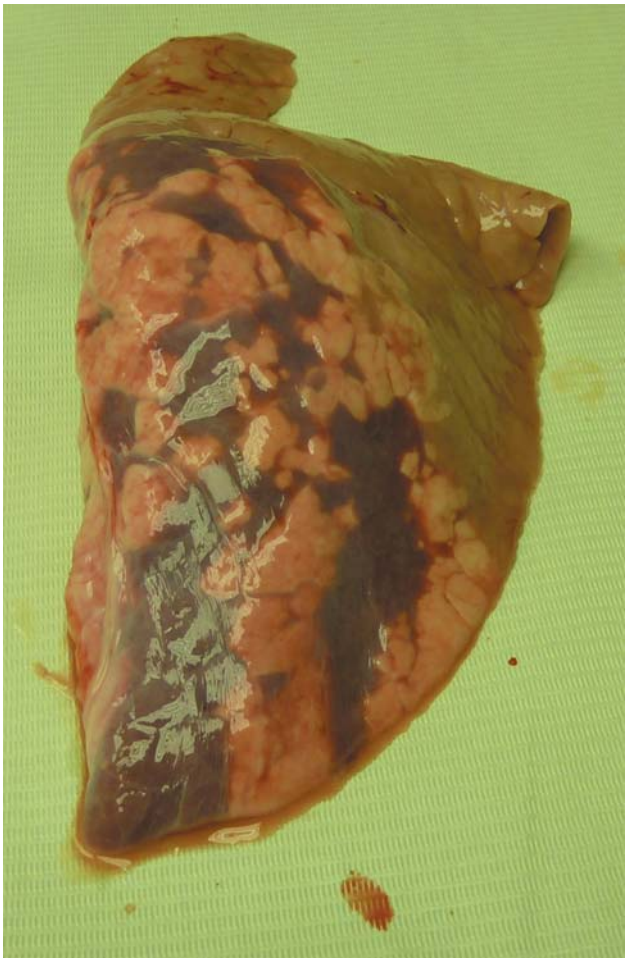

Figure 2. *A macroscopic image of the right lung from an animal of group B (Vt 10 ml/kg). Focal hyperinflation and condensation with vascular congestion of lung tissue can be seen.*

12-h ventilation period (Table I). This trend was not observed in the higher-Vt group (Table I).

The level of oxaemia was stable during the study in group A. Conversely, 12 h of mechanical ventilation with the higher Vt was associated with mild decreases in dynamic lung compliance and arterial oxygen concentration, as documented by significant changes in the ventilator indices ($AaDO_2$, OI, PaO_2/FiO_2 ratio). Progressive hypercapnia and respiratory acidosis were probably caused by higher ratio of dead volume ventilation. Similar findings were published by Wolthuis *et al*., who ventilated healthy mice for 5 h using two different tidal volumes (7.5 ml/kg *vs*. 15 ml/kg). Those receiving the low Vt ventilation developed hypercapnia during the study, compared to spontaneously-ventilated controls or animals ventilated with a high Vt. Oxaemia was stable throughout the study in both groups, with a higher level of oxygen accompanying ventilation with a high Vt (19). In the study performed by Gurkan *et al*., arterial blood gas analysis demonstrated a trend towards developing respiratory acidosis in mice ventilated with a Vt of 6 ml/kg for 4 h compared to mice ventilated with 17 ml/kg, which was even more pronounced when the mice of the low Vt group were exposed to hydrochloric acid aspiration (20). Permissive hypercapnia is an inherent element of accepted protective lung ventilatory strategy. It has been proposed that hypercapnia may have beneficial effects in patients with acute lung injury, and the concepts of permissive and even "therapeutic" hypercapnia have emerged (21, 22). However, recent work has raised concerns about the potentially deleterious effects of hypercapnia (23-25). The clinical implications of hypercapnia and hypercapnic acidosis are still not entirely clear.

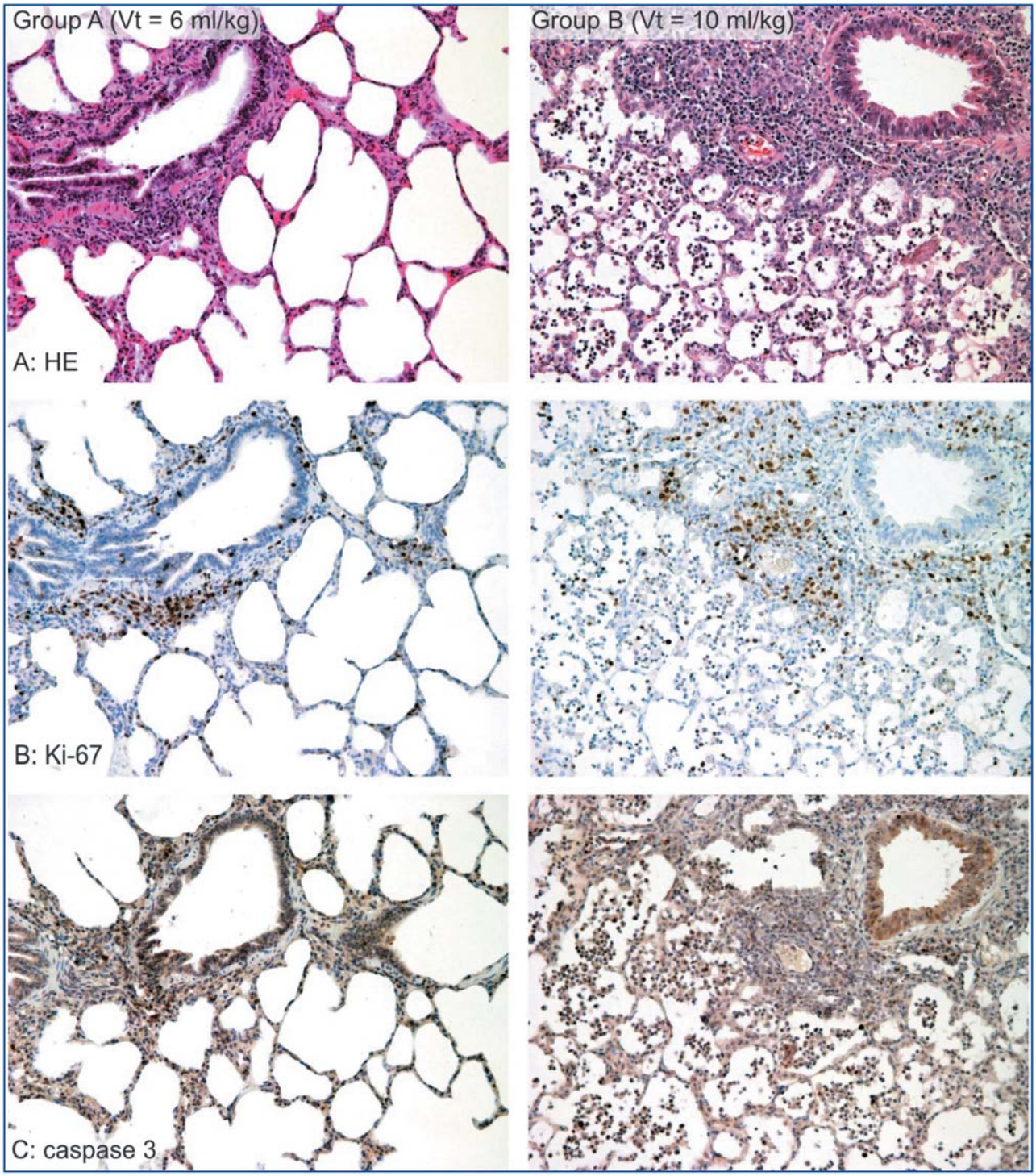

Figure 3. *Histological assessment of the tissue samples. A: Haematoxylin-eosin stain; B: immunohistochemical detection of the Ki-67 antigen, a marker of cellular proliferation, positively-stained cells are dark brown; C: immunohistochemical detection of caspase 3, a marker of apoptosis, positively-stained cells are dark brown. Micrographs demonstrate the overall morphology (A), cellular proliferation (B) and apoptosis (C) in group A animals (Vt=6 ml/kg) on the left vs. group B animals (Vt=10 ml/kg) on the right. Both samples represent terminal bronchioli and adjacent alveolar ducts, sacs, and individual alveoli within the first vertical West zone. Note the more dense infiltration surrounding the bronchiole and occupying the intra-alveolar space (A), more Ki-67-positive cells (B), and more caspase 3-positive cells in of group B when compared with group A. Scale bar=100 μm.*

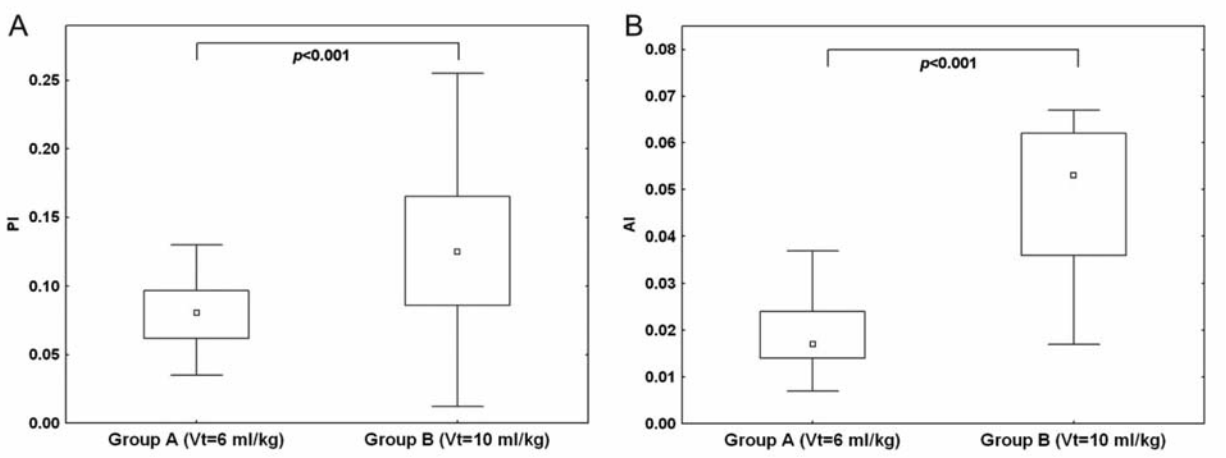

Figure 4. Histological quantification of proliferation and apoptosis in samples with different tidal volumes (Vt). A: The proliferative index (PI), calculated as a ratio between the nuclear Ki-67-positive cells and the total number of cells examined, was higher in group B than in group A (Mann-Whitney U-test p<0.001). B: The apoptotic index (AI) calculated as a ratio between nuclear caspase 3-positive cells and the total number of cells examined was higher in group B than in group A (Mann-Whitney U-test p<0.001). Data are presented as medians. The boxes span the limits of the first and third quartiles, the whiskers show the non-outlier range for each group.

Another objective of the study was to evaluate the influence of mechanical ventilation on circulation and haemodynamics. All animals were haemodynamically-stable throughout the 12 h of ventilation; no medical circulatory support was used. Invasive blood pressure monitoring and repeated echocardiographic examination revealed no changes during the study (data not published).

The last objective of the study was to evaluate the impact of mechanical ventilation on lung tissue structure and assess histological changes. We tested the hypothesis that conventional mechanical ventilation induces lung cell apoptosis and that protective mechanical ventilation prevents lung tissue apoptotic changes. Bem *et al*. showed that mechanical ventilation enhances the activation of inflammatory cytokine pathways and caspase 3 cell death pathways in response to pneumovirus infection (26). Labelling cells for activated caspase 3 in our study revealed pronounced induction of general lung cell apoptosis in conventionally-ventilated animals in comparison to insignificant apoptotic changes in lung tissue exposed to protective ventilation. These apoptotic changes were associated with concomitant proliferation, demonstrated by labelling the cells for Ki-67 (Figure 3). The role of neutrophil granulocytes and monocytes in the development of ventilatory-induced lung injury (VILI) has been widely discussed in the literature (27-29). Under light microscopy and haematoxylin-eosin staining, dense infiltration surrounding the bronchioles and vascular structures and occupying the alveolar space was pronounced in conventionally ventilated animals. A mild septal inflammatory infiltration was expressed in animals exposed to a protective ventilation strategy (Figure 3). Dense inflammatory infiltration with enhanced apoptotic and proliferative changes in conventionally-ventilated animals (group B) explain a concomitant decrease of dynamic compliance and a trend towards decreasing oxygenation (Table I). More pronounced changes would probably develop with ongoing mechanical ventilation under the same settings.

Study limitations. Twelve-hour ventilation does not correspond to the typical clinical scenario of patients in ICU, who are typically ventilated for longer time periods. There are also model- and species-specific limitations that prevent direct extrapolation of our findings to other species. We failed to raise the respiratory rate in the group of animals ventilated with a lower Vt to compensate for minute ventilation in the higher Vt group. We did not specifically describe the type of cells affected by apoptosis and did not assess VILI changes using a scoring system.

Conclusion

Mechanical ventilation with a Vt of 6 ml/kg induces minimal histological lung parenchymal changes in terms of proliferation and apoptosis. Positive pressure mechanical ventilation with Vt of 10 ml/kg does not protect lung tissue and induces substantial proliferative and apoptotic changes within the lung parenchyma. Positive pressure mechanical ventilation with Vt of 10 ml/kg does not guarantee protection of healthy pulmonary tissue in the absence of a priming pulmonary insult.

Competing Interests

The Authors declare that they have no competing interests

Acknowledgements

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