RESPIRATORY MECHANICS IN INFANT AND ADULT MICE MODELLING VENTILATOR-INDUCED LUNG INJURY

PhD Thesis

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SUMMARY

Mechanical ventilation is critical in the management of patients suffering respiratory failure. However, mechanical ventilation has also the potential to aggravate or induce lung injury. This injury is referred to as ventilator-induced lung injury (VILI). To better understand different aspects and mechanisms involved in VILI age-specific animal models are desirable. However, animal models investigating VILI do not include measurements of accurate respiratory system mechanics and inadequately consider effects of confounding factors such as positive end-expiratory pressure (PEEP), oxygen, and lung volume recruitment maneuvers.

The current thesis was designed to investigate major determinants of VILI, namely high tidal volume (V_T), inadequate PEEP, high oxygen concentrations, and stress and strain-induced release of inflammatory mediators. Thus, we aimed at investigating effects of high-V_T ventilation and PEEP in infant mice, impact of supplemental oxygen in both infant and adult mice, and outcome of lung volume recruitment maneuvers (RM) in adult mice.

Different ventilation strategies in healthy infant and adult mice were compared in an interventional controlled manner. The following outcome variables were assessed: a) *respiratory system impedance*, partitioned into components representing the conducting airways and lung parenchyma, representing dynamic lung function measurements, b) thoracic gas volume, c) *pressure-volume curves*, characterizing quasi-static lung function measurements, d) *inflammatory response*, measuring differential cell counts, protein content, and cytokines in lung lavage fluid and serum, and e) *histology*, quantifiying structural changes and inflammation.

While high-V_T ventilation produced lung injury in infant mice presumably via overdistension and loss of lung volume, high oxygen concentrations had no impact on respiratory system mechanics in either age group. In addition, we found in adult mice that PEEP increase alone and application of RMs producing peak airway opening pressures <25 cmH_2O did not prevent or reverse changes in lung mechanics, whereas frequent application of substantial RMs on top of elevated PEEP levels produced stable lung mechanics without signs of lung injury.

These findings underline the need for age-specific small animal models and require that specification of ventilator settings are reported in all studies investigating effects of mechanical ventilation in mice.

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GLOSSARY OF TERMS (ABBREVIATIONS)

ANOVA	Analysis of variance
BALF	Bronchoalveolar lavage fluid
EELV	End-expiratory lung volume
$F_{I_{O_2}}$	Fraction of inspired oxygen
FOT	Forced oscillation technique
G	Coefficient of tissue damping
H	Coefficient of tissue elastance
HVP	High tidal volume and PEEP
HVZ	High tidal volume and ZEEP
I_{aw}	Airway inertance
ID	Internal diameter
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IM	Inflation maneuver
LVP	Low tidal volume and PEEP
MIP-2	Macrophage inflammatory protein-2
OD	Outer diameter
P_{ao}	Airway opening pressure
PBF	Phosphate buffered formalin
PEEP	Positive end-expiratory pressure
P_{rs}	Transrespiratory pressure
P_{tp}	Transpulmonary pressure
PV	Pressure-volume
R_{aw}	Airway resistance
RM	Recruitment maneuver
R_N	Newtonian resistance
RR	Respiratory rate
SD	Standard deviation
SEM	Standard error of the mean
TNF-α	Tumor necrosis factor alpha

VALI	. . . .	Ventilator-associated lung injury
VILI	. . . .	Ventilator-induced lung injury
V_T	. . . .	Tidal volume
ZEEP	. . . .	Zero end-expiratory pressure
Z_{rs}	. . . .	Respiratory system impedance

LIST OF PAPERS INCLUDED IN THE THESIS

1. Cannizzaro V, Zosky GR, Hantos Z, Turner DJ, Sly PD. High tidal volume ventilation in infant mice. *Respir Physiol Neurobiol* 2008; 162: 93-9.

2. Cannizzaro V, Berry LJ, Zosky GR, Turner DJ, Hantos Z, Sly PD. Impact of supplemental oxygen in mechanically ventilated adult and infant mice. *Respir Physiol Neurobiol* 2009; 165: 61-6.

3. Cannizzaro V, Berry LJ, Nicholls PK, Zosky GR, Turner DJ, Hantos Z, Sly PD. Lung volume recruitment maneuvers and respiratory system mechanics in mechanically ventilated mice. *Respir Physiol Neurobiol* 2009; 169: 243-51.

Paper related to the subject of this thesis

4. Zosky GR, Janosi TZ, Adamicza A, Bozanich EM, **Cannizzaro V**, Larcombe AN, Turner DJ, Sly PD, Hantos Z. The bimodal quasi-static and dynamic elastance of the murine lung. *J Appl Physiol* 2008; 105: 685-92.

1. INTRODUCTION

1.1 Ventilator-induced lung injury

Clinical and experimental studies have demonstrated that artificial ventilation can promote lung injury (Slutsky, 1999; Gajic et al., 2004; Hubmayr, 2005). As a result, research on the field of acute lung injury and ventilator-induced lung injury (VILI) has led to 'protective' ventilation strategies, which comprise the application of low tidal volumes (V_T), limitation of peak pressures, and adequate setting of positive end-expiratory pressures (PEEP) (The ARDS Network, 2000; Jardin and Vieillard-Baron, 2006; Villar et al., 2006). This approach is supposed to minimize the adverse influences involved in VILI, including overdistension of alveolar units (Vlahakis and Hubmayr, 2005), repeated opening and closing of peripheral lung units resulting in shear stress at the interface between aerated and non-aerated airspaces (Lapinsky and Mehta, 2005), and damage due to release of inflammatory mediators (Dos Santos and Slutsky, 2006).

1.2 VILI in age-specific animal models

The potential harm of mechanical ventilation is magnified in infants because of a more compliant chest wall with greater risk of volutrauma, smaller lung volumes promoting atelectasis, and the delicate developing lung structures (Vitali and Arnold, 2005). Infants undergoing mechanical ventilation may develop long lasting clinical problems such as chronic lung disease, pulmonary hypertension, prolonged supplemental oxygen requirement, nutritional problems, developmental delay, and hospital readmissions with severe psychosocial consequences for the child and the parents, and financial implications, for the family and the health care system (Schibler, 2006).

Given the difficulties in performing mechanical ventilation studies in human infants and the consequent lack of data (Turner and Arnold, 2007), animal models have emerged as useful tools for studying VILI. Rodent models are widely used because of their availability and well-characterized respiratory mechanics (Gomes et al., 2000). These models have provided important insights into the mechanisms of VILI (Wilson et al., 2003; Allen et al., 2006), but have predominantly used adult animals with fewer studies in neonates (Martinez et al., 2004; Copland et al., 2004). In adult mice, high-V_T ventilation resulted in lung injury and decrease in lung compliance (Wilson et al., 2003; Allen et al., 2006). Interesting results were found

after high-V_T mechanical ventilation in newborn rats. In spite of similar ventilator settings one study showed an improvement in lung compliance (Martinez et al., 2004) whereas another study demonstrated a small decrease in compliance only after 180 min of ventilation (Copland et al., 2004). The latter study also found that adult rats were more susceptible to high-V_T-induced lung injury than newborn rats. When looking at the impact of high V_T in infant (non-neonatal) rats, Kornecki et al. (2005) observed no alteration of lung compliance after 90 min of artificial ventilation.

Recent advances in techniques for measuring thoracic gas volume and respiratory system mechanics in small intact animals (Lundblad et al., 2004; Janosi et al., 2006) have the potential to provide further insights for the specific age group of infants. Establishment and study of an infant mouse model of VILI is particularly promising because of the potential to use genetically altered mice in future studies that may allow more specific mechanisms to be explored. In terms of lung development and maturation 2-week-old mice can be compared to 2-year-old human infants (Dietert et al., 2000).

1.3 Ventilation protocols in small animals – supplemental oxygen

Supplemental oxygen is frequently used to improve survival during prolonged periods of mechanical ventilation. In addition, the lack of an oxygen blender often results in application of pure oxygen during mechanical ventilation protocols. The pro-inflammatory properties of inhaling high concentrations of oxygen are well known (Zaher et al., 2007). Also, local high oxygen concentrations may hasten atelectasis in lung units communicating poorly with the airway opening (Duggan et al., 2005; Aboab et al., 2006) and alter the function of the pulmonary surfactant system (Zenri et al., 2004), both leading to a decrease in lung compliance. Hence, a high fraction of inspired oxygen ($F_{I_{O_2}}$) may impair lung mechanics, contribute to additional lung injury and confound interpretation of the main study design.

Spontaneously breathing infant mice and rats have been shown to be more resistant to hyperoxic exposure of several days' duration than adult animals. Factors involved in this relative resistance to hyperoxia may include: greater antioxidant enzyme activity (Frank et al., 1978), decreased generation of reactive oxygen species (Ischiropoulos et al., 1989), and an increased ability to clear lung water (Laudert et al., 1994).

In the context of mechanical ventilation combination of ventilation with high V_T and/or low PEEP and hyperoxia resulted in lung injury and increased inflammatory response in adult (Quinn et al., 2002; Duggan et al., 2005; Desai et al., 2007; Li et al., 2007) and infant (Copland et al., 2004; Bland et al., 2007) rodents. However, it is not well known how short-term exposure to oxygen affects respiratory system mechanics during mechanical ventilation with low-V_T and PEEP.

Rapid-onset effects of oxygen include alteration of systemic and pulmonary vascular resistance (Rousseau et al., 2005), but also formation of atelectasis and modulation of airway smooth muscle tone and bronchomotor tone via oxygen-sensitive airway receptors (Peers and Kemp, 2001). Moreover, immature and developing lungs seem to have a higher distribution and concentration of oxygen-sensitive receptors (van Lommel, 2001). Hence, it is possible that younger animals with their evolving airway and alveolar structures and vascularization may react differently to oxygen exposure.

1.4 Ventilation protocols in small animals – recruitment maneuvers

The use of mechanically ventilated rodents to study lung injury and airway disease has increased markedly in recent times (Finkelman and Wills-Karp, 2008; Matute-Bello et al., 2008). With this has come a recognition that the ventilator settings have the potential to affect respiratory mechanics (Rich et al., 2003; Sly et al., 2003; Duggan et al., 2005, Allen et al., 2006; Tsuchida et al., 2006). Tidal volume, respiratory rate, $F_{I_{O_2}}$, airway pressures (peak inspiratory and mean airway pressure) and PEEP levels, and inspiratory to expiratory time ratio are commonly reported in mechanical ventilation studies using *in vivo* rodent models to investigate acute and chronic lung diseases. It is essential to provide information on ventilator settings in order to facilitate future studies. However, apart from specific "recruitment maneuver studies" (Allen et al., 2002; Allen et al., 2004; Frank et al., 2005; Koh et al., 2005; Farias et al., 2005; Allen et al., 2006; Allen et al., 2007; Ko et al., 2008; Jonasson et al., 2008) few experimental studies using mechanical ventilation protocols provide detailed information on application, frequency, and type of lung volume recruitment maneuvers (RM). Given that the mechanical properties of the respiratory system are specific to the lung volume at which their measurements are made (Sly et al., 2003)

and to the lung volume history (Zosky et al., 2008), it is surprising that details of RMs are not always reported.

In clinical practice, based on the concept of "open up the lung and keep the lung open" (Lachmann, 1992), recruitment refers to a dynamic process of reopening non-aerated peripheral lung units through a substantial and sustained increase of transpulmonary pressure including elevation of PEEP (Fan et al., 2005). In lung function studies conducted in murine models of respiratory diseases, RMs aim at establishing similar lung volume history and often precede baseline measurement of lung function. Generally, these RMs consist of a series of inflation maneuvers that are either volume- or pressure-controlled and do not include elevation of PEEP. Application of different types of RMs reflects different views on how to best recruit non-aerated lung units without producing lung injury (Hjoberg et al., 2004; Frank et al., 2005; Allen et al., 2007; Ito et al., 2007). In a recent study, Jonasson et al. (2008) showed that application of deep inflations also protects against bronchoconstriction and affects outcome respiratory system mechanics in healthy and allergen-challenged mice.

1.5 Aims and hypotheses of the present thesis

1.5.1 Study 1: High tidal volume ventilation in infant mice

The aim of the study reported in this section was to investigate the effects of high-V_T ventilation in a novel *in vivo* infant mouse model for VILI. In line with adult mouse studies we hypothesized that high-V_T ventilation without PEEP may cause the most significant changes in lung mechanical parameters and lung injury.

1.5.2 Study 2: Impact of supplemental oxygen in mechanically ventilated adult and infant mice

Study 2 was undertaken to determine whether high oxygen concentrations alter respiratory system mechanics and inflammatory response, and whether this interaction differs between infant (2 week old) and adult (8 week old) rodents.

1.5.3 Study 3: Lung volume recruitment maneuvers and respiratory system mechanics in mechanically ventilated mice

The respective impacts of PEEP elevation, inflation maneuvers without PEEP elevation, and RMs (i.e. inflation maneuvers plus PEEP elevation) on lung function are not clear in mice. The aim of study 3 was to establish how these maneuvers affect respiratory system mechanics and whether they induce or exacerbate lung injury in mechanically ventilated mice. We hypothesized that frequent and large RMs provide stable respiratory system mechanics, but at the expense of lung injury.

2. METHODS

2.1 Study animals

For the studies included in the thesis 2 and 8 week old specific pathogen-free female BALB/c mice were purchased from the Animal Resource Centre (Murdoch, Western Australia). Two week old mice were kept with their dam until the day of experiment. The experimental procedures were approved by the Telethon Institute for Child Health Research Animal Experimentation and Ethics Committee and conform to the guidelines of the National Health and Medical Research Council of Australia.

2.2 Animal preparation

Mice were anaesthetised with an intraperitoneal injection of a solution containing ketamine and xylazine (both Troy Laboratories, N.S.W, Australia). To induce surgical level of anaesthesia 8 week old mice were given 160 μg/g ketamine and 8 μg/g xylazine, followed by a top-up of 100 μg/g ketamine and 5 μg/g xylazine after 10 min. Two week old mice were given 160 μg/g ketamine and 8 μg/g xylazine without top-up after 10 min. Further anaesthetic was given as required throughout the experiment. Once adequately anaesthetised, a tracheotomy was performed and a 10 mm long polyethylene cannula (OD: 1.27 mm, ID: 0.86 mm) or a metal cannula (21-gauge) for 8 and 2 week old mice, respectively, was inserted and secured with suture. Mice were then connected to a computer-controlled ventilator (*flexiVent,* Scireq, Montreal, Canada).

2.3 Measurement of lung volume and respiratory system mechanics via whole body plethysmography and wave-tube technique, respectively (Study 1)

Animals were placed in the supine position in a custom-built whole body plethysmograph (180 mL volume) and connected to the small animal ventilator via a specially designed connector that passed through the plethysmograph wall. Body temperature was monitored with a rectal thermocouple and maintained at 36.5 to 37.5 ºC with the use of a heat lamp.

Lung volume history was standardised as follows: lung volume was increased by lowering plethysmographic box pressure via a regulated vacuum line from 0 to -20 cmH_2O at a constant rate, while the tracheal cannula was open to atmosphere through

the box wall. This was followed by a slow passive expiration to 0 cmH_2O P_{rs}. By this means two slow (~30 s) inflation-deflation maneuvers, separated by 5 min of ventilation were applied. Once lung volume history was standardised, baseline measurements of end-expiratory lung volume (EELV) and respiratory system impedance (Z_{rs}) were taken. EELV was measured using the whole-body plethysmographic technique based on Boyle's law, as described in detail previously (Janosi et al., 2006). Briefly, the airway was occluded at 0 cmH_2O transrespiratory pressure (P_{rs}) and breathing efforts were induced using electrical stimulation of the intercostal muscles.

Z_{rs} was measured using the miniature wave-tube version of the low-frequency forced oscillation technique (Hantos et al., 1995; Bozanich et al., 2007). A pseudorandom oscillatory signal ranging from 4 to 38 Hz was delivered by a loudspeaker-in-box system to the tracheal cannula via a wave-tube at P_{rs} = 0 cmH_2O, and Z_{rs} was measured as the load impedance on the wave-tube. The "constant-phase" model (Hantos et al., 1992) was then fitted to the resulting Z_{rs}, allowing the estimation of airway resistance (R_{aw}) and inertance (I_{aw}), and the coefficients of tissue damping (G) and elastance (H):

$$Z_{rs} = R_{aw} + j\omega I_{aw} + (G-jH)/\omega^{\alpha}$$

where $\alpha=(2/\pi)\tan^{-1}(H/G)$, j is the imaginary unit, and ω is angular frequency. Values of R_{aw} and I_{aw} were corrected for the resistance and inertance, respectively, of the tracheal cannula. After the subtraction of the impedance of the tracheal cannula the values of I_{aw} became insignificantly low and hence not reported.

2.4 Measurement of respiratory system mechanics with the *flexiVent*® (Studies 2 and 3)

After placing the mouse in the supine position on a heating mat and connecting the animal to the ventilator lung volume history was standardized by three pressure-limited (20 cmH_2O) linear inflation-deflation maneuvers applied within 5 min. Then, baseline measurement of Z_{rs} was performed using the low-frequency FOT provided by the *flexiVent*® system. In study 2, Z_{rs} was obtained during a 16 s pause from mechanical ventilation during which a broadband signal of 19 mutually prime frequencies from 0.25 to 20 Hz was applied to the airway opening of the mouse. In study 3, Z_{rs} was obtained by a 4-s oscillation signal of 13 mutually prime frequencies

from 1.0 to 20.5 Hz. During Z_{rs} measurement PEEP level remained unchanged at the pre-measurement level. The resulting input impedance data were analysed using the constant-phase model (Hantos et al., 1992), which allows distinction between central and peripheral respiratory mechanics. R_{aw}, I_{aw}, G, and H were determined by fitting the model to the Z_{rs} data. I_{aw} values are considered insignificantly low and hence are not reported. Except for data points coinciding with the heart rate or its harmonics, the constant-phase model fitted well to impedance data. The coefficient of determination, a quality control parameter reflecting the goodness of the model fit, was > 0.985 in all studies.

2.5 Correction for differences in gas composition (Study 2)

In addition to routine dynamic calibration procedure of the *flexiVent*®, which accounts for gas compression and pressure drop across tubing (including the tracheal tube), the effect of the different gas compositions had to be taken into consideration. Due to the fact that pure oxygen has a higher viscosity than nitrogen, correction for viscosity change was mandatory when using different $F_{I_{O_2}}$ levels. Correction factors were obtained by using published values for gas viscosity (Turney and Blumenfeld, 1973). Accordingly, theoretical calculations from equations for viscosities of oxygen and nitrogen predicted increases in viscosity and hence airway resistance by 1.013, 1.056, and 1.117 for a $F_{I_{O_2}}$ of 0.3, 0.6, and 1.0, respectively, relative to room air. These factors were calculated with the assumption of an average gas temperature in the airways of ~30°C. The correction factors were determined experimentally in our equipment (data not shown) and used to divide the values of the R_{aw} measured with the corresponding gas mixture. Only corrected values of R_{aw} are reported.

2.6 Study designs and experimental protocols

2.6.1 Study 1: High tidal volume ventilation in infant mice

Before allocation to study groups animals were ventilated with room air at a respiratory rate (RR) of 360/min with a delivered V_T of 8 mL/kg (when accounting for gas compression and/or friction in the cylinder) and PEEP of 3 cmH_2O. PEEP level was regulated via depth of a water column that was connected to the expiratory port of the ventilator. After baseline measurements, mice were randomized to receive one of three ventilation strategies: (1) high V_T with zero end-expiratory pressure (HVZ): RR

= 150/min, delivered V_T of 20 mL/kg, no sighs (n=12), (2) high V_T with PEEP (HVP): RR = 150/min, delivered V_T of 20 mL/kg, PEEP of 3 cmH_2O, and no sighs (n=12), and (3) low-V_T with PEEP (LVP): RR = 360/min, delivered V_T of 8 mL/kg, PEEP of 3 cmH_2O, and 2 sighs of 20 mL/kg which did not exceed 20 cmH_2O inspiratory peak pressure, applied shortly after and 5 min before each measurement of respiratory mechanics (n=12). Mice were ventilated for 60 min with measurements of EELV and Z_{rs} at baseline and every 10 min during the ventilation period. Ventilation frequencies in each strategy were matched for minute ventilation.

2.6.2 Study 2: Impact of supplemental oxygen in mechanically ventilated adult and infant mice

Eight week old mice were ventilated with room air at a RR = 300/min with PEEP of 3 cmH_2O, and a delivered V_T of 7.3 ± 0.1 mL/kg. Two week old mice were ventilated as follows: room air, RR of 240/min with PEEP of 3 cmH_2O, and a delivered V_T of 8.8 ± 0.5 mL/kg. Minute ventilation was similar in both age groups and high enough to ensure that mice remained apneic during measurements of respiratory system mechanics. After baseline measurement, mice were assigned to one of the following groups: (1) $F_{I_{O_2}}$=0.21 (control group), (2) $F_{I_{O_2}}$=0.3, (3) $F_{I_{O_2}}$=0.6, and (4) $F_{I_{O_2}}$=1.0; n = 8 mice per group. $F_{I_{O_2}}$ levels of 0.3, 0.6, and 1.0 were arbitrarily chosen based on the common clinical assumption that these levels are “safe”, “borderline”, and “harmful”, respectively. Oxygen concentrations were controlled by a gas blender (Bird 3M, Palm Springs, California, USA) that was supplied by the Research Institute’s air and oxygen lines. Constant flow of 2 L/min was adjusted with a flow meter and delivered via hose to the top end of the inspiratory port of the ventilator. Mice were ventilated for 120 min. Moderate RMs, designed to prevent extensive atelectasis were delivered shortly after and 5 min before each Z_{rs} measurement (every 10 min during the ventilation period). The RM was pressure-limited and lasted 6 s, partitioned into 3 s ramp duration and 3 s hold at 20 cmH_2O, followed by passive deflation to a PEEP of 3 cmH_2O.

2.6.3 Study 3: Lung volume recruitment maneuvers and respiratory system mechanics in mechanically ventilated mice

Before baseline measurement and allocation to study groups mice were ventilated with the following settings: $F_{I_{O_2}}$=0.5, RR=180/min, delivered V_T of ~8 mL/kg, and PEEP=2 cmH_2O. Heart rate and transcutaneous oxygen saturation were monitored via a small animal pulse oximeter (MouseOx™, STARR Life Sciences Corporation™, Oakmont PA, USA) by placing the non-invasive sensor on the tail. Animals were then allocated to study groups differing in PEEP level and inflation maneuver (IM) frequency and mode (n=7 mice/group). Six study groups (1a-6a) received different IMs while the PEEP level was unchanged at 2 cmH_2O throughout the protocol: 20 mL/kg every 5 min (Group 1a) or every 75 min (Group 2a), 40 mL/kg every 5 min (Group 3a) or every 75 min (Group 4a), and IMs to 25 cmH_2O every 5 min (Group 5a) or every 75 min (Group 6a). In another six study groups (1b-6b) PEEP was increased from 2 to 6 cmH_2O shortly before application of the first IM and then remained at 6 cmH_2O throughout the study: 20 mL/kg every 5 min (Group 1b) or every 75 min (Group 2b), 40 mL/kg every 5 min (Group 3b) or every 75 min (Group 4b), and IMs to 25 cmH_2O every 5 min (Group 5b) or every 75 min (Group 6b). Two additional groups were ventilated at a PEEP of 2 cmH_2O (Group 7a) or 6 cmH_2O (Group 7b) without any application of IMs. In the latter group (Group 7b) PEEP was increased from 2 to 6 cmH_2O shortly after the first baseline measurement.

Lung volume recruitment was achieved by a combination of changes in PEEP level (i.e. 2 and 6 cmH_2O) and application of IM at different time points (i.e. every 5 or 75 min) during the 150-min protocol. IMs were delivered either in a volume-controlled manner without pressure limit (20 mL/kg or 40 mL/kg) or in a pressure-controlled mode (25 cmH_2O). The IMs consisted of a 3-s ramp duration to reach preset volume or plateau pressure and a 3-s hold followed by passive deflation to the predefined PEEP level.

Z_{rs} measurements were performed every 15 min and included pre- and post-IM measurements for the study groups 1a-6a and 1b-6b. At each time point four Z_{rs} spectra were collected within 90 s and the corresponding values for R_{aw}, G, and H were averaged. The PEEP level was left unchanged at either 2 or 6 cmH_2O to prevent lung derecruitment during measurements and the oscillations were delivered on top of these PEEP levels.

2.7 Sampling and processing of blood and bronchoalveolar lavage fluid

Blood and BALF samples were taken from and analyzed in all animals, including non-ventilated controls. At the end of the study blood samples were obtained by cardiac puncture. Blood was allowed to clot and centrifuged at 2000 r/min for 10 min and serum frozen for later analyses of interleukin-6 (IL-6) and macrophage inflammatory protein-2 (MIP-2). After blood sampling, lungs were lavaged via tracheostomy with 0.5 and 0.2 mL of sterile 0.9% saline solution for adult and infant mice, respectively. The lavage solution was instilled in and out of the lung three times and ice cooled until centrifugation at 2000 r/min for 4 min. Supernatant was collected and frozen for later analysis of IL-6, MIP-2, tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and total protein. The cell pellet was resuspended in phosphate buffered saline and an aliquot was stained with Trypan blue to obtain a total cell count using a haemocytometer. A second aliquot was centrifuged onto a slide and stained with Leishmann's to obtain a differential cell count using light microscopy by counting 300 cells from each slide. Concentrations of IL-6, IL-1β, MIP-2, and TNF-α were measured in all samples by using enzyme linked immunosorbent assays, following the manufacturer's instructions (BD Biosciences, San Diego, California). Total protein was analysed using a colorimetric Bio-Rad protein assay (Bio-Rad, Regents Park, New South Wales, Australia).

2.8 Analysis of lung tissue by morphology and morphometry (Study 3)

Lungs were fixed with 10% phosphate buffered formalin (PBF) instilled via the endotracheal tube at a pressure of 10 cmH_2O. Two hours later the lungs and heart were removed en bloc from the thoracic cavity and stored in a PBF filled container overnight. At the time of processing the heart was dissected free and the remaining tissues were processed whole in paraffin, and embedded with the caudoventral aspect down. Sections were cut at 5 μm intervals from the caudoventral aspect to include as many lung lobes as possible, and stained routinely with haematoxylin and eosin. Inflammatory cells in the histological sections of lung were counted by blindly selecting ten fields at x100 (oil immersion) from each section. In each of these ten fields, the number of erythrocytes, alveolar macrophages, alveolar neutrophils, and septal neutrophils was counted. To determine the degree of lung inflation by morphometry, ten fields from each lung section were blindly selected and digitally

captured under the x40 objective. A 100-point grid was superimposed over each image, and the number of grid intersection points that coincided with an alveolar wall was determined. For each animal, the sum of the grid counts over the ten digitized images was taken as the relative inflation score. The pathologist was blinded to the study groups. Five lungs in each of the following groups (study 3) were rated: PEEP 2 – no IM (group 7a), PEEP 6 – no IM (group 7b), PEEP 2 – inflation with 40 mL/kg every 5 min (group 3a) or every 75 min (group 4a), and PEEP 6 – inflation with 40 mL/kg every 5 min (group 3b) and every 75 min (group 4b). The selection of these groups was based on the assumption that comparison between control groups (7a and 7b) and groups undergoing large inflations would be adequate to demonstrate lung injury in tissue samples.

2.9 Statistical analysis

All statistics were conducted using SigmaStat 3.5 (Systat Software Inc, Richmond, California, USA). One-way analysis of variance (ANOVA) with Holm-Sidak post-hoc tests was used for baseline comparisons of Z_{rs} data, peak airway opening pressure levels, BALF and serum outcome parameters, and histological lung injury scores. For repeated physiological measurements repeated measures ANOVA with Holm-Sidak post-hoc tests were used. Unless specifically stated data are expressed as group means ± standard error of the mean and were transformed where appropriate to ensure the assumptions of normality and equal variance were satisfied. Where this was not possible equivalent non-parametric comparisons were used. Statistical significance was set at $p<0.05$.

3. RESULTS

3.1 Study 1: High tidal volume ventilation in infant mice

3.1.1 End-expiratory lung volume (EELV)

As demonstrated in Fig. 1A, lung volume in both the HVP and LVP groups did not change throughout the 60-min ventilation period. In contrast, the HVZ group showed a significant reduction in EELV after 20 min of mechanical ventilation, which was sustained for the duration of the study period and approximated to a volume loss of one third.

Figure 1

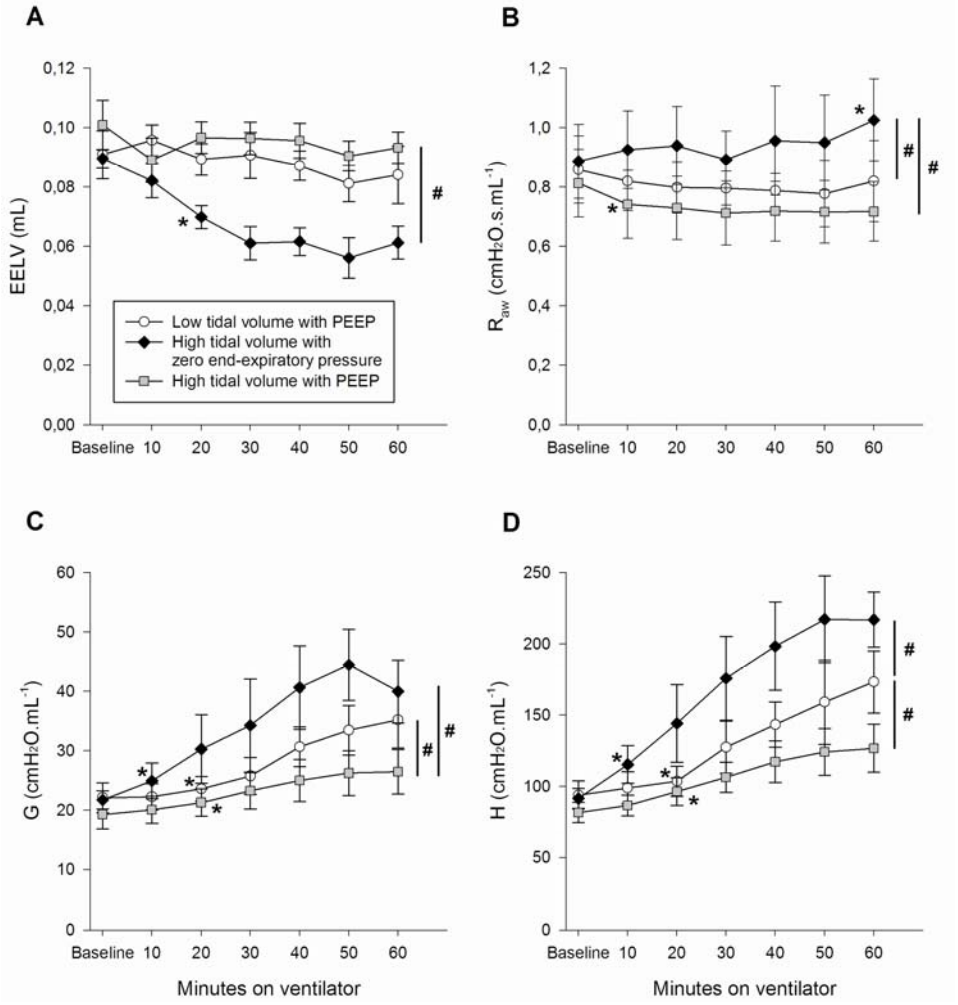

Fig. 1. Respiratory mechanics as a function of time on the ventilator in the 3 experimental groups. A: end-expiratory lung volume (EELV); B: airway resistance (R_{aw}); C: coefficient of tissue damping (G); D: coefficient of tissue elastance (H). Data are expressed as group means ± standard error of the mean. Significant change ($p<0.05$) when compared with baseline and between groups, are indicated by * and $^{\#}$, respectively.

3.1.2 Respiratory system mechanics

Fig. 1B shows changes in R_{aw} during the ventilation period in the 3 groups of mice. A significant increase in R_{aw} was measured after 60 min in the HVZ group whereas R_{aw} significantly dropped after 10 min in the HVP group and remained unchanged until the end of the experiment. Alterations of G and H are illustrated in Fig. 1C and D, respectively. Values for both G and H increased significantly after 10 min in the HVZ group and by 20 min for the HVP and LVP groups when compared with their respective baselines. At the end of the study protocol G and H in the HVZ group increased by 86% and 148%, respectively, which was significantly greater than in the HVP (38% and 56%, respectively) and LVP groups (62% and 88%, respectively).

3.1.3 Relationship between EELV and lung tissue mechanics

In order to explore the relationship between change in lung volume and parenchymal mechanics, the relationship between EELV and 1/H (compliance) was investigated. As the changes in EELV and H over the ventilation period were small for the HVP and LVP groups, this relationship could not be examined. However, in the HVZ group where there was a significant decrease in EELV and increase in H, there was a strong linear relationship between 1/H and EELV ($p_{slope} < 0.001$; $R^2 = 0.965$) (Fig. 2).

Figure 2

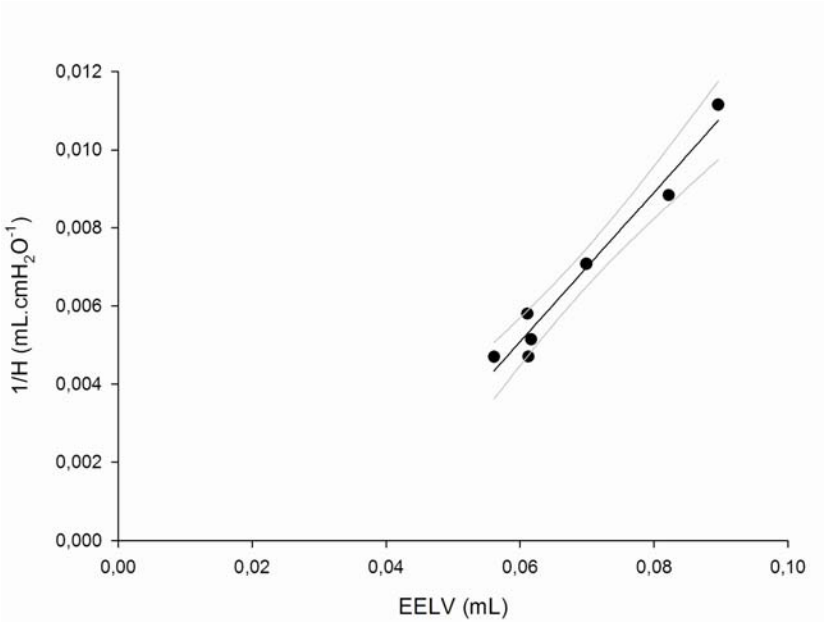

Fig. 2. Relationship between 1/H (1/tissue elastance) and end-expiratory lung volume (EELV) for the HVZ group. Shown are the group mean data, fitted linear regression line and 95% CI.

3.1.4 Peak and mean airway opening pressure levels

Fig. 3A and B show peak values of airway opening pressure (P_{ao}) and mean P_{ao} levels from baseline to the end of the study. After switching to the allocated ventilation pattern peak P_{ao} levels of the LVP, HVP and HVZ groups increased to 11.8, 16.9, and 13.3 cmH_2O, respectively. Thereafter, P_{ao} values of the study groups with PEEP (LVP and HVP) rose to 14.4 and 20.2 cmH_2O, respectively, whereas peak P_{ao} in the HVZ group increased to 20.6 cmH_2O by the end of the experiment. Similarly, after switching to the allocated ventilation pattern mean P_{ao} levels of LVP and HVP groups increased to 7.4 and 9.9 cmH_2O, respectively, whereas mean P_{ao} decreased to 6.6 cmH_2O in the HVZ group as a result of PEEP withdrawal. In LVP, HVP, and HVZ groups mean P_{ao} further increased to 8.7, 11.6, and 10.3 cmH_2O, respectively, by the end of the study protocol. Periodically applied sighs in the LVP group were below P_{ao} levels of 20 cmH_2O.

Figure 3

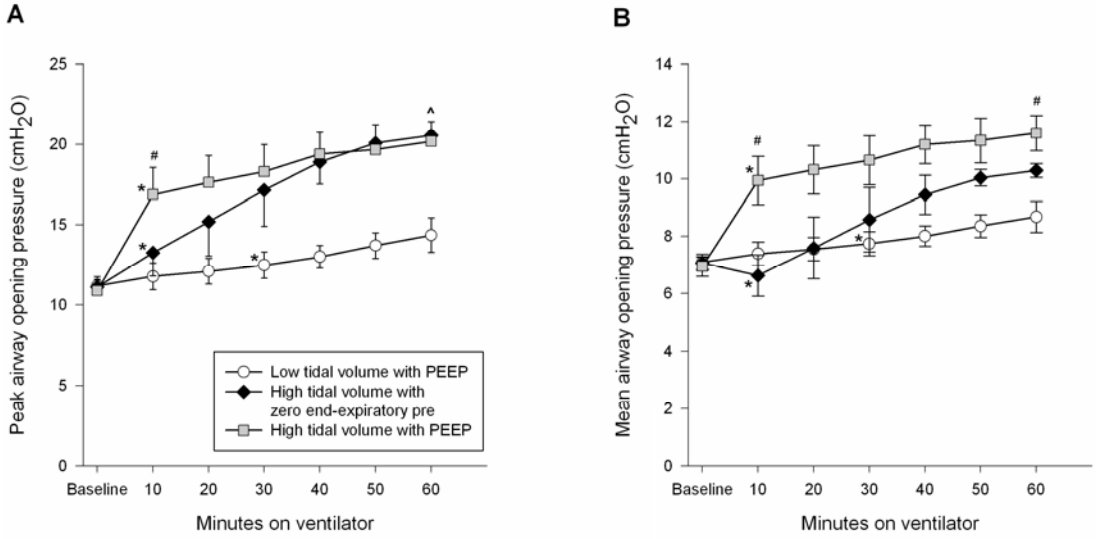

Fig. 3. Peak (A) and mean airway opening pressure (B) during ventilation with low-V_T and PEEP (LVP), high V_T and PEEP (HVP), and high V_T and zero end-expiratory pressure (HVZ) at different time points from baseline (before allocation to study groups) to end of the study. Data of each group are means ± SD. * indicates a significant change when compared with respective baseline, # indicates a significant difference between all the study groups, ^ indicates a significant difference between the two high-V_T groups and the low-V_T group ($p < 0.05$).

3.1.5 Inflammatory cells, cytokines and total protein in BALF and interleukin-6 in serum

There was no difference between any of the ventilation groups and the non-ventilated controls for total cells and differential cell counts (macrophages and neutrophils) obtained from the BALF. Similarly, as shown in Fig. 4A we found no significant differences in levels of IL-6 in BALF ($p = 0.09$). In contrast, there was a significant increase in total protein levels of mice ventilated with the HVP strategy compared to all other groups (Fig. 4C). The level of IL-1ß was below the range of detection (<30 pg/mL) for control and ventilated mice. The serum concentration of IL-6 was significantly higher in the HVZ group when compared with all other groups (Fig. 4B).

Figure 4

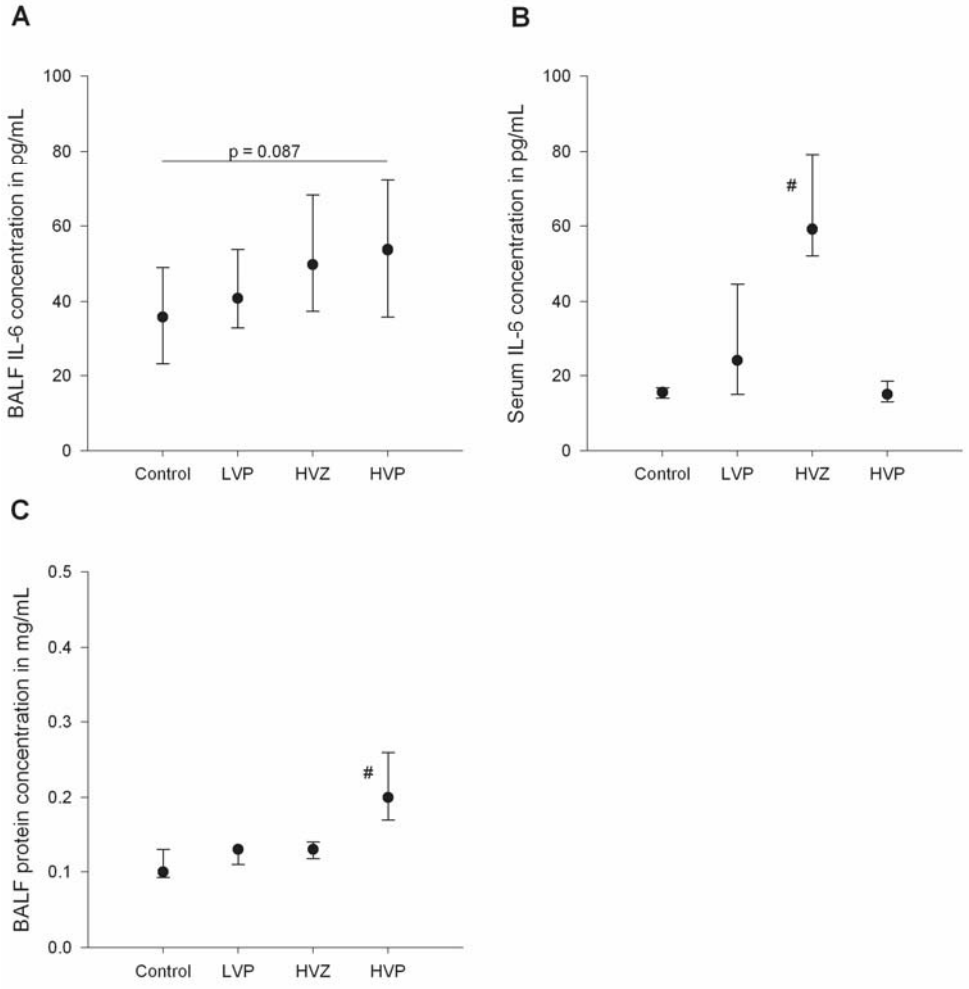

Fig. 4. Interleukin-6 (A) and total protein (C) concentrations in bronchoalveolar lavage fluid; interleukin-6 (B) concentration in serum. Symbols with error bars display median with interquartile ranges. #Statistically significant change between groups ($p<0.05$).

3.2 Study 2: Impact of supplemental oxygen in mechanically ventilated adult and infant mice

3.2.1 Airway resistance (R_{aw}) after correction for change in gas mixture

Baseline measurements for R_{aw} were not different between $F_{I_{O_2}}$ groups in both 8 and 2 week old mice. We found no difference in R_{aw} between $F_{I_{O_2}}$ groups after 120 min of mechanical ventilation in both age groups (Fig. 5A and B, respectively). When compared with baseline values, R_{aw} significantly increased after 90 min in 8 week old mice, whereas it significantly decreased after 10 min in 2 week old mice ($p < 0.05$, respectively). However, these differences were small and considered to be physiologically insignificant.

3.2.2 Coefficients of lung tissue damping (G) and elastance (H)

Baseline measurements for G and H were not different between groups in both age groups (Fig. 5C - F). After 120 min of mechanical ventilation no difference between $F_{I_{O_2}}$ groups was found for both G and H in 8 and 2 week old mice. Values for G and H significantly increased after 30 and 10 min, respectively, in both age groups when compared with their respective baselines. At the end of the study protocol G increased on average by 27 and 29% in 8 and 2 week old mice, respectively, while H increased by 49 and 41% in 8 and 2 week old mice, respectively.

3.2.3 Inflammatory cells, cytokines, and total protein in BALF and cytokines in serum

After 120 min we found no difference in total cells and differential cell counts (e.g. macrophages, neutrophils, and lymphocytes) obtained from the BALF between $F_{I_{O_2}}$ groups in both age groups ($p > 0.41$ in all cases). As shown in Figure 6A - F there also was no statistically significant difference between levels of oxygen concentrations in both age groups for IL-6, MIP-2, and total protein concentrations. IL-6 and MIP-2 serum concentrations were below the assay's limits of detection in all mice (IL-6 < 34.8 pg/mL, MIP-2 < 26.5 pg/mL).

Figure 5

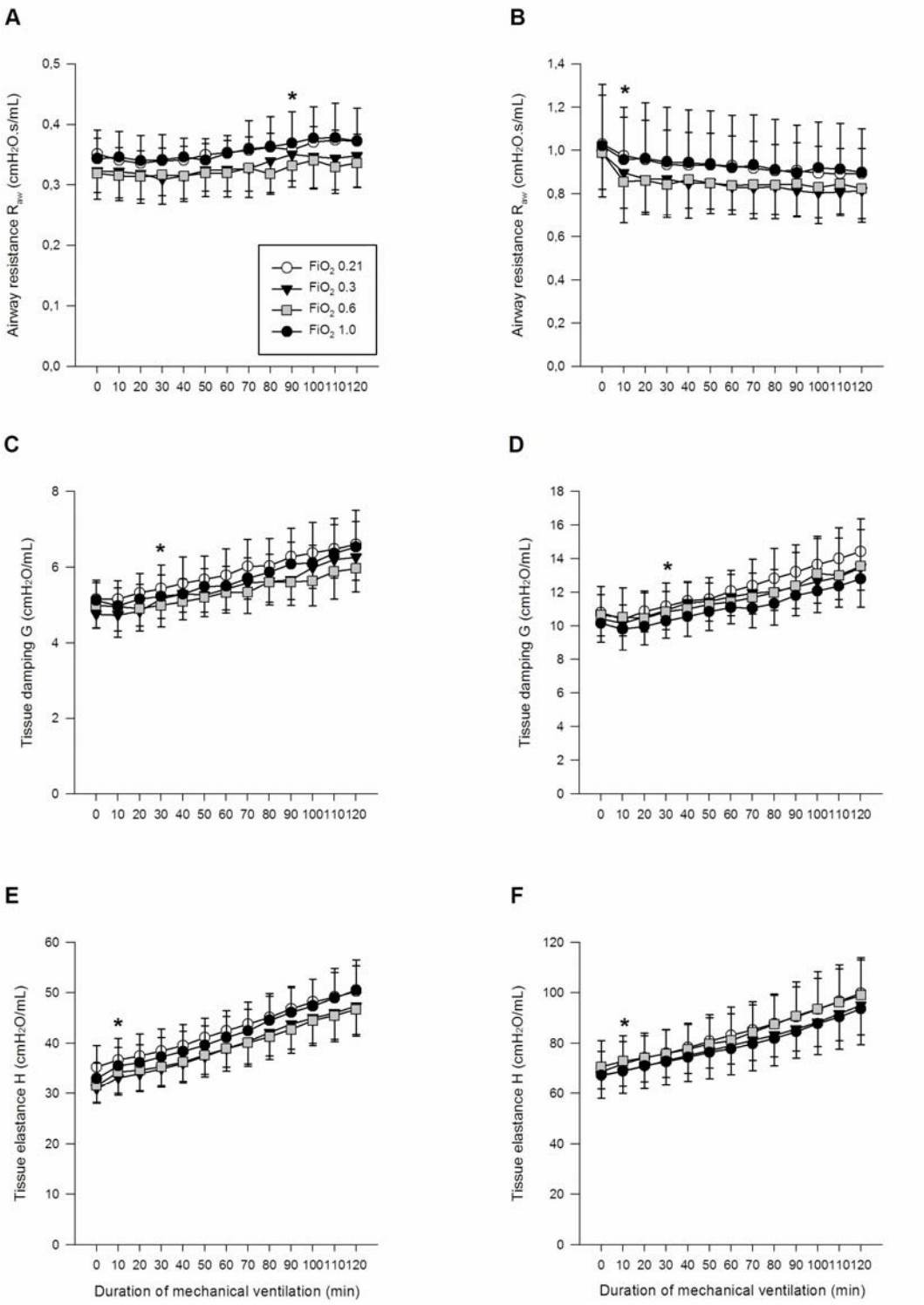

Fig. 5. Airway resistance (R_{aw}) (panels A and B), coefficients of lung tissue damping (G) (panels C and D) and elastance (H) (panels E and F) as a function of time on the ventilator in 8 week old (panels A, C, and E) and 2 week old mice (panels B, D, and F). Data are expressed as group means ± standard error of the mean. * indicates significant changes when compared with baseline R_{aw}, G, and H values of 8 and 2 week old mice, respectively ($p < 0.05$). No significant differences were found between study groups ($p > 0.17$ in all cases).

Figure 6

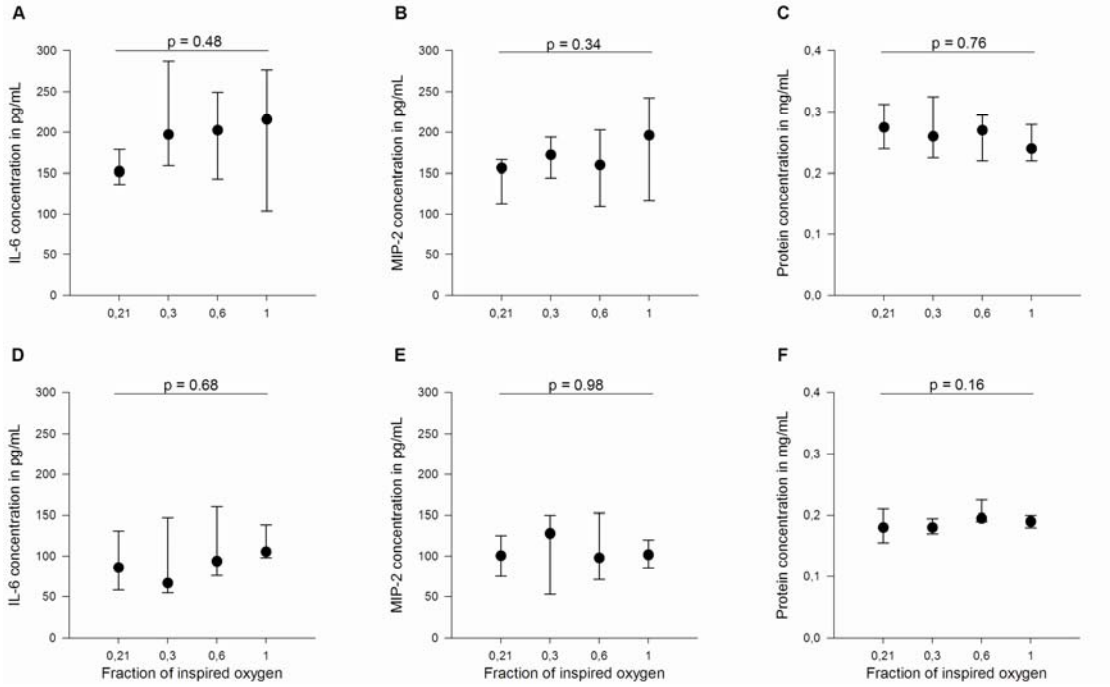

Fig. 6. Interleukin-6, macrophage inflammatory protein-2, and total protein concentrations in bronchoalveolar lavage fluid after 120 min of mechanical ventilation. Panels A-C and D-F refer to 8 and 2 week old mice, respectively. Symbols with error bars display median with interquartile ranges. Concentration levels between groups were not statistically different.

3.3 Study 3: Lung volume recruitment maneuvers and respiratory system mechanics in mechanically ventilated mice

3.3.1 Z_{rs} values and peak airway opening pressure levels at baseline

After standardization of lung volume history and before allocation to study groups no differences were found for R_{aw}, G, H, and peak P_{ao} levels between study groups ($p>0.49$ in all cases) (Figs. 7 and 8, Table 1).

3.3.2 R_{aw} after lung volume recruitment at PEEP level of 2 cmH_2O

Compared with baseline values R_{aw} statistically significantly increased at 150 min in the control group (Fig. 7A). IMs with 20 mL/kg resulted in steady R_{aw} values ($p>0.17$ in both cases) while frequent (i.e. every 5 min) application of IMs to 25 cmH_2O or with 40 mL/kg produced a statistically significant but physiologically unimportant decrease in R_{aw} over time. At the end of the protocol we found small but statistically significantly higher R_{aw} in controls when compared to groups receiving pressure controlled IMs or IMs of 40 mL/kg.

Table 1

Peak airway opening pressure (P_{ao}) in cmH_2O before, during, and after lung volume recruitment maneuvers (RMs) at selected time points (mean, SD)

Study group	P_{ao} at baseline	P_{ao} at 74 min (before RM)	P_{ao} during RM	P_{ao} at 75 min (after RM)	P_{ao} at 149 min (before RM)	P_{ao} during RM	P_{ao} at 150 min (after RM)
1a	7.9 (0.5)	9.7 (0.8)	17.4 (2.7)	9.6 (0.8)	11.0 (0.9)	22.3 (1.8)	10.8 (0.8)[a,*]
2a	7.8 (0.2)	9.4 (0.6)	16.6 (1.6)	9.3 (0.5)	11.2 (1.0)	22.0 (1.7)	10.5 (0.6)[a,*]
3a	7.9 (0.5)	7.3 (0.4)	30.6 (0.9)	7.2 (0.4)	7.3 (0.4)	30.8 (0.8)	7.2 (0.4)[b,*]
4a	8.0 (0.3)	9.8 (0.9)	33.0 (1.6)	7.8 (0.3)	9.2 (0.5)	33.5 (1.5)	7.7 (0.3)[b,*]
5a	8.0 (0.3)	8.5 (0.4)	25	8.3 (0.3)	8.7 (0.4)	25	8.5 (0.4)[c,*]
6a	7.8 (0.2)	9.5 (0.4)	25	8.4 (0.5)	10.5 (0.9)	25	8.8 (0.7)[a,*]
7a	7.8 (0.3)			9.5 (0.5)			11.6 (1.0)[a,*]
1b	7.9 (0.3)	12.6 (0.3)	24.8 (1.1)	12.5 (0.3)	12.8 (0.3)	25.8 (1.1)	12.6 (0.4)[c,*]
2b	8.1 (0.4)	9.9 (0.7)	24.3 (0.5)	13.3 (0.6)	15.0 (0.9)	26.7 (0.6)	14.0 (0.6)[a,*]
3b	8.0 (0.5)	10.5 (0.2)	41.7 (0.6)	10.3 (0.2)	10.3 (0.2)	41.4 (0.8)	10.2 (0.2)[c,*]
4b	8.0 (0.2)	9.7 (0.4)	35.9 (1.6)	11.0 (0.1)	11.9 (0.3)	40.7 (1.5)	10.7 (0.2)[a,*]
5b	7.7 (0.4)	11.9 (0.9)	25	11.8 (0.9)	12.1 (1.1)	25	12.0 (1.0)[a,*]
6b	8.2 (0.3)	9.9 (0.3)	25	12.7 (0.9)	14.1 (1.1)	25	13.2 (1.2)[a,*]
7b	8.0 (0.2)			13.3 (0.3)			14.6 (0.2)[a,*]

For definitions of abbreviations 1-7a and 1-7b see legends of Fig. 7 and 8 on page 29 and 30, respectively. "a" indicates statistically significant differences between selected time points at baseline, 75, and 150 min; "b" denotes a statistically significant P_{ao} decrease between baseline and both 75 and 150 min, but no significant difference between 75 and 150 min, and "c" indicates a statistically significant P_{ao} increase between baseline and both 75 and 150 min with no difference between 75 and 150 min ($p>0.06$ in all cases). "*" denotes a statistically significant P_{ao} decrease when comparing the time points 149 min and 150 min, i.e. before and after a RM ($p\leq0.03$, paired t-test).

3.3.3 G and H after lung volume recruitment at PEEP level of 2 cmH_2O

G steadily and significantly increased from baseline to the time points 75 and 150 min in controls and study groups receiving IMs of 20 mL/kg (Fig. 7B). Application of more substantial and frequent IMs (25 cmH_2O and 40 mL/kg) resulted in stable G values. Similarly, to significantly decrease the steady rise of G in groups receiving intermittent IMs, large IMs were necessary. A general pattern of progressive increase in H over time can be seen in all groups apart from those given large IMs (40 mL/kg or 25 cmH_2O) every 5 min (Fig. 7C). Where a single IM was given every 75 min, an abrupt decrease in H was seen; however H subsequently increased along the same trajectory.

Figure 7

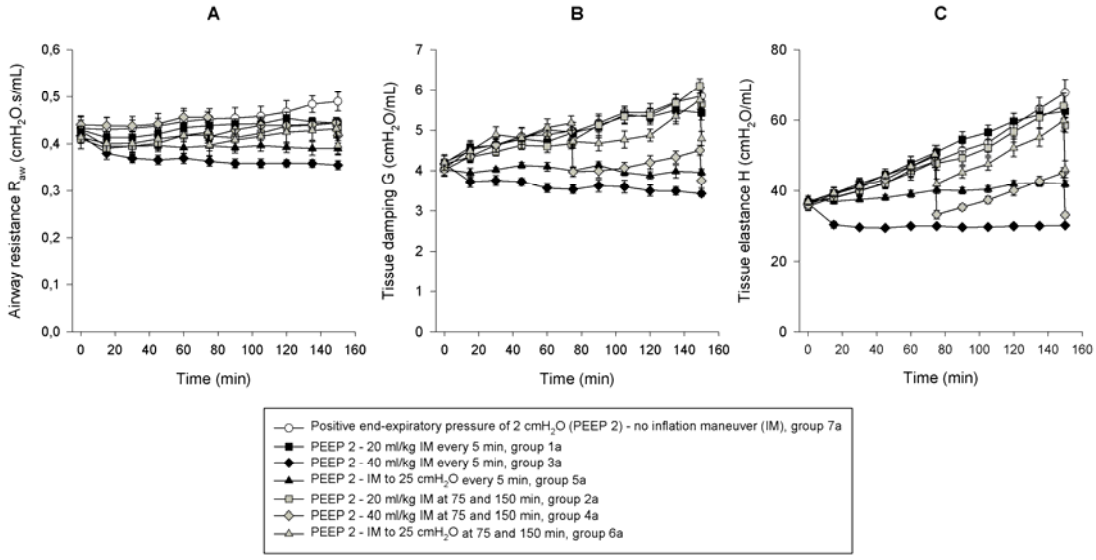

Fig. 7. Impact of inflation maneuvers on respiratory mechanics. Airway resistance (R_{aw}, Panel A) and coefficients of tissue damping (G) and tissue elastance (H) (Panels B and C, respectively) are displayed as a function of time on the ventilator. PEEP was left unchanged at 2 cmH_2O. Data are expressed as group means ± standard error of the mean.

3.3.4 R_{aw} after lung volume recruitment with PEEP level of 6 cmH_2O

In the study groups 1b, 3b, 5b, and 7b PEEP was increased to 6 cmH_2O after baseline measurement, whereas in study groups 2b, 4b, and 6b PEEP was only elevated to 6 cmH_2O during application of the first IM at 75 min. When PEEP was increased from 2 to 6 cmH_2O R_{aw} decreased in all groups, regardless of the timing of the increase in PEEP (Fig. 8A). From the time point 15 min on, R_{aw} statistically significantly rose in the control group with 6 cmH_2O PEEP. Frequent IMs with 20 mL/kg and 40 mL/kg produced a statistically significant but physiologically unimportant rise and fall, respectively, in R_{aw}. After 150 min R_{aw} values of controls were significantly higher when compared to all other groups, except for infrequent IMs with 20 mL/kg ($p=0.08$).

Figure 8

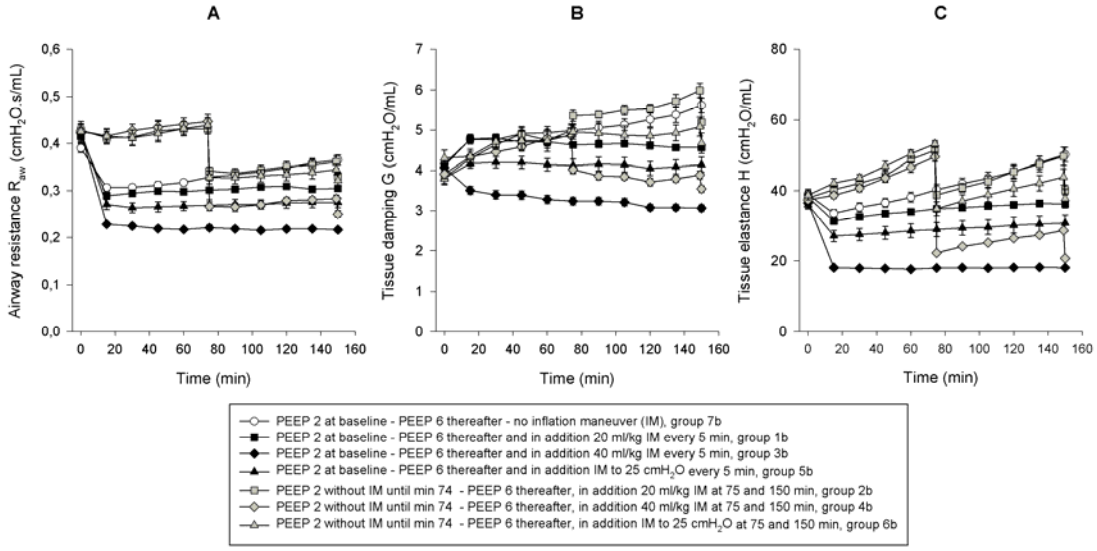

Fig. 8. Impact of inflation maneuvers and PEEP elevation on respiratory mechanics. Airway resistance (R_{aw}, Panel A) and coefficients of tissue damping (G) and tissue elastance (H) (Panels B and C, respectively) are illustrated as a function of time on the ventilator. Data are expressed as group means ± standard error of the mean.

3.3.5 G and H after lung volume recruitment at PEEP level of 6 cmH_2O

In the control group G steadily and significantly increased until the end of the protocol (Fig. 8B). After the time point 15 min, irrespective of the magnitude, frequent application of IMs produced stable G values. When a sporadic IM was given every 75 min, large IMs (40 mL/kg) were required to significantly decrease G values. IMs given every 5 min on top of a PEEP of 6 produced a stable H over the ventilation period (Fig. 8C), regardless of the type of magnitude of the IM. Less frequent IMs were associated with a progressive increase in H up until the time the IM was applied; with a subsequent increase in H following a similar trajectory (Fig. 8C).

3.3.6 Pressure-volume relation during the 3-s ramp inflation

In Figures 9A to D typical examples of the pressure-volume relation during the first 3-s of the ramp inflation maneuver are given for one animal per study group (1a-6a and 1b-6b). Delayed application of the first RM at the time point 75 min (Figs 9B and 9D) resulted in lower values of inflation volume at a given P_{ao} when compared with early application of RMs. Irrespective of the time point of first RM application and the PEEP level, inflation above ~25 cmH_2O was followed by another steep rise of the inflation limb without signs of flattening up to a peak P_{ao} of 35 cmH_2O.

Figure 9

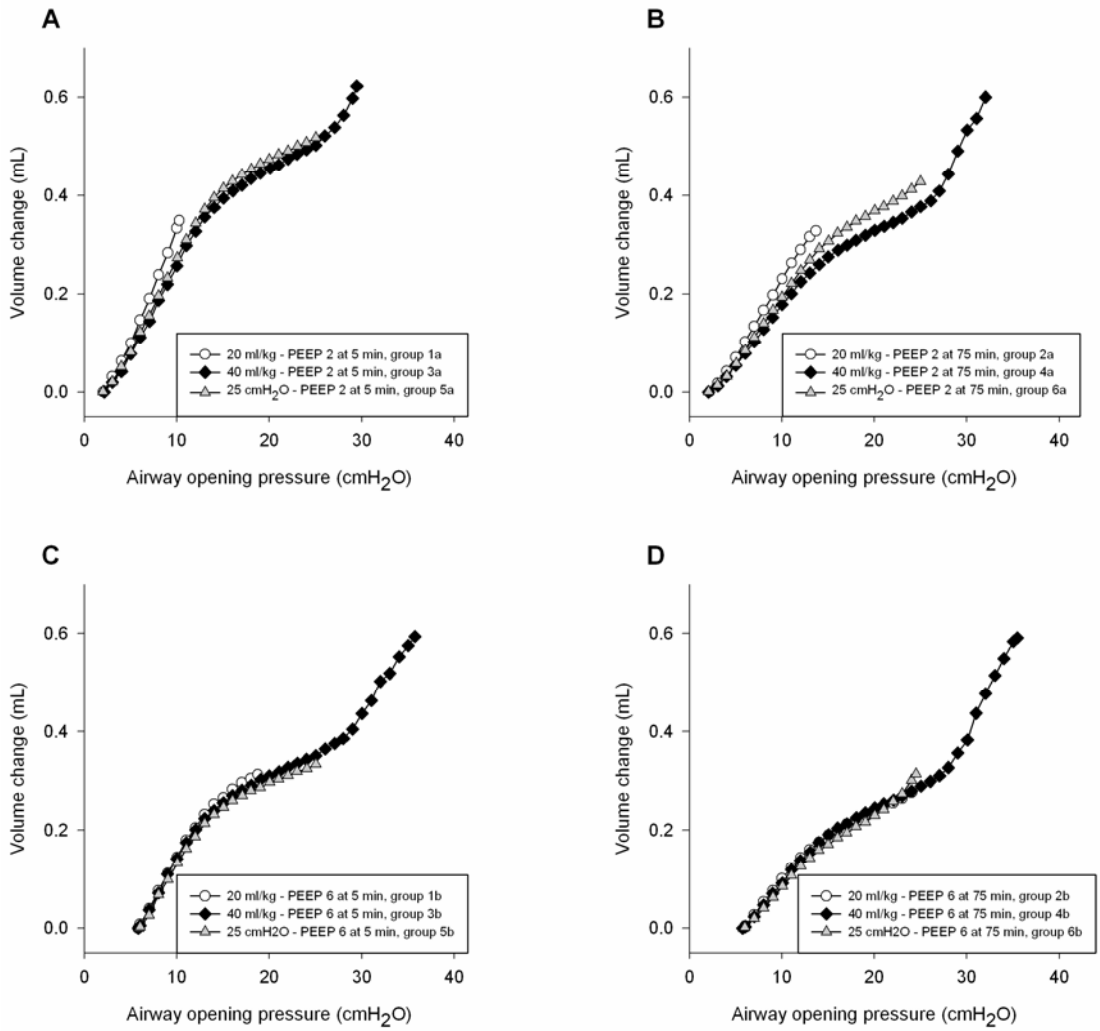

Fig. 9. Inflation volume versus airway opening pressure recorded during the first part of the inflation maneuver (3-s ramp). Panels A and B show inflation limbs during the first recruitment maneuver (RM) applied on top of PEEP 2 cmH_2O at the time points 5 or 75 min, respectively. Panels C and D illustrate inflation limbs during the first RM at the time points 5 or 75 min, respectively, obtained shortly after PEEP elevation from 2 to 6 cmH_2O. One typical example for each group is presented.

3.3.7 Heart rate and transcutaneous oxygen saturation

No differences were found between study groups at baseline and at the time points 75 and 150 min ($p>0.13$ in all cases) (Table 2). Over time heart rate significantly decreased when compared to baseline values.

Table 2

Heart rate and transcutaneous oxygen saturation (S_{tcO2}) at selected time points. Values: mean (SD)

Study group	Heart rate baseline	Heart rate 75 min	Heart rate 150 min	S_{tcO2} baseline	S_{tcO2} 75 min	S_{tcO2} 150 min
1a	241 (38.2)	227 (31.3)	232 (38.6)	96.2 (1.4)	95.2 (2.1)	95.6 (1.1)
2a	238 (47.3)	206 (21.1)	190 (17.5)	96.8 (1.9)	96.9 (0.6)	96.2 (0.7)
3a	278 (13.7)	220 (20.2)	214 (17.8)	94.9 (2.4)	96.9 (0.9)	96.9 (0.8)
4a	236 (45.5)	220 (21.9)	234 (25.2)	95.7 (2.6)	94.8 (2.9)	95.1 (0.8)
5a	261 (39.8)	226 (22.1)	235 (13.4)	94.5 (1.2)	95.2 (1.7)	95.2 (0.8)
6a	221 (44.1)	204 (35.0)	207 (37.0)	97.2 (1.6)	96.4 (1.3)	96.1 (0.9)
7a	251 (37.7)	201 (14.6)	214 (15.5)	97.6 (2.3)	97.2 (1.5)	96.6 (1.2)
1b	249 (20.4)	210 (14.8)	224 (22.8)	95.2 (3.4)	94.5 (1.4)	95.0 (1.2)
2b	247 (36.9)	231 (26.9)	233 (14.5)	95.3 (2.1)	95.9 (0.7)	96.1 (0.8)
3b	263 (20.5)	225 (34.4)	231 (30.8)	96.1 (3.3)	96.9 (1.5)	96.5 (0.7)
4b	253 (34.4)	204 (31.7)	214 (42.6)	97.5 (1.5)	94.8 (1.9)	95.3 (0.9)
5b	218 (16.9)	222 (34.2)	224 (45.0)	96.4 (2.3)	96.4 (0.8)	96.0 (1.2)
6b	253 (47.0)	217 (38.9)	210 (23.2)	96.0 (1.9)	94.8 (2.2)	94.9 (1.6)
7b	233 (19.6)	207 (12.9)	220 (21.2)	97.2 (1.5)	96.5 (2.2)	96.2 (0.8)

3.3.8 Cell counts, total protein and cytokines concentrations in BALF

BALF analysis for total (p=0.14) and differential cell counts (macrophages p=0.10, neutrophils p=0.73) produced no difference between groups. Results from measurement of TNF-α, MIP-2, IL-6, and total protein are displayed in Table 3.

Table 3

Tumor necrosis factor alpha (pg/mL), macrophage inflammatory protein-2 (pg/mL), interleukin-6 (pg/mL), and total protein (mg/mL) in bronchoalveolar lavage fluid.

Study groups	TNF-α	MIP-2	IL-6	Total protein
1a	120 (103-133)	130 (104-153)	263 (215-284)	0.18 (0.17-0.22)
2a	155 (125-174)	149 (135-173)	283 (234-371)	0.19 (0.18-0.20)
3a	189 (161-195)	194 (142-221)	347 (288-459)	0.19 (0.18-0.21)
4a	158 (129-181)	188 (150-200)	425 (262-452)	0.22 (0.20-0.27)
5a	129 (120-142)	165 (154-177)	253 (226-327)	0.17 (0.17-0.22)
6a	152 (140-155)	169 (148-173)	277 (229-354)	0.20 (0.20-0.21)
7a	156 (151-198)	162 (152-211)	229 (217-253)	0.18 (0.18-0.19)
1b	136 (131-156)	150 (147-159)	222 (206-304)	0.17 (0.15-0.20)
2b	129 (118-135)	140 (115-157)	218 (192-232)	0.20 (0.18-0.24)
3b	158 (142-162)	192 (150-224)	264 (248-388)	0.22 (0.20-0.43)
4b	151 (127-169)	160 (136-188)	207 (187-340)	0.21 (0.20-0.24)
5b	159 (130-184)	167 (141-184)	272 (204-316)	0.18 (0.17-0.19)
6b	136 (119-152)	169 (167-174)	309 (255-373)	0.18 (0.18-0.24)
7b	151 (120-176)	124 (110-135)	333 (283-365)	0.21 (0.21-0.25)

Data are displayed as median (interquartile range). Differences between groups were statistically not significant (p = 0.16, 0.10, 0.48, and 0.11 for TNF-α, MIP-2, IL-6, and total protein, respectively).

3.3.9 Analysis of lung tissues by morphology and morphometry

Numbers of alveolar macrophages and erythrocytes, and alveolar and septal neutrophils, as well as lung inflation scores, determined by morphometry (Fig. 10), are presented in Table 4.

Figure 10

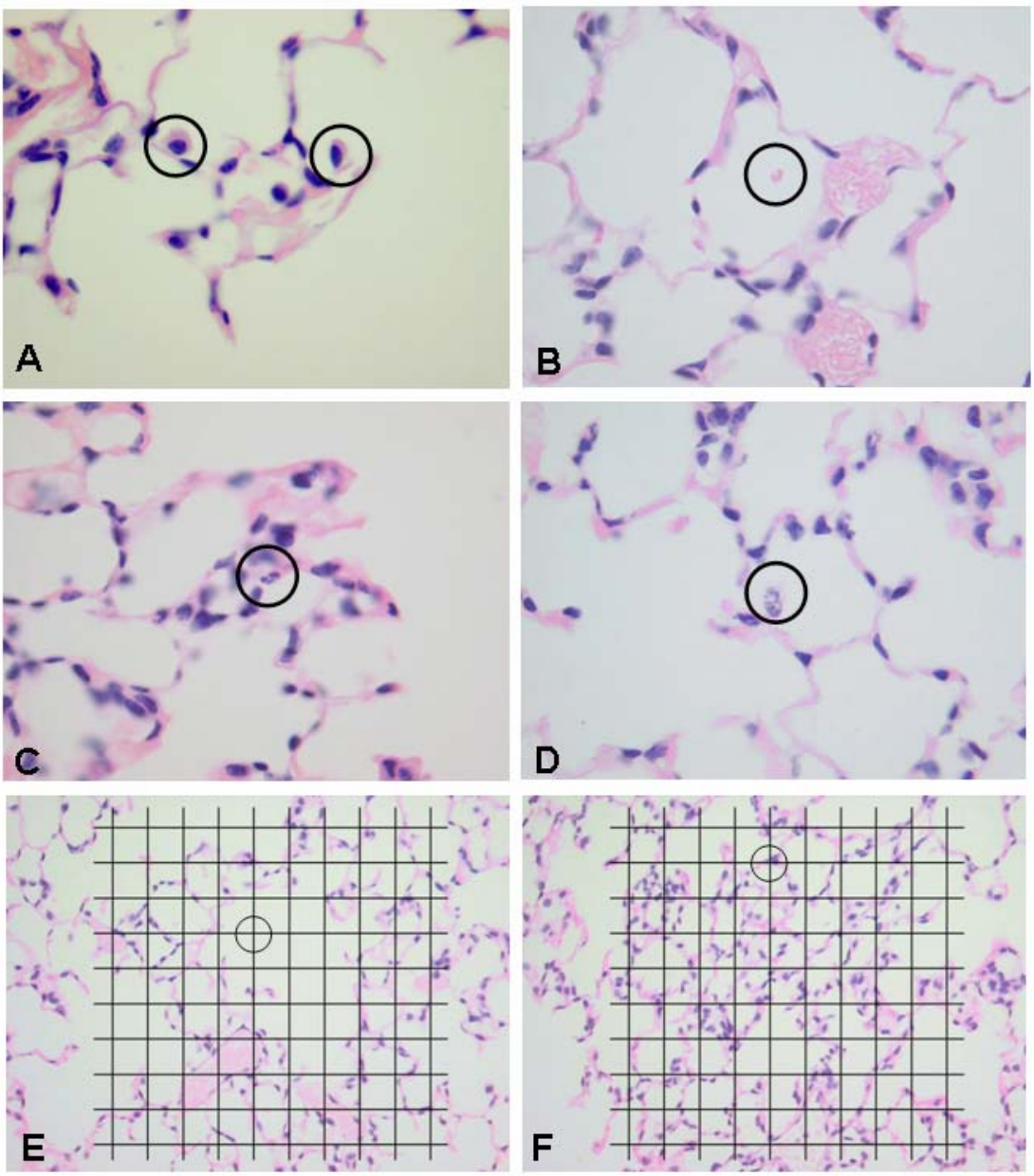

Fig. 10. Photomicrographs of representative lung features. A: Example of alveolar macrophages, encircled (x100 oil). B: Alveolar red cell, encircled (x100 oil). C: Neutrophil, encircled, within alveolar septum (x100 oil). D: Alveolar neutrophil, encircled (x100 oil). E: Grid superimposed for counting alveolar septal intersects, intersection without alveolar septum encircled (x40). F: Intersection with alveolar septum encircled (x40).

Table 4

Lung injury and inflation scores in selected histological lung sections.

Study groups	Alveolar macrophages	Alveolar neutrophils	Septal neutrophils	Alveolar erythrocytes	Inflation score
PEEP 2, no IM	15 (3)	1.4 (1.7)	26 (9)	26 (12)	303 (22)
PEEP 2, 40 mL/kg every 5 min	14 (6)	0.6 (0.9)	37 (12)	28 (15)	310 (17)
PEEP 2, 40 mL/kg every 75 min	13 (4)	0.8 (0.8)	35 (7)	27 (21)	326 (25)
PEEP 6, no IM	14 (6)	1.0 (0.7)	37 (6)	26 (14)	324 (13)
PEEP 6, 40 mL/kg every 5 min	14 (5)	1.2 (0.8)	33 (12)	24 (23)	294 (36)
PEEP 6, 40 mL/kg every 75 min	17 (5)	0.5 (0.6)	31 (7)	22 (10)	320 (40)

Data are displayed as mean (standard deviation). Differences between groups were statistically not significant ($p = 0.92$, 0.72, 0.69, 0.99, and 0.40 for alveolar macrophages, alveolar neutrophils, septal neutrophils, alveolar erythrocytes, and inflation score, respectively). Definition of abbreviations: PEEP: positive end-expiratory pressure; IM: inflation maneuver.

4. DISCUSSION

4.1 Major findings emerging from this thesis

The most important findings of the presented studies include the following. First, alterations of respiratory system mechanics during artificial ventilation without PEEP can be attributed to loss of lung volume. Second, application of PEEP during short-term high-V_T ventilation prevents atelectasis but induces lung injury in infant mice. Third, short-term exposure to levels of oxygen up to 100% does not increase changes in respiratory system mechanics induced by mechanical ventilation. Fourth, frequent application of substantial inflation maneuvers (IM) on top of elevated PEEP levels results in stable respiratory system mechanics without causing lung injury after short-term ventilation with low V_T. Lastly, both an increase in PEEP (without use of IMs) and application of IMs resulting in peak P_{ao} below 25 cmH_2O are insufficient to prevent or reverse increases in R_{aw}, G, and H during low-V_T ventilation.

4.2 Forced oscillation technique and estimates of respiratory system mechanics

Experimental research on ventilator-induced lung injury (VILI) primarily relies on the use of small animal models. Assessment of respiratory system mechanics is essential because of the potential to gain insight into development and mechanisms involved in VILI. The dynamic measurement of respiratory system mechanics via application of forced oscillations provides information about resistive and elastic properties of the respiratory system. Impedance data generated by the low-frequency forced oscillation technique (FOT) has best been described by the constant-phase model (Hantos et al., 1992). Parameters estimated from model fitting include Newtonian resistance (R_N), tissue damping (G), and tissue elastance (H). In species with relatively low chest wall impedance, such as mice, R_N is essentially equal to airway resistance (R_{aw}) and basically reflects a change in central airway calibre, whereas G and H can be considered as parenchymal parameters and characterize dissipative and elastic properties, respectively, of the respiratory system (Sly et al., 2003). A proportionate increase of G and H results from lung volume derecruitment (Allen and Bates, 2004), while a disproportionate, i.e. higher rise in G compared to H reflects an increase in regional heterogeneity (Lutchen et al., 1996; Ito et al., 2007). Hence, the parameters obtained from low-frequency Z_{rs} data allow one to differentiate changes in airway

diameter from changes related to loss of lung volume or regional ventilation heterogeneities.

4.3 Assessment of inflammatory response

We assessed the inflammatory response to the various ventilation patterns using counts of inflammatory cells and measurements of TNF-α, MIP-2, IL-1β, and IL-6 in BALF and serum. The relevance of cytokine production in the context of VILI has been challenged by Dreyfuss et al. (2003). This is not surprising since several factors such as species, pre-treatment, and ventilation strategy influence duration of experiments and outcomes in animal models. Furthermore, it is evident that short-lived and clinically relevant, that is less extreme, animal study protocols using primary healthy lungs will not cause massive cytokine and protein production. Nonetheless, more recent studies continue to measure cytokines in an attempt to explain VILI (Halbertsma et al., 2005; Frank et al., 2006). In the present study, we measured TNF-α, MIP-2, IL-1β, and IL-6 in BALF, since these chemoattractants have been shown to be important mediators in the development of VILI (Allen et al., 2006; Frank et al., 2006; Li et al., 2007).

4.4 High tidal volume ventilation in infant mice (Study 1)

In accordance with adult rodent data we hypothesized that a HVZ strategy would cause the most significant changes in lung mechanics and lung injury (Wilson et al., 2003; Choudhury et al., 2004; Walder et al., 2005; Allen et al., 2006). Our data only partially support this hypothesis in infant mice. HVZ caused the fastest and steepest increase in G and H. The proportionate increase in G and H in the HVZ group is indicative of a peripheral process (Irvin and Bates, 2003), namely atelectasis. The linear relationship between the measure of tissue compliance (1/H) and EELV is consistent with the predominant mechanism for the increase in G and H as a result of loss of lung volume. It should be noted that this relationship does not point to the origin, i.e. the fall in compliance appears to be somewhat faster than the loss in lung volume. However, this tendency should be interpreted carefully because of the assumption of the linear relationship and the relatively long distance to the origin not covered by measurement data.

Application of a moderate PEEP level in the HVP and LVP groups prevented loss of lung volume. Periodic delivery of mild RMs with V_T of 20 mL/kg in the LVP group resulted in a pronounced increase of G and H. However, these changes were not associated with an increased inflammatory response or lung injury.

Allen et al. (2006) used comparable ventilation strategies and also accurate tools for measuring lung mechanics in adult mice but reported different results. High-V_T ventilation with or without PEEP produced no change in R_{aw} of adult mice, whereas in the present study infant mice showed an increase in R_{aw} with HVZ and a decrease with HVP. Similarly, after 60 min of ventilation G and H were unaltered in adult mice ventilated with high V_T (Allen et al., 2006), but were elevated in infant mice ventilated with HVZ and HVP in the present study. Apart from developmental and structural differences between lungs of infant and adult mice we cannot exclude that differences in mouse strain, lung volume history standardization after the start of mechanical ventilation, respiratory rate, and slightly higher V_T (25 mL/kg versus 20 mL/kg in our study) may have caused more alveolar recruitment in the adult mice and hence different patterns of R_{aw}, G, and H.

In contrast to findings in adult rodents, newborn rats ventilated with extreme V_T (40 mL/kg) and zero PEEP showed an increase in total respiratory compliance (Martinez et al., 2004). This finding persisted until 120 min of mechanical ventilation and was associated with increase in alveolar surfactant pools including a rise in the functionally active form of large aggregate surfactant subfraction. Copland et al. (2004) investigated the effects of high-V_T ventilation with zero PEEP in newborn and adult rats and found that adults were more susceptible to high-V_T-induced lung injury. Interestingly, only newborn rats ventilated with a V_T of 40 mL/kg, but not with 25 mL/kg, showed a decrease in respiratory system compliance by 180 min of artificial ventilation. Hence, newborn rat lungs seem to be resistant to a supposedly injurious ventilation mode (25 mL/kg and zero PEEP). This finding may be due to a different response of surfactant production and composition after mechanical stretch and a relatively immature immune system when compared to older rodents.

Kornecki et al. (2005) applied a pressure-limited ventilation mode with a PEEP level of 1 cmH_2O in 17 days old infant rats with almost full alveolarization. Ventilation with peak pressure levels of 20 cmH_2O and 30 cmH_2O resulted in V_T of ~37 mL/kg and ~48 mL/kg, respectively. Total lung compliance assessed by constructed static pressure-volume curves was not even altered after 90 min of

ventilation with peak pressures of 30 cmH_2O. Clearly, this is in contrast to our findings in 14 days old infant mice ventilated with both the HVZ and HVP strategy. A comparison of the effects of high-V_T ventilation between infant mice and rats is difficult and may be biased by different ventilation modes and lung function techniques used. However, infant mice and rats produce a different pattern of respiratory system mechanics and inflammation and lung injury when compared with adult rodents after artificial ventilation.

4.4.1 Inflammatory response and lung injury

Unlike Allen et al. (2006) we did not find elevated concentrations of IL-6 in BALF, but increased values of IL-6 in serum of the HVZ group. A similar result was also shown in adult rats after a high pressure zero PEEP strategy (Haitsma et al., 2003). Overexpression of IL-6 in response to lung stretch during high-V_T ventilation has been shown in adult animal studies, but its role as a purely proinflammatory cytokine has been questioned (Copland et al., 2005), since several animal studies have also demonstrated lung protective characteristics of IL-6. In our study, we assume that the reason for elevated serum IL-6 levels was largely related to substantial atelectasis leading to ventilation-perfusion-mismatch, deterioration in cardiac output and eventually a systemic inflammatory response.

Measurement of total protein in BALF is not a specific marker of lung injury; however, it has been used to track progression of lung injury and as an indicator of loss of endothelial and epithelial barrier function (Parker and Townsley, 2004). In our study, only HVP strategy caused increased total protein concentrations in BALF. High V_T was delivered on top of PEEP and may have caused overstretching of lung units resulting in lung injury. This is in line with results from adult animal studies where alveolar overdistension has been shown to be an important mechanism in the pathogenesis of VILI (Vlahakis and Hubmayr, 2005). However, one may wonder why our HVZ strategy did not lead to elevated protein levels, such as with comparable HVZ strategies in adult animal studies (Allen et al., 2005; Ricard et al., 2001; Belperio et al., 2002), even though similar peak P_{ao} levels were achieved as within the HVP group. This is surprising because derecruited lungs exposed to high V_T and zero PEEP have been shown to be particularly prone to repeated opening and closing of alveoli causing shear stress between adjacent aerated and non-aerated alveoli, and eventually leading to lung injury (Lapinsky and Mehta, 2005). While in our HVZ group shear

stress may have occurred because of proven loss of lung volume with resulting atelectasis, we have no direct evidence for repeated opening and closing of lung units and doubt that the peak P_{ao} values achieved in the HVZ group were sufficient enough to reopen collapsed peripheral lung units.

However, the degree of lung distension and with it the risk of VILI does not depend on peak P_{ao}, but rather on transpulmonary pressure (P_{tp}), tissue elastance, and lung volume history (Gattinoni et al., 2004). In theory, application of PEEP during HVP ventilation may have lessened P_{tp}. However, due to the difficulties in measuring pleural or oesophageal pressure in infant mice, it remains unclear how changes in respiratory system mechanics, EELV, and PEEP affect P_{tp}. More importantly, application of high V_T on top of PEEP resulted in both higher EELV and better lung compliance when compared with the HVZ group. Due to the relatively small contribution of the chest wall on respiratory system mechanics reported in mice (Frappell and Mortola, 1989; Sly et al., 2003; Ito et al., 2007), it is unlikely that a higher state of inflation (as in HVP) may have attenuated alveolar distension in infant mice, where chest wall tissue is even thinner. Furthermore, the use of PEEP also resulted in consistently higher mean P_{ao} values throughout the study, probably reflecting the higher state of inflation. Consequently, higher EELV, lower R_{aw}, and better lung compliance most likely resulted in alveolar overdistension during HVP ventilation when compared with HVZ ventilation. Conversely, despite similar peak P_{ao} values at the end of the study, lower EELV, high lung elastance, and moderately increased R_{aw} is likely to have resulted in less alveolar distension in the HVZ group.

4.5 Impact of supplemental oxygen in mechanically ventilated adult and infant mice (Study 2)

Airway smooth muscle tone and airway diameter are influenced by partial oxygen pressure (Skogvall et al., 1999). Local hypoxia leads to bronchodilatation and local hyperoxia can induce bronchoconstriction. Thus, pulmonary ventilation can rapidly be modulated to optimise oxygen supply to tissues (Peers and Kemp, 2001). In our study, we found no physiologically significant oxygen-related changes of R_{aw} in either age group. Also, the increase in G was associated with a similar elevation in H and reflects development of atelectasis rather than an increase in ventilation heterogeneity as a result of inhomogeneous regional airway narrowing. Given the absence of hyperoxia-related alterations of R_{aw}, G, and H, it is possible that the role and function of oxygen-

sensitive chemoreceptors are less important *in vivo* when compared to *in vitro* and *ex vivo* experiments.

4.5.1 Inflammatory response and lung injury

We did not find elevations in either cytokine in response to increased $F_{I_{O_2}}$. These data are consistent with those of Quinn et al. (2002) who found no oxygen-dependent alterations in BALF MIP-2 and neutrophils after 2 h of mechanical ventilation with V_T of 7 mL/kg in a rat model of VILI. Also, after 6 h of ventilation, there were no oxygen-related increases in inflammatory cytokines for the groups ventilated with 7 mL/kg but a significant neutrophil increase only with hyperoxia and high V_T. Similarly, Copland et al. (2004) applied an injurious ventilation strategy in newborn rats and found no oxygen-related MIP-2 increase after 180 min. As to IL-6 production during mechanical ventilation with low V_T, we found no association with supplemental oxygen. Other research groups (Bailey et al., 2003; Copland et al., 2004) only reported increased IL-6 concentrations after high-V_T ventilation with no difference between room air and hyperoxia groups. In addition, the lack of increase in total protein in the BALF we observed in the present study suggests that short-term exposure to high $F_{I_{O_2}}$ *per se* does not compromise epithelial or endothelial barrier function (Parker and Townsley, 2004). Taken together, our findings underscore the lack of significant oxygen-associated pulmonary inflammation and lung injury after non-injurious short-term mechanical ventilation.

4.6 Lung volume recruitment maneuvers and respiratory system mechanics in mechanically ventilated mice (Study 3)

In murine models, absence of IMs or application of mild IMs that do not result in P_{ao} >25 cmH_2O throughout mechanical ventilation, leads to gradual development of atelectasis, particularly during low-V_T ventilation with low PEEP (Allen et al., 2006; Thammanomai et al., 2007). Progressive atelectasis may lead to ventilation-perfusion mismatch, ventilation inhomogeneity, shear stress, and eventually lung injury (Duggan et al., 2003; Lapinsky and Mehta, 2005). When using a volume-controlled ventilation mode, reduction in lung volume not only results in a fall in compliance and higher peak P_{ao} levels, but also causes lung injury by overdistension of the remaining open lung units (Tsuchida et al., 2006). Benefits of RMs include prevention and re-opening

of atelectasis, improvement of compliance and gas exchange, and prevention of derecruitment-associated lung injury (Koh et al., 2005). However, RMs have also been reported to be transient, ineffective, and injurious (Musch et al., 2004); the harmful effects include overdistension of open lung units, low ventilation-to-perfusion ratio, and reduced venous return via increased intrathoracic pressure.

Mechanical ventilation with low V_T and low PEEP in the present study resulted in a physiologically unimportant increase of R_{aw} (~10%) and in a significant increase in G and H (~45% and ~90%, respectively) over time. The almost linear rise of G and H, reflecting gradual airway closure (Irvin and Bates, 2003), is consistent with a progressive loss of lung volume secondary to atelectasis. In order to prevent progressive atelectasis we applied IMs in a volume-controlled manner without pressure limit or in a pressure-controlled mode. Volume-controlled IMs delivered a fixed V_T and resulted in peak P_{ao} determined by lung compliance. In contrast, pressure-controlled IMs ensured that a selected peak P_{ao} was not exceeded, but provided different V_T depending on respiratory mechanics.

PEEP alone was not able to prevent the progressive rise in R_{aw}, G, and H in the present study, although the rate of apparent loss of lung volume appeared to be slower when a PEEP of 6 cmH_2O was employed. This finding is in line with results from recent studies (Farias et al., 2005; Ko et al., 2008). Although the PEEP level of 6 cmH_2O was chosen arbitrarily, we anticipated that this level was likely to be adequate to prevent derecruitment in mice with healthy lungs ventilated for a short period. However, it is conceivable that higher PEEP values may have prevented loss of lung volume. Comparison of pressure-controlled RMs showed a greater decrease in H after IMs with PEEP 6 when compared with PEEP 2. However, when comparing the course of R_{aw} and H after the time point 15 min, we found similar rates of relative changes at both PEEP levels. These results suggest that during pressure-controlled RMs a PEEP of 6 cmH_2O is more effective than a PEEP of 2 cmH_2O at reversing estimates of respiratory mechanics initially, but equally inadequate to impede alterations thereafter.

The stability of H after application of frequent and large RMs indicates that full recruitment of lung units was probably achieved after the first RM. Infrequent application of large RMs had transient effects on H; however the pattern of progressive increase in H after the IM followed a similar trajectory to that seen before the IM. This finding is in line with those from other studies in animals with healthy and pre-injured lungs (Mead and Collier, 1959; Allen et al., 2002; Allen et al., 2004;

Frank et al., 2005; Allen et al., 2006; Ko et al., 2008) and may reflect inadequate PEEP during low-V_T ventilation.

In the present study only large IMs resulting in peak P_{ao} above 30 cmH_2O produced overall improvements in respiratory mechanics, whereas IMs reaching peak P_{ao} values at or below 25 cmH_2O did not reverse the ventilation-induced increases in R_{aw}, G, and H. By contrast, repetitive IMs with 40 mL/kg (especially superimposed on a higher PEEP), producing peak P_{ao} values above 35 cmH_2O, provided the most significant improvement in lung function and stable respiratory system mechanics with little intra-group variability. This finding is closely linked to the development of a "secondary" pressure-volume sigmoid with lung inflation beyond 20 cmH_2O (Zosky et al., 2008), which makes total lung capacity difficult to define in mice (Soutiere and Mitzner, 2004). This bi-modal pressure-volume behaviour was demonstrated in some small species long time ago (Leith, 1976); however, its importance was not recognised until recently (Soutiere and Mitzner, 2004; Zosky et al., 2008). The findings in the present study therefore support the view that the improvement of estimates of respiratory system mechanics after large RMs is due to fundamental changes in quasi-static and dynamic lung compliance, as substantiated by the inflation PV curve and the values of R_{aw}, G, and H, respectively. Though the mechanisms responsible for the increased lung compliance above P_{ao} of 25 cmH_2O are not clear, alveolar unfolding and surfactant redistribution have been proposed as possible explanations (Soutiere and Mitzner, 2004; Escolar and Escolar, 2004), while others suggest that alveolar mouths, previously closed by a surfactant-lined liquid film, open during recruitment of peripheral lung units at high transpulmonary pressures providing a new population of available alveoli (Scarpelli, 1998; Kitaoka et al., 2007; Namati et al., 2008). A structural reorganization of peripheral lung units resulting in an increased number of alveoli and homogeneous ventilation may explain why our initial hypothesis, i.e. frequent and large RMs would induce lung injury via overdistension, could not be confirmed in this study.

4.6.1 Inflammatory response and lung injury

In line with results from others (Frank et al., 2005; Allen et al., 2006) application of RMs producing transient elevations in peak P_{ao} did not increase cytokine concentrations in BALF. Similarly, total protein concentration did not differ between study groups, indicating that RM-induced stretch of airways and alveoli did not

adversely affect epithelial-endothelial barrier function (Parker and Townsley, 2004). Also, absence or application of RMs had no impact on transcutaneous oxygen saturation; however, it should be noted that healthy lungs were ventilated over a short period of time and $F_{I_{O_2}}$ of 0.5 was delivered in order to avoid survival problems.

4.7 Limitations of the studies presented in this thesis

The following study limitations should be acknowledged. Firstly, the duration of our experiments was short (ranging from 60 to 150 min). Though it is possible to extend the time on the ventilator keeping mice alive, we experienced survival issues during preliminary studies with longer study protocols. Secondly, lung inflammatory response is a time dependent process. Therefore, it is uncertain whether longer mechanical ventilation may have produced a different pattern of inflammatory response. Thirdly, given the animal size and technical difficulties we were unable to analyse arterial blood gas samples to determine partial pressures of oxygen and carbon dioxide. Fourthly, our studies were not designed to assess biotrauma and only measured a limited number of potential mediators in VILI. Our selection of cytokines was based on results from other research groups with comparable duration of mechanical ventilation protocols and limited by available BALF after lavage. Fifthly, given that there is no consensus on how to recruit non-aerated lungs we arbitrarily chose different types of RMs. It is likely that the time interval between RMs, duration of RMs, ramp to plateau ratio, and degree of PEEP and IMs result in different findings. Lastly, though it is true that development of derecruitment and response to PEEP and IMs depend on the type and the degree of a pre-existing lung injury (Allen et al., 2004), our study aim was to investigate the role of V_T, oxygen, and RMs in healthy lungs since lung function measurements are often performed in healthy mice.

5. CONCLUSIONS

The presented *in vivo* infant and adult mouse studies involving measurements of airway and tissue mechanics, absolute lung volumes, construction of PV curves, and assessment of inflammatory response show that infant mice lungs behave differently from adult and neonatal rodent lungs. This finding underlines the need for age-specific animal models and asks for caution when data and conclusions from adult animal research are extrapolated for infant animals and eventually human infants.

Our first study provides evidence that mechanical ventilation with high V_T with or without PEEP is deleterious in infant mice. We propose that overdistension of peripheral lung units and atelectasis leading to ventilation-perfusion mismatch, respectively, are the main mechanisms involved in the development of VILI.

Also, our results show that short-time exposure to high $F_{I_{O_2}}$ in the presence of non-injurious mechanical ventilation does not exacerbate lung injury in either infant or adult mice. This finding may not have a direct translational value for clinical practice, but has valuable implications for experimental VILI studies, which are often performed in mice and use short-term mechanical ventilation protocols. In addition, due to technical advances in measuring respiratory system mechanics in small rodents, infant mouse models have the potential to provide insights into paediatric ventilator-associated lung injury, thus limiting extrapolation from adult data.

Finally, we demonstrated that infrequent application of large RMs is sufficient to reverse increases in bronchial resistance and lung elastance in healthy mice. To maximize lung volume recruitment throughout study protocols using ventilation strategies of low V_T and "adequate" PEEP, repetitive IMs reaching peak P_{ao} values >25 cmH_2O are required to provide stable respiratory mechanics. This is particularly useful in animal model studies where similar baseline conditions after standardized procedures are desirable. Furthermore, frequent application of substantial RMs resulting in peak P_{ao} of 35 cmH_2O and above provides stable respiratory mechanics without histological signs of lung injury and without elevation of TNF-α, IL-6, MIP-2, and total protein concentrations in BALF during short-term mechanical ventilation in mice. Given the impact of RMs on respiratory system mechanics documented by the present study requires that the specifications of PEEP and IMs used are reported in all studies investigating the effects of mechanical ventilation in mice.

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8. ANNEX

Full papers published in international journals referenced by Science Citation Index.

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High tidal volume ventilation in infant mice

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ABSTRACT

Infant mice were ventilated with either high tidal volume (V_T) with zero end-expiratory pressure (HVZ), high V_T with positive end-expiratory pressure (PEEP) (HVP), or low V_T with PEEP. Thoracic gas volume (TGV) was determined plethysmographically and low-frequency forced oscillations were used to measure the input impedance of the respiratory system. Inflammatory cells, total protein, and cytokines in bronchoalveolar lavage fluid (BALF) and interleukin-6 (IL-6) in serum were measured as markers of pulmonary and systemic inflammatory response, respectively. Coefficients of tissue damping and tissue elastance increased in all ventilated mice, with the largest rise seen in the HVZ group where TGV rapidly decreased. BALF protein levels increased in the HVP group, whereas serum IL-6 rose in the HVZ group. PEEP keeps the lungs open, but provides high volumes to the entire lungs and induces lung injury. Compared to studies in adult and non-neonatal rodents, infant mice demonstrate a different response to similar ventilation strategies underscoring the need for age-specific animal models.

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1. Introduction

Clinical and experimental studies have demonstrated that artificial ventilation can promote lung injury (Slutsky, 1999; Gajic et al., 2004; Hubmayr, 2005). As a result, research on the field of acute lung injury and ventilator-induced lung injury (VILI) has led to 'protective' ventilation strategies, which comprise the application of low tidal volumes (V_T), limitation of peak pressures, and adequate positive end-expiratory pressures (PEEP) (The ARDS Network, 2000; Jardin and Vieillard-Baron, 2006; Villar et al., 2006). This approach is supposed to minimize the adverse influences involved in VILI, including overdistension of alveolar units (Vlahakis and Hubmayr, 2005), repeated opening and closing of peripheral lung units resulting in shear stress at the interface between aerated and non-aerated airspaces (Lapinsky and Mehta, 2005), and damage due to release of inflammatory mediators (Dos Santos and Slutsky, 2006).

The potential harm of mechanical ventilation is magnified in infants because of a more compliant chest wall with greater risk of volutrauma, smaller lung volumes promoting atelectasis, and developing lung structures (Vitali and Arnold, 2005). Infants undergoing mechanical ventilation may develop long lasting clinical problems such as chronic lung disease, pulmonary hypertension, prolonged supplemental oxygen requirement, nutritional problems, developmental delay, and hospital readmissions with severe psychosocial and financial implications for the child, the parents, and the health care system (Schibler, 2006).

Given the lack of data (Turner and Arnold, 2007) and the difficulties in performing mechanical ventilation studies in human infants, animal models have emerged as useful tools for studying VILI. Rodent models are widely used because of their availability and well-characterized respiratory mechanics (Gomes et al., 2000). These models have provided important insights into the mechanisms of VILI, but have predominantly used adult animals with fewer studies in neonates (Wilson et al., 2003; Martinez et al., 2004; Copland et al., 2004; Allen et al., 2006). In adult mice, high V_T ventilation resulted in lung injury and decrease in lung compliance (Wilson et al., 2003; Allen et al., 2006). Interesting results were found after high V_T mechanical ventilation in newborn rats. In spite of similar ventilator settings one study showed an improvement in lung compliance (Martinez et al., 2004) whereas

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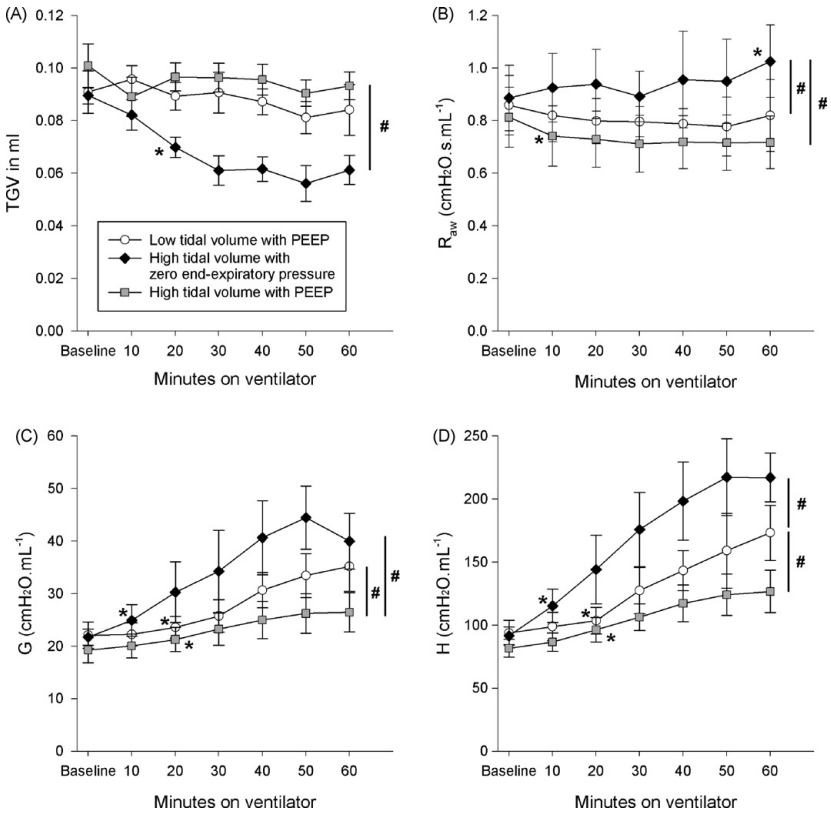

Fig. 1. Respiratory mechanics as a function of time on the ventilator in the three experimental groups. (A) Thoracic gas volume (TGV); (B): airway resistance (R_{aw}); (C) coefficient of tissue damping (G); (D) coefficient of tissue elastance (H). Data are expressed as group means ± standard error of the mean. Significant change when compared with baseline * and between groups #, $p < 0.05$, respectively.

another study demonstrated a small decrease in compliance only after 180 min of ventilation (Copland et al., 2004). The latter study also found that adult rats were more susceptible to high V_T-induced lung injury than newborn rats. When looking at the impact of high V_T in infant (non-neonatal) rats Kornecki et al. (2005) found no alteration of lung compliance after 90 min of artificial ventilation.

Recent advances in techniques for measuring thoracic gas volume (TGV) and respiratory system mechanics in small intact animals (Janosi et al., 2006) have the potential to provide further insights for the specific age group of infants. Establishment and study of an infant mouse model of VILI is particularly promising because of the potential to use genetically altered mice in future studies that may allow more specific mechanisms to be explored. In terms of lung development and maturation 2-week-old mice can be compared to 2-year-old human infants (Dietert et al., 2000). The purpose of this study was to investigate the effects of high V_T ventilation in a novel *in vivo* infant mouse model for VILI. In contrast to newborn and infant rat studies, but in line with adult mouse studies we hypothesized that high V_T ventilation without PEEP may cause the most significant changes in lung mechanical parameters and lung injury.

2. Materials and methods

2.1. Animals

Two-week-old specific pathogen-free female BALB/c mice were purchased from the Animal Resource Centre (Murdoch, Western Australia). The experimental procedures were approved by the Telethon Institute for Child Health Research Animal Experimentation and Ethics Committee and conform to the guidelines of the National Health and Medical Research Council of Australia.

2.2. Measurement of lung volume and respiratory mechanics

Thoracic gas volume was measured using the whole-body plethysmographic technique based on Boyle's law, as described in detail previously (Janosi et al., 2006). Briefly, the airway was occluded at 0 cm H_2O transrespiratory pressure (P_{rs}) and breathing efforts were induced using electrical stimulation of the intercostal muscles.

Respiratory system impedance (Z_{rs}) was measured using the miniature wave-tube version of the low-frequency forced oscillation technique (Hantos et al., 1995; Bozanich et al., 2007). A pseudorandom oscillatory signal ranging from 4 to 38 Hz was deliv-

ered by a loudspeaker-in-box system to the tracheal cannula via a wave-tube at P_{rs} = 0 cm H_2O, and Z_{rs} was measured as the load impedance on the wave-tube. Airway resistance (R_{aw}) and inertance (I_{aw}) and the coefficients of tissue damping (G) and tissue elastance (H) were estimated from Z_{rs} by model fitting (Hantos et al., 1992). Although theoretically, R_{aw} incorporates all frequency-independent (Newtonian) resistances, the contributions from the chest wall were found negligible in mice (Sly et al., 2003). Similarly, G and H can be considered as parenchymal parameters. After the subtraction of the impedance of the tracheal cannula the values of I_{aw} became insignificantly low and hence not reported.

2.3. Experimental protocol

Mice were anaesthetised with an intraperitoneal injection of a solution containing ketamine (20 mg/ml, Troy Laboratories, N.S.W., Australia) and xylazine (1 mg/ml, Troy Laboratories, N.S.W., Australia). Mice received 0.02 ml/g of this mixture with two thirds of the dose given to induce a surgical level of anaesthesia and the remaining third given once the mouse was on the ventilator. Further anaesthetic was given as required throughout the experiment. Once anaesthetised, a tracheotomy was performed and a 10 mm long 21-gauge metal cannula was inserted and secured with suture. The mouse was then placed in the supine position in a custom-built whole body plethysmograph (180 ml volume), connected to a computer-controlled ventilator (*flexiVent*, Scireq, Montreal, Canada) via a specially designed connector that passed through the plethysmographic wall and ventilated with room air at a frequency (f) of 360 min^{-1} with a delivered V_T of 8 ml/kg (when accounting for gas compression and/or friction in the cylinder) and PEEP of 3 cm H_2O. Lung volume history was standardised as follows: lung volume was increased by lowering plethysmographic box pressure via a regulated vacuum line from 0 to −20 cm H_2O at a constant rate, while the tracheal cannula was open to atmosphere through the box wall. This was followed by a slow passive expiration to 0 cm H_2O P_{rs}. By this means two slow (approximately 30 s) inflation–deflation maneuvers, separated by 5 min of ventilation were applied. Once lung volume history was standardised, baseline measurements of TGV and Z_{rs} were taken. Mice were then randomized to receive one of three ventilation strategies:

1) high V_T with zero end-expiratory pressure (HVZ): f = 150 min^{-1}, delivered V_T of 20 ml/kg, no PEEP, no sighs (n = 12);
2) high V_T with PEEP (HVP): f = 150 min^{-1}, delivered V_T of 20 ml/kg, PEEP of 3 cm H_2O, and no sighs (n = 12), and;
3) low V_T with PEEP (LVP): f = 360 min^{-1}, delivered V_T of 8 ml/kg, PEEP of 3 cm H_2O, and 2 sighs of 20 ml/kg which did not exceed 20 cm H_2O inspiratory peak pressure, applied shortly after and 5 min before each measurement of respiratory mechanics (n = 12).

Mice were ventilated for 60 min with TGV and Z_{rs} measured at baseline and every 10 min during the ventilation period. Ventilation frequencies in each strategy were matched for minute ventilation. Body temperature was monitored with a rectal thermocouple and maintained at 36.5–37.5 °C with the use of a heat lamp.

2.4. Sampling of bronchoalveolar lavage fluid and serum

At the end of the study period blood samples were obtained by cardiac puncture and bronchoalveolar lavage fluid (BALF) was collected by gently washing 0.2 ml of pathogen free 0.9% saline in and out of the lung three times. Blood and BALF samples were taken from and analyzed in all animals, including a non-ventilated control group (n = 8). Blood was allowed to clot and centrifuged at 2000 r min^{-1} for 10 min and serum frozen for later analysis of interleukin-6 (IL-6). BALF samples were centrifuged at 2000 r min^{-1} for 4 min. Supernatant was collected and frozen for later analysis of IL-6, interleukin-1 beta (IL-1β) and total protein. Early phase "proinflammatory" cytokines IL-6 and IL-1 beta were chosen because of their role as neutrophil chemoattractants and inflammatory markers in airways and lung tissue. The cell pellet was resuspended in phosphate buffered saline and an aliquot was stained with Trypan blue to obtain a total cell count using a haemocytometer. A second aliquot was centrifuged onto a slide and stained with Leishmann's to obtain a differential cell count using light microscopy by counting 300 cells from each slide.

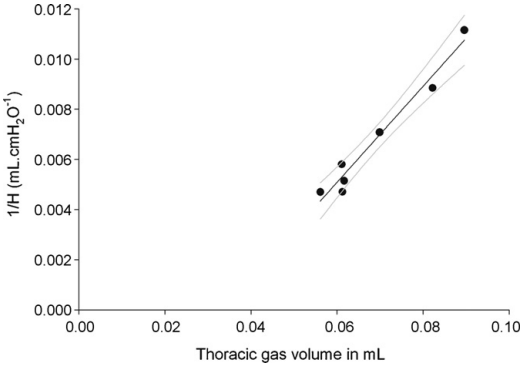

Fig. 2. Relationship between 1/H (1/tissue elastance) and thoracic gas volume (TGV) for the HVZ group. There was a strong (R^2 = 0.965) linear relationship between 1/H and TGV (1/H = −0.0064 + 0.1915 × TGV). Shown are the group mean data, fitted linear regression line and 95% CI.

Enzyme linked immunosorbent assays for the pro-inflammatory cytokines IL-6 and IL-1β were conducted as per the manufacturer's instructions (BD Biosciences, San Diego, CA). Total protein was analysed using a colorimetric Bio-Rad protein assay (Bio-Rad, Regents Park, N.S.W., Australia).

2.5. Statistical analysis

All statistics were conducted using SigmaStat 3.5 (Systat Software Inc., Richmond, CA, U.S.A.). For repeated physiological measurements repeated measures analysis of variance with Holm-Sidak post hoc tests were used. Comparisons of BALF and serum outcomes were made using one-way analysis of variance with Holm-Sidak post hoc tests. Data were transformed where appropriate to ensure the assumptions of normality and equal variance were satisfied. Where this was not possible equivalent non-parametric comparisons were used.

3. Results

3.1. Thoracic gas volume

As demonstrated in Fig. 1A, lung volume in both the HVP and LVP groups did not change throughout the 60 min ventilation period. In contrast, the HVZ group showed a significant reduction in TGV after 20 min of mechanical ventilation, which was sustained for the duration of the study period and approximated to a volume loss of one third.

3.2. Lung mechanics

Fig. 1B shows changes in R_{aw} during the ventilation period in the three groups of mice. A significant increase in R_{aw} was measured after 60 min in the HVZ group whereas R_{aw} significantly dropped after 10 min in the HVP group and remained unchanged until the end of the experiment. Alterations of G and H are illustrated in Fig. 1C and D, respectively. Values for both G and H increased significantly after 10 min in the HVZ group and by 20 min for the HVP and LVP groups when compared with their respective baselines. At the end of the study protocol G and H in the HVZ group increased by 86% and 148%, respectively, which was significantly greater than in the HVP (38% and 56%, respectively) and LVP groups (62% and 88%, respectively).

3.3. Relationship between TGV and tissue mechanics

In order to explore the relationship between change in lung volume and parenchymal mechanics, the relationship between TGV and $1/H$ (compliance) was investigated. As the changes in TGV and H over the ventilation period were small for the HVP and LVP groups, this relationship could not be examined. However, in the HVZ group where there was a significant decrease in TGV and increase in H, there was a strong linear relationship between $1/H$ and TGV ($p_{slope} < 0.001$; $R^2 = 0.965$) (Fig. 2).

3.4. Peak and mean airway opening pressure levels

Fig. 3A and B shows peak airway opening pressure (P_{ao}) and mean P_{ao} levels from baseline to the end of the study. After switching to the allocated ventilation pattern peak P_{ao} levels of the LVP, HVP and HVZ groups increased to 11.8, 16.9, and 13.3 cm H_2O, respectively. Thereafter, P_{ao} values of the study groups with PEEP (LVP and HVP) rose to 14.4 and 20.2 cm H_2O, respectively, whereas peak P_{ao} in the HVZ group increased to 20.6 cm H_2O by the end of the experiment. Similarly, after switching to the allocated ventilation pattern mean P_{ao} levels of LVP and HVP groups increased to 7.4 and 9.9 cm H_2O, respectively, whereas mean P_{ao} decreased to 6.6 cm H_2O in the HVZ group as a result of PEEP withdrawal. In LVP, HVP, and HVZ groups mean P_{ao} further increased to 8.7, 11.6, and 10.3 cm H_2O, respectively, by the end of the study protocol. Periodically applied sighs in the LVP group were below P_{ao} levels of 20 cm H_2O.

3.5. Inflammatory cells, cytokines and total protein in BALF

There was no difference between any of the ventilation groups and the non-ventilated control groups for total cells and differential cell counts (macrophages and neutrophils) obtained from the BALF. Similarly, as shown in Fig. 4A we found no differences in levels of IL-6 in BALF ($p = 0.087$). In contrast, there was a significant increase in total protein levels of mice ventilated with the HVP strategy compared to all other groups (Fig. 4C). The level of IL-1β was below the range of detection (<30 pg/ml) for control and ventilated mice.

3.6. Interleukin-6 in serum

The serum concentration of IL-6 was significantly higher in the HVZ group when compared with all other groups (Fig. 4B).

4. Discussion

The following two major findings emerge from the current study. First, alterations of respiratory mechanics during high V_T ventilation without PEEP can be attributed to loss of lung volume. Second, application of PEEP during high V_T ventilation prevents atelectasis but induces lung injury.

In accordance with adult rodent data we hypothesized that a HVZ strategy would cause the most significant changes in lung mechanics and lung injury (Wilson et al., 2003; Choudhury et al., 2004; Walder et al., 2005; Allen et al., 2006). Our data only partially support this hypothesis in infant mice. HVZ caused the fastest and steepest increase in the coefficients of tissue damping and tissue elastance. However, these changes can largely be explained by loss of lung volume. G reflects both tissue mechanical properties and regional airway heterogeneity, whereas H represents lung stiffness (Allen et al., 2005). The proportionate increase in G and H in the HVZ group is indicative of a peripheral process (Irvin and Bates, 2003), namely atelectasis. The linear relationship between tissue compliance ($1/H$) and lung volume (TGV) is consistent with the predominant mechanism for the increase in G and H being loss of lung volume. It should be noted that this relationship does not point to the origin, i.e. the fall in compliance appears to be somewhat faster than the loss in lung volume. However, this tendency should be interpreted carefully because of the assumption of the linear relationship and the relatively long distance to the origin not covered by measurement data.

Application of a moderate PEEP level in the HVP and LVP groups prevented loss of lung volume. As opposed to standard ventilation with V_T of 20 ml/kg during the HVP strategy, periodic V_T delivery of 20 ml/kg, as a mild recruitment maneuver (RM) in the LVP group, resulted in a pronounced increase of G and H. However, these changes were not associated with an increased inflammatory response or lung injury.

Allen et al. (2006) used comparable ventilation strategies and also accurate tools for measuring lung mechanics in adult mice but reported different results. High V_T ventilation with or without PEEP produced no change in R_{aw} of adult mice, whereas in the present study infant mice showed an increase in R_{aw} with HVZ and a decrease with HVP. Similarly, after 60 min of ventilation G and H were unaltered in adult mice ventilated with high V_T (Allen et al., 2006), but were elevated in infant mice ventilated with HVZ and HVP in the present study. Apart from developmental and structural differences between lungs of infant and adult mice we cannot exclude that differences in mouse strain, lung volume history standardization after the start of mechanical ventilation, respiratory rate, and slightly higher V_T (25 ml/kg versus 20 ml/kg in our study) may have caused more alveolar recruitment in the adult mice and hence different patterns of R_{aw}, G, and H.

In contrast to findings in adult rodents, newborn rats ventilated with extremely high V_T (40 ml/kg) and zero PEEP showed an increase in total respiratory compliance (Martinez et al., 2004). This finding persisted until 120 min of mechanical ventilation and was associated with increase in alveolar surfactant pools including a rise in the functionally active form of large aggregate surfactant subfraction. Copland et al. (2004) investigated the effects of high V_T ventilation with zero PEEP in newborn and adult rats and found that adults were more susceptible to high V_T-induced lung injury. Interestingly, only newborn rats ventilated with a V_T of 40 ml/kg, but not with 25 ml/kg, showed a decrease in respiratory system compliance by 180 min of artificial ventilation. Hence, newborn rat lungs seem to be resistant to a supposedly injurious ventilation mode (25 ml/kg and zero PEEP). This finding may be due to a different response of surfactant production and composition after mechanical stretch and a relatively immature immune system when compared to older rodents.

Kornecki et al. (2005) applied a pressure-limited ventilation mode with a PEEP level of 1 cm H_2O in 17-day-old infant rats with almost full alveolarization. Ventilation with peak pressure levels of 20 and 30 cm H_2O resulted in a V_T of ~37 and ~48 ml/kg,

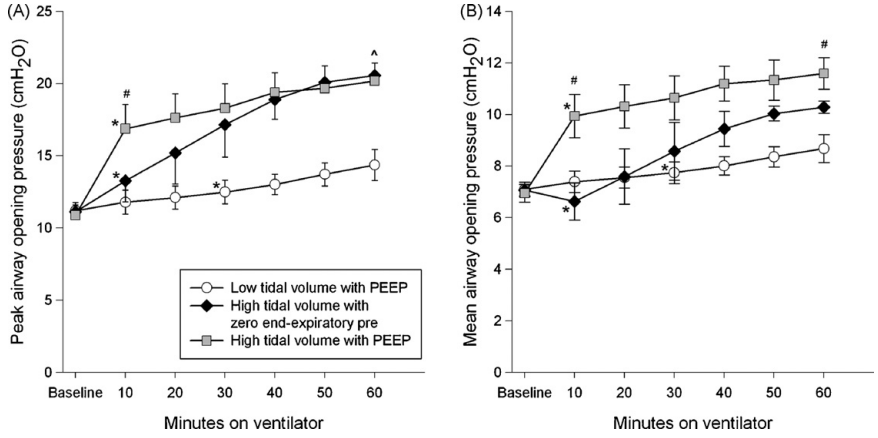

Fig. 3. Peak (A) and mean airway opening pressure (B) during ventilation with low V_T and PEEP (LVP), high V_T and PEEP (HVP), and high V_T and zero end-expiratory pressure (HVZ) at different time points from baseline (before allocation to study groups) to end of the study. Data of each group are means ± S.D. *Indicates a significant change when compared with respective baseline, # displays a difference between all the study groups, ^ indicates a difference between the two high V_T groups and the low V_T group ($p < 0.05$).

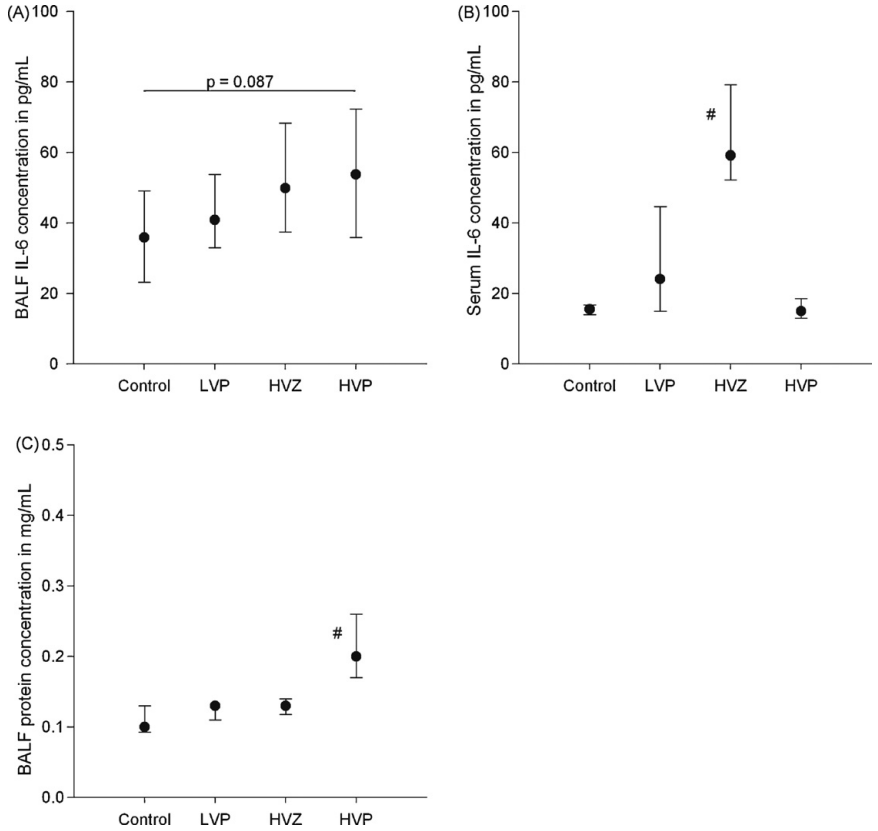

Fig. 4. Interleukin-6 (A) and total protein (C) concentrations in bronchoalveolar lavage fluid; interleukin-6 (B) concentration in serum. Scatter plots with error bars display median with interquartile ranges. Significant change between groups # ($p < 0.05$).

respectively. Total lung compliance assessed by constructed static pressure–volume curves was not even altered after 90 min of ventilation with peak pressures of 30 cm H_2O. Clearly, this is in contrast to our findings in 14-day-old infant mice ventilated with both the HVZ and HVP strategy. A comparison of the effects of high V_T ventilation between infant mice and rats is difficult and may be biased by different ventilation modes (e.g. pressure versus volume-controlled, respiratory rate, PEEP levels) and lung function techniques used. However, infant mice and rats produce a different pattern of respiratory system mechanics and inflammation and lung injury when compared with adult rodents after artificial ventilation.

The role of inflammatory mediators in the development of VILI is unclear and under continuous debate (Frank et al., 2006). This is not surprising since several factors such as species, pre-treatment, and ventilation strategy influence duration of experiments and outcomes in animal models. Furthermore, it is evident that temporary and clinically relevant, that is less extreme, animal study protocols using primary healthy lungs will not cause massive cytokine and protein production. However, as opposed to Allen et al. (2006) we did not find elevated concentrations of IL-6 in BALF of ventilated infant mice, but increased values of IL-6 in serum of the HVZ group. A similar result was also shown in adult rats after a high pressure zero PEEP strategy (Haitsma et al., 2003). Although overexpression of IL-6 in response to lung stretch during high V_T ventilation has been shown in adult animal studies, its role as a purely proinflammatory cytokine has been questioned (Copland et al., 2004), since several animal studies have also demonstrated lung protective characteristics of IL-6. In our study, we assume that the reason for elevated serum IL-6 levels is largely related to substantial atelectasis leading to ventilation–perfusion–mismatch, deterioration in cardiac output and eventually a systemic inflammatory response.

Measurement of total protein in BALF is not a specific marker of lung injury; however, it has been used to track progression of lung injury and as an indicator of loss of endothelial and epithelial barrier function (Parker and Townsley, 2004). An interesting finding of our study is that only the HVP strategy caused increased total protein concentrations in BALF. In the HVP group, high V_T was delivered on top of PEEP and may have caused overstretching of lung units resulting in lung injury. This is in line with results from adult animal studies where alveolar overdistension has been shown to be an important mechanism in the pathogenesis of VILI (Vlahakis and Hubmayr, 2005). However, one may wonder why our HVZ strategy did not lead to elevated protein levels like comparable HVZ strategies in other adult animal studies (Allen et al., 2005, 2006; Ricard et al., 2001; Belperio et al., 2002), even though similar peak P_{ao} levels were achieved as within the HVP group. This is surprising because derecruited lungs exposed to high V_T and zero PEEP have been shown to be particularly prone to repeated opening and closing of alveoli causing shear stress between adjacent aerated and non-aerated alveoli, and eventually leading to lung injury (Lapinsky and Mehta, 2005). While in our HVZ group shear stress may have occurred because of proven loss of lung volume with resulting atelectasis, we have no direct evidence for repeated opening and closing of lung units and doubt that the peak P_{ao} values achieved in the HVZ group were sufficient enough to reopen collapsed peripheral lung units.

However, the degree of lung distension and with it the risk of VILI does not depend on peak P_{ao}, but rather on transpulmonary pressure (P_{tp}), tissue elastance, and lung volume history (Gattinoni et al., 2004). In theory, application of PEEP during HVP ventilation may have lessened P_{tp}. However, due to the difficulties in measuring pleural or oesophageal pressure in infant mice, it remains unclear how changes in respiratory system mechanics, TGV, and PEEP affect P_{tp}. More importantly, application of high V_T on top of PEEP resulted in both higher end-expiratory lung volume (EELV) and better lung compliance when compared with the HVZ group. Due to the relatively small contribution of the chest wall on respiratory system mechanics reported in mice (Frappell and Mortola, 1989; Sly et al., 2003; Ito et al., 2007), it is unlikely that a higher state of inflation (as in HVP) may have attenuated alveolar distension in infant mice, where chest wall tissue is even thinner. Furthermore, the use of PEEP also resulted in consistently higher mean P_{ao} values throughout the study, probably reflecting the higher state of inflation. Consequently, higher EELV, lower R_{aw}, and better lung compliance most likely resulted in alveolar overdistension during HVP ventilation when compared with HVZ ventilation. Conversely, despite similar peak P_{ao} values at the end of the study, lower EELV, high lung elastance, and moderately increased R_{aw} is likely to have resulted in less alveolar distension in the HVZ group.

Some limitations of the presented study should be conceded. First, the duration of our experiment was short. Though it may be possible to extend the time on the ventilator keeping infant mice alive by applying oxygen and periodic RM both interventions have been shown to induce lung injury (Halbertsma and van der Hoeven, 2005; Altemeier and Sinclair, 2007). Secondly, lung inflammatory response may be time dependent, it is therefore uncertain whether longer mechanical ventilation may have produced a different pattern of inflammatory response. Lastly, given the infant animal size and technical difficulties we did not measure oxygen saturation and partial pressures of arterial oxygen or carbon dioxide. Nonetheless, our infant mouse model proved to be a useful tool for the investigation of age-specific mechanisms of VILI and produced distinct short-term outcomes.

In conclusion, our *in vivo* infant mouse study involving separate measurements of airway and tissue mechanics and absolute lung volumes shows that overall mechanical ventilation with high V_T with or without PEEP is deleterious, albeit via different mechanisms. In addition, this study provides evidence that mechanically ventilated infant mice lungs behave differently from adult and neonatal rodent lungs. This finding underlines the need for age-specific animal models and asks for caution when data and conclusions from adult animal research are extrapolated for infant animals and eventually human infants.

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Impact of supplemental oxygen in mechanically ventilated adult and infant mice

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ABSTRACT

The aim of the present study was to determine the short-term effects of hyperoxia on respiratory mechanics in mechanically ventilated infant and adult mice. Eight and two week old BALB/c mice were exposed to inspired oxygen fractions (FI_{O_2}) of 0.21, 0.3, 0.6, and 1.0, respectively, during 120 min of mechanical ventilation. Respiratory system mechanics and inflammatory responses were measured. Using the low-frequency forced oscillation technique no differences were found in airway resistance between different FI_{O_2} groups when corrected for changes in gas viscosity. Coefficients of lung tissue damping and elastance were not different between groups and showed similar changes over time in both age groups. Inflammatory responses did not differ between groups at either age. Hyperoxia had no impact on respiratory mechanics during mechanical ventilation with low tidal volume and positive end-expiratory pressure. Hence, supplemental oxygen can safely be applied during short-term mechanical ventilation strategies in infant and adult mice.

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1. Introduction

Supplemental oxygen is frequently used to improve survival during prolonged periods of mechanical ventilation. In addition, the lack of an oxygen blender often results in application of pure oxygen during mechanical ventilation protocols. The pro-inflammatory properties of inhaling high concentrations of oxygen are well-known (Zaher et al., 2007). Also, local high oxygen concentrations may hasten atelectasis in lung units communicating poorly with the airway opening (Duggan et al., 2005; Aboab et al., 2006) and alter the function of the pulmonary surfactant system (Zenri et al., 2004), both leading to a decrease in lung compliance. Hence, a high fraction of inspired oxygen (FI_{O_2}) may impair lung mechanics, contribute to additional lung injury and confound interpretation of the main study design.

Spontaneously breathing infant mice and rats have been shown to be more resistant to hyperoxic exposure of several days' duration than adult animals. Factors involved in this relative resistance to hyperoxia may include: greater antioxidant enzyme activity (Frank et al., 1978), decreased generation of reactive oxygen species (Ischiropoulos et al., 1989), and an increased ability to clear lung water (Laudert et al., 1994).

In the context of mechanical ventilation combination of high tidal volume (V_T) and/or low positive end-expiratory pressure (PEEP) ventilation and hyperoxia resulted in lung injury and increased inflammatory response in adult (Quinn et al., 2002; Duggan et al., 2005; Desai et al., 2007; Li et al., 2007) and infant (Copland et al., 2004; Bland et al., 2007) rodents. However, it is not well-known how short-term exposure to oxygen affects respiratory system mechanics during mechanical ventilation with low V_T and PEEP.

Fast-acting oxygen effects include not only alteration of systemic and pulmonary vascular resistance (Rousseau et al., 2005), but also formation of atelectasis and modulation of airway smooth muscle tone and bronchomotor tone via oxygen-sensitive airway receptors (Peers and Kemp, 2001). Moreover, immature and developing lungs seem to have a higher distribution and concentration of oxygen-sensitive receptors (Van Lommel, 2001). Hence, it is possible that younger animals with their evolving airway and alveolar structures and vascularization may react differently to oxygen exposure.

We have recently demonstrated the importance of age-specific animal models for investigating ventilator-induced lung injury (Cannizzaro et al., 2008). The different response of infant rodents to mechanical ventilation together with the different responses to hyperoxia highlights the need to understand the effects of high FI_{O_2} and mechanical ventilation in both adult and infants. The present

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study was undertaken to determine whether high oxygen concentrations alter respiratory system mechanics and inflammatory response, and whether this interaction differs between infant (2 week old) and adult (8 week old) rodents.

2. Materials and methods

2.1. Study animals

Experimental protocols were approved by the Telethon Institute for Child Health Research Animal Experimentation Ethics Committee and conformed to the guidelines of the National Health and Medical Research Council of Australia. Eight and two week old female BALB/c mice were purchased from the Animal Resource Centre (Murdoch, Western Australia). Two week old mice were kept with their dam until the day of experiment.

2.2. Animal preparation

Mice were anaesthetised with an intraperitoneal injection of a solution containing ketamine (Troy Laboratories, N.S.W, Australia), xylazine (Troy Laboratories, N.S.W, Australia), and saline. To induce surgical level of anaesthesia 8 week old mice (18.6 ± 1.0 g) were given 160 μg/g ketamine and 8 μg/g xylazine, followed by a top-up of 100 μg/g ketamine and 5 μg/g xylazine after 10 min. Two week old mice (8.7 ± 1.0 g) were given 160 μg/g ketamine and 8 μg/g xylazine without top-up after 10 min. Once adequately anaesthetised, a tracheotomy was performed and a 10 mm long polyethylene cannula (OD: 1.27 mm, ID: 0.86 mm) or a metal cannula (21 gauge) for 8 and 2 week old mice, respectively, was inserted and secured with suture. The mouse was then placed in the supine position on a heating mat and connected to a computer-controlled ventilator (*flexiVent*®, Scireq, Montreal, Canada) using a volume-controlled mechanical ventilation mode.

2.3. Ventilator settings

Eight week old mice were ventilated with room air at a respiratory rate (RR) of 300/min with PEEP of 3 cmH_2O, and a target tidal V_T of 10 mL/kg, which resulted in a delivered V_T of 7.3 ± 0.13 mL/kg when accounting for gas compression and/or friction in the cylinder. For 2 week old mice, modifications of respirator settings were necessary to facilitate survival rates over the ventilation period of 120 min. After preliminary studies (data not shown) 2 week old mice were ventilated as follows: room air, RR of 240/min with PEEP of 3 cmH_2O, and a target V_T of 15 mL/kg (delivered V_T of 8.8 ± 0.48 mL/kg). Minute ventilation was similar in both age groups and high enough to ensure that mice remained apneic during measurements of respiratory system mechanics (outlined below). PEEP level was regulated via depth of a water column that was connected to the expiratory port of the ventilator.

2.4. Measurement of respiratory system mechanics

Before standardization of lung volume history an anaesthetic top-up containing 100 μg/g ketamine and 5 μg/g xylazine was administered to both age groups (further anaesthetic was given as required throughout the experiment). Lung volume history was standardized by a pressure-limited (20 cmH_2O) linear inflation–deflation maneuver over a time period of 30 and 20 s for 8 and 2 week old mice, respectively. A total of three maneuvers were applied within 5 min. Then, baseline measurement of respiratory system input impedance (Z_{rs}) was performed using the low-frequency forced oscillation technique (FOT) provided by the *flexiVent*® system. Z_{rs} was obtained during a 16 s pause from mechanical ventilation during which a broadband signal of 19 mutually prime frequencies from 0.25 to 20 Hz was applied to the airway opening of the mouse. During Z_{rs} measurement PEEP level remained unchanged at 3 cmH_2O. The resulting input impedance data were analysed using the constant-phase model (Hantos et al., 1992), which allows distinction between central and peripheral respiratory mechanics. Airway resistance (R_{aw}), inertance (I_{aw}), tissue damping (G) and elastance (H) were determined by fitting the model to the Z_{rs} data. I_{aw} values got insignificantly low and hence are not reported. Except for data points coinciding with the heart rate or its harmonics, the constant-phase model fitted well to impedance data. The coefficient of determination, a quality control parameter reflecting the goodness of the model fit, was >0.990 and >0.985 for 8 and 2 week old mice, respectively. Moderate recruitment maneuvers (RM), designed to prevent extensive atelectasis were delivered shortly after and 5 min before each Z_{rs} measurement (every 10 min during the ventilation period). The RM were pressure-limited and lasted 6 s, partitioned into 3 s ramp duration and 3 s hold at 20 cmH_2O, followed by passive deflation to a PEEP of 3 cmH_2O.

After the first Z_{rs} baseline measurement, mice were assigned to one of the following groups: (1) FI_{O_2} 0.21 (control group), (2) FI_{O_2} 0.3, (3) FI_{O_2} 0.6, and (4) FI_{O_2} 1.0; $n = 8$ mice per group. FI_{O_2} levels of 0.3, 0.6, and 1.0 were arbitrarily chosen based on the common clinical assumption that these levels are "safe", "borderline", and "harmful", respectively. Oxygen concentrations were controlled by a gas blender (Bird 3 M, Palm Springs, CA, USA) that was supplied by the Research Institute's air and oxygen lines. Constant flow of 2 L/min was adjusted with a flow meter and delivered via hose to the top end of the inspiratory port of the ventilator.

2.5. Correction for differences in gas composition

In addition to routine dynamic calibration procedure of the *flexiVent*®, which accounts for gas compression and pressure drop across tubing (including the tracheal tube), the effect of the different gas compositions had to be taken into consideration. Due to the fact that pure oxygen has a higher viscosity than nitrogen, correction for viscosity change was mandatory when using different FI_{O_2} levels. Correction factors were obtained by using published values for gas viscosity (Turney and Blumenfeld, 1973). Accordingly, theoretical calculations from equations for viscosities of oxygen and nitrogen predicted increases in viscosity and hence airway resistance by 1.013, 1.056, and 1.117 for a FI_{O_2} of 0.3, 0.6, and 1.0, respectively, relative to room air. These factors were determined with the assumption of an average gas temperature in the airways of ~30 °C. The correction factors were verified experimentally in our equipment (data not shown) and used to divide the values of the R_{aw} measured with the corresponding gas mixture. Only corrected values of R_{aw} are reported.

2.6. Sampling of bronchoalveolar lavage fluid (BALF) and serum

At the end of the study period blood samples were obtained by cardiac puncture. Blood was allowed to clot and centrifuged at 2000 rpm for 10 min and serum frozen for later analysis of interleukin-6 (IL-6) and macrophage inflammatory protein-2 (MIP-2). After blood sampling, lungs were lavaged via tracheostomy with 0.5 and 0.2 mL of sterile 0.9% saline solution for adult and infant mice, respectively. The lavage solution was instilled in and out of the lung three times and ice cooled until centrifugation at $400 \times g$ for 4 min. Supernatant was collected and frozen for later analysis of IL-6, MIP-2 and total protein. The cell pellet was resuspended in phosphate buffered saline and an aliquot was stained with Trypan

blue to obtain a total cell count using a haemocytometer. A second aliquot was centrifuged onto a slide and stained with Leishmann's to obtain a differential cell count using light microscopy by counting 300 cells from each slide.

Concentrations of IL-6 and MIP-2 were measured in all samples by using specific enzyme linked immunosorbent assays, following the manufacturers' instructions (BD Biosciences, San Diego, CA). Total protein was analysed using a colorimetric Bio-Rad protein assay (Bio-Rad, Regents Park, New South Wales, Australia).

2.7. Statistical analysis

For repeated physiological measurements repeated measures analysis of variance with Holm-Sidak post-hoc tests were used.

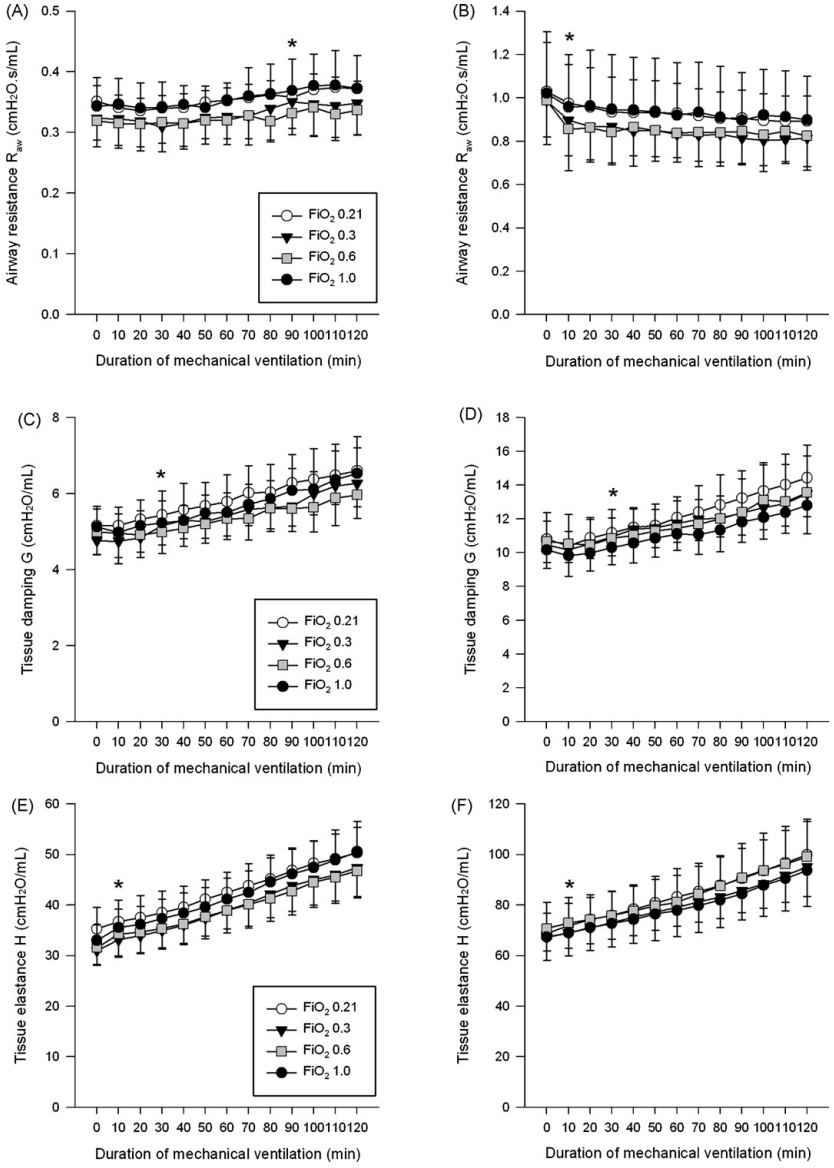

Fig. 1. Airway resistance (R_{aw}) (panels A and B), coefficients of lung tissue damping (G) (panels C and D) and elastance (H) (panels E and F) as a function of time on the ventilator in 8 week old (panels A, C, and E) and 2 week old mice (panels B, D, and F). Data are expressed as group means ± standard error of the mean. (*) indicates significant changes when compared with baseline R_{aw}, G, and H values of 8 and 2 week old mice, respectively ($p < 0.05$). No significant differences were found between study groups ($p > 0.17$ in all cases).

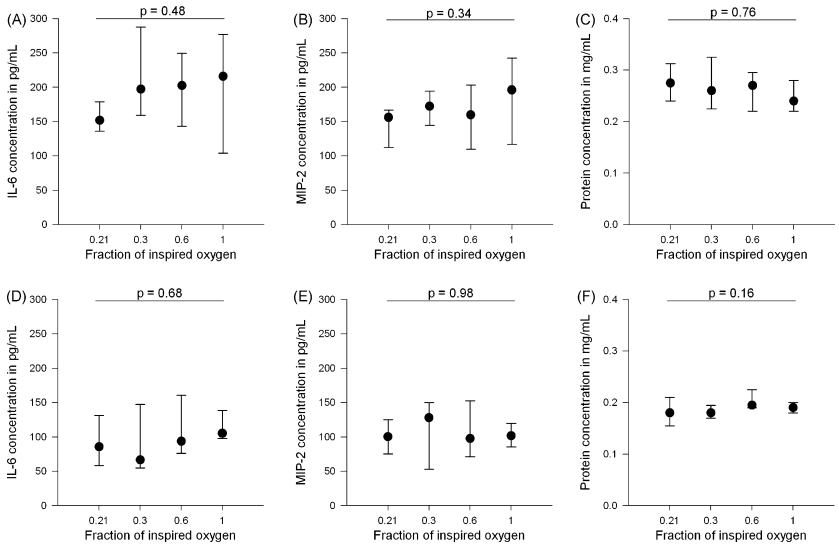

Fig. 2. Panels A–C and D–F refer to 8 and 2 week old mice, respectively. Panels A and D, B and E, and C and F show interleukin-6, macrophage inflammatory protein-2, and total protein concentrations in bronchoalveolar lavage fluid after 120 min of mechanical ventilation, respectively. Scatter plots with error bars display median with interquartile ranges. Concentration levels between groups were not statistically different.

Comparisons of BALF outcome parameters were made using one-way analysis of variance with Holm-Sidak post-hoc tests. Data are expressed as group means ± standard error of the mean and were transformed where appropriate to ensure the assumptions of normality and equal variance were satisfied. Where this was not possible equivalent non-parametric comparisons were used. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Airway resistance (R_{aw}) after correction for change in gas mixture

Baseline measurements for R_{aw} were not different between FI_{O_2} groups in both 8 and 2 week old mice. We found no difference in R_{aw} between FI_{O_2} groups after 120 min of mechanical ventilation in both age groups (Fig. 1A and B, respectively). When compared with baseline values, R_{aw} significantly increased after 90 min in 8 week old mice, whereas it significantly decreased after 10 min in 2 week old mice ($p < 0.05$, respectively). However, these differences were small and considered to be physiologically insignificant.

3.2. Coefficients of lung tissue damping (G) and elastance (H)

Baseline measurements for G and H were not different between groups in both age groups (Fig. 1C–F). After 120 min of mechanical ventilation no difference between FI_{O_2} groups was found for both G and H in 8 and 2 week old mice. Values for G and H significantly increased after 30 and 10 min, respectively, in both age groups when compared with their respective baselines. At the end of the study protocol G increased on average by 27 and 29% in 8 and 2 week old mice, respectively, while H increased by 49 and 41% in 8 and 2 week old mice, respectively.

3.3. Inflammatory cells, cytokines, and total protein in BALF

After 120 min we found no difference in total cells and differential cell counts (e.g. macrophages, neutrophils, and lymphocytes) obtained from the BALF between FI_{O_2} groups in both age groups ($p > 0.41$ in all cases). As shown in Fig. 2A–F there also was no statistically significant difference between levels of oxygen concentrations in both age groups for IL-6, MIP-2, and total protein concentrations.

3.4. Cytokines in serum

IL-6 and MIP-2 serum concentrations were below the assay's limits of detection in all mice (IL-6 < 34.8 pg/mL, MIP-2 < 26.5 pg/mL).

4. Discussion

The data from the present study demonstrate that short-term exposure to levels of oxygen up to 100% do not increase the changes in respiratory system mechanics induced by mechanical ventilation in either infant or adult mice. This finding is particularly useful in commonly used rodent animal models of ventilator-induced lung injury (VILI) when it comes to hypoxia-related tissue damage and survival issues as reported by Duggan et al. (2005).

Low-frequency FOT allows a separate estimation of changes in mechanical properties of the airways and parenchyma during exposure to different oxygen concentrations. Respiratory system mechanical parameters G and H characterize the dissipative and elastic properties of the respiratory tissues, respectively (Hantos et al., 1992). G reflects both tissue mechanical properties and regional airway heterogeneity, whereas H represents lung elastance. Moreover, a strong correlation between rise in H and decrease in thoracic gas volumes assessed by computed tomography has been demonstrated (Allen et al., 2007). Hence, the almost linear increase of G

and *H* in our study most likely reflects derecruitment of lung volume over time, especially because the changes in *G* and *H* were proportionate. These changes were similar in both age groups and were not altered by FI_{O_2} concentrations. However, we cannot exclude that application of mild recruitment maneuvers may have mitigated the increase of *G* and *H* modifying the development of atelectasis.

Airway smooth muscle tone and airway diameter are influenced by partial oxygen pressure (Skogvall et al., 1999). Local hypoxia leads to bronchodilatation and local hyperoxia can induce bronchoconstriction. Thus, pulmonary ventilation can rapidly be modulated to optimise oxygen supply to tissues (Peers and Kemp, 2001). The low-frequency FOT has the potential to detect changes in central airways and airway heterogeneity, via R_{aw} and *G*, respectively.In our study, we found no physiologically significant oxygen-related changes of R_{aw} in either age group. Also, the increase in *G* was associated with a similar increase in *H* and reflects development of atelectasis rather than an increase in ventilation heterogeneity as a result of inhomogeneous regional airway narrowing. Given the absence of hyperoxia-related alterations of R_{aw}, *G*, and *H*, it is possible that the role and function of oxygen-sensitive chemoreceptors is less important *in vivo* when compared to *in vitro* and *ex vivo* experiments.

The relevance of cytokine production in the context of VILI has been challenged by Dreyfuss et al. (2003). Nonetheless, more recent studies continue to measure cytokines in an attempt to explain VILI (Halbertsma et al., 2005; Frank et al., 2006). In the present study, we measured MIP-2 and IL-6 in BALF, since these chemoattractants have been shown to be important mediators in the development of VILI (Frank et al., 2006; Li et al., 2007). We did not find elevations in either cytokine in response to increased FI_{O_2}. These data are consistent with those of Quinn et al. (2002) who found no oxygen-dependent alterations in BALF MIP-2 and neutrophils after 2 h of mechanical ventilation with V_T of 7 mL/kg in a rat model of VILI. In addition, after 6 h of mechanical ventilation, there were no oxygen-related increases in inflammatory cytokines for the groups ventilated with 7 mL/kg, but a significant neutrophil increase only with hyperoxia and high V_T. Similarly, Copland et al. (2004) applied an injurious ventilation strategy in newborn rats and found no oxygen-related MIP-2 increase after 180 min. As to IL-6 production during mechanical ventilation with low V_T, we found no association with supplemental oxygen. Other research groups (Bailey et al., 2003; Copland et al., 2004) only reported increased IL-6 concentrations after high V_T ventilation with no difference between room air and hyperoxia groups. In addition, the lack of increase in total protein in the BALF we observed in the present study suggests that short-term exposure to increased FI_{O_2} *per se* does not compromise epithelial or endothelial barrier function (Parker and Townsley, 2004). Taken together, our findings underscore the lack of significant oxygen-associated pulmonary inflammation and lung injury after non-injurious and short-term mechanical ventilation.

The following study limitations should be acknowledged: first, duration of the study was limited to 120 min. Though it is possible to extend the time on the ventilator keeping adult mice alive, we experienced decreased survival rates with 2 week old mice during preliminary studies with longer study protocols. Second, our study was not designed to assess biotrauma and only measured a limited number of potential mediators in VILI. Our selection of cytokines was based on results from other research groups with comparable duration of mechanical ventilation protocols and limited by available BALF after lavage in infant mice weighing less than 10 g. Third, it is conceivable that longer mechanical ventilation may have produced a different pattern of inflammatory response.

In conclusion, the results of the present study demonstrate that short-time exposure to increased FI_{O_2} in the presence of non-injurious mechanical ventilation does not exacerbate lung injury in either infant or adult mice. Our results may not have a direct translational value for clinical practice, but have valuable implications for experimental VILI studies, which are often performed in mice and use short-term mechanical ventilation protocols. In addition, due to technical advances in measuring respiratory system mechanics in small rodents, infant mouse models have the potential to provide insights into pediatric ventilator-associated lung injury, thus limiting extrapolation from adult data.

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PAPER 3 INCLUDED IN THE THESIS

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Lung volume recruitment maneuvers and respiratory system mechanics in mechanically ventilated mice

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ABSTRACT

The study aim was to establish how recruitment maneuvers (RMs) influence lung mechanics and to determine whether RMs produce lung injury. Healthy BALB/c mice were allocated to receive positive end-expiratory pressure (PEEP) at 2 or 6 cmH_2O and volume- (20 or 40 mL/kg) or pressure-controlled (25 cmH_2O) RMs every 5 or 75 min for 150 min. The low-frequency forced oscillation technique was used to measure respiratory input impedance. Large RMs resulting in peak airway opening pressures (P_{ao}) > 30 cmH_2O did not increase inflammatory response or affect transcutaneous oxygen saturation but significantly lowered airway resistance, tissue damping and tissue elastance; the latter changes are likely associated with the bimodal pressure–volume behavior observed in mice. PEEP increase alone and application of RMs producing peak P_{ao} below 25 cmH_2O did not prevent or reverse changes in lung mechanics; whereas frequent application of substantial RMs on top of elevated PEEP levels produced stable lung mechanics without signs of lung injury.

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1. Introduction

The use of mechanically ventilated rodents to study lung injury and airway disease has increased markedly in recent times (Finkelman and Wills-Karp, 2008; Matute-Bello et al., 2008). With this has come a recognition that the ventilator settings used have the potential to affect respiratory mechanics (Rich et al., 2003; Sly et al., 2003; Duggan et al., 2005; Allen et al., 2006; Tsuchida et al., 2006). Tidal volume (V_T), respiratory rate, fraction of inspired oxygen (FIO_2), airway pressure levels (peak inspiratory, mean airway, and positive end-expiratory pressure (PEEP)) levels, and inspiratory to expiratory time ratio are commonly reported in mechanical ventilation studies using *in vivo* rodent models to investigate acute and chronic lung diseases. It is essential to provide information on ventilator settings in order to facilitate future studies. However, apart from specific "recruitment maneuver studies" (Allen et al., 2002, 2006, 2007; Allen and Bates, 2004; Frank et al., 2005; Koh et al., 2005; Farias et al., 2005; Ko et al., 2008; Jonasson et al., 2008) few experimental studies using mechanical ventilation protocols provide detailed information on application, frequency, and type of lung volume recruitment maneuvers (RMs). Given that the mechanical properties of the respiratory system are specific to the lung volume at which their measurements are made (Sly et al., 2003) and to the lung volume history (Zosky et al., 2008), it is surprising that details of RMs are not always reported.

In clinical practice, based on the concept of "open up the lung and keep the lung open" (Lachmann, 1992), recruitment refers to a dynamic process of reopening non-aerated peripheral lung units through a substantial and sustained increase in transpulmonary pressure in combination with PEEP elevation (Fan et al., 2005). In lung function studies conducted in murine models of respiratory diseases, RMs aim at establishing similar lung volume history and often precede baseline measurement of lung function. Generally, these RMs consist of a series of inflation maneuvers that are either volume- or pressure-controlled and do not include elevation of PEEP. Application of different types of RMs reflects different views on how to best recruit non-aerated lung units without producing lung injury (Hjoberg et al., 2004; Frank et al., 2005; Allen et al., 2007; Ito et al., 2007). In a recent study, Jonasson et al. (2008) showed that application of deep inflations also protects against bronchoconstriction and affects outcome respiratory system mechanics in healthy and allergen-challenged mice.

The respective impacts of PEEP elevation, inflation maneuvers without PEEP elevation, and RMs (i.e. inflation maneuvers plus PEEP

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elevation) on lung function are not clear in mice. The aim of this study was to establish how these maneuvers affect respiratory system mechanics and whether they induce or exacerbate lung injury in mechanically ventilated mice. We hypothesized that frequent and large RMs provide stable respiratory system mechanics, but at the expense of lung injury.

2. Materials and methods

2.1. Animal preparation

The study protocol was approved by the institutional Animal Experimentation Ethics Committee and conformed to guidelines of the National Health and Medical Research Council of Australia. Eight weeks old female BALB/c mice (19.2 ± 0.9 g) were anaesthetised with an i.p. injection of a solution containing ketamine (160 μg/g), xylazine (8 μg/g) in saline. After 10 min a half dose of the anesthetic was administered (the same dose was given later throughout the protocol as required). A tracheostomy was performed and a 10 mm polyethylene cannula (ID: 0.86 mm) inserted. The mouse was then placed in supine position on a heating mat and connected to a computer-controlled ventilator (*flexiVent*®, Scireq, Montreal, Canada) using the following settings: FIO_2 0.5, respiratory rate 180/min, V_T of ~8.0 mL/kg, and PEEP 2 cmH_2O. PEEP was regulated by submerging the expiratory line from the ventilator into a water column. Heart rate and transcutaneous oxygen saturation were monitored via a small animal pulse oximeter (MouseOx™, STARR Life Sciences Corporation™, Oakmont, PA, USA) by placing the non-invasive sensor on the tail.

2.2. Measurement of respiratory system mechanics

Lung volume history was standardized by three pressure-limited (up to 20 cmH_2O) inflation–deflation maneuvers applied within 5 min. Then, baseline measurement of respiratory system input impedance (Z_{rs}) was performed using the low-frequency forced oscillation technique (FOT) provided by *flexiVent*® system. Z_{rs} was obtained by a 4-s oscillation signal of 13 mutually prime frequencies from 1.0 to 20.5 Hz applied to the airway opening of the mouse during a pause from mechanical ventilation. The "constant-phase" model was fitted to the resulting Z_{rs} (Hantos et al., 1992), allowing the estimation of airway resistance (R_{aw}) and inertance (I_{aw}), and the coefficients of tissue damping (G) and elastance (H). Except for data points coinciding with the heart rate or its harmonics, the constant-phase model fitted well to impedance data.

2.3. Experimental protocol and allocation of animals

Lung volume recruitment was achieved by a combination of changes in PEEP level (i.e. 2 and 6 cmH_2O) and application of inflation maneuvers (IM) at different time points (i.e. every 5 or 75 min) during the 150 min protocol. IMs were delivered either in a volume-controlled manner without pressure limit (20 or 40 mL/kg) or in a pressure-controlled mode (25 cmH_2O). The IMs consisted of 3-s ramp duration to reach preset volume or plateau pressure and a 3-s hold followed by passive deflation to the predefined PEEP level.

After the first Z_{rs} baseline measurement, animals were allocated to study groups differing in PEEP level and IM frequency and mode ($n = 7$ mice/group). Six study groups (1a–6a) received different IMs while the PEEP level was unchanged at 2 cmH_2O throughout the protocol: 20 mL/kg every 5 min (Group 1a) or every 75 min (Group 2a), 40 mL/kg every 5 min (Group 3a) or every 75 min (Group 4a), and IMs to 25 cmH_2O every 5 min (Group 5a) or every 75 min (Group 6a). In another six study groups (1b–6b) PEEP was increased from 2 to 6 cmH_2O shortly before application of the first IM and then remained at 6 cmH_2O throughout the study: 20 mL/kg every 5 min (Group 1b) or every 75 min (Group 2b), 40 mL/kg every 5 min (Group 3b) or every 75 min (Group 4b), and IMs to 25 cmH_2O every 5 min (Group 5b) or every 75 min (Group 6b). Two additional groups were ventilated at a PEEP of 2 cmH_2O (Group 7a) or 6 cmH_2O (Group 7b) without any application of IMs. In the latter group (Group 7b) PEEP was increased from 2 to 6 cmH_2O shortly after the first baseline measurement.

Z_{rs} measurements were performed every 15 min and included pre- and post-IM measurements for the study groups 1a–6a and 1b–6b. At each time point four Z_{rs} spectra were collected within 90 s and the corresponding values for R_{aw}, G, and H were averaged. The PEEP level was left unchanged at either 2 or 6 cmH_2O to prevent lung derecruitment during measurements and the oscillations were delivered on top of these PEEP levels.

2.4. Sampling and processing of bronchoalveolar lavage fluid (BALF)

At the end of the study period lungs were lavaged via the tracheal cannula with 0.5 mL of sterile 0.9% saline solution. The lavage solution was instilled in and out of the lung three times and ice cooled until centrifugation at $400 \times g$ for 4 min. Supernatant was collected and frozen for later analysis of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), macrophage inflammatory protein-2 (MIP-2), and total protein. The cell pellet was resuspended in phosphate buffered saline and an aliquot was stained with Trypan blue to obtain a total cell count using a haemocytometer. A second aliquot was centrifuged onto a slide and stained with Leishmann's to obtain a differential cell count using light microscopy by counting 300 cells from each slide. Concentrations of TNF-α, IL-6, and MIP-2 were measured in all samples by using specific enzyme linked immunosorbent assays, following the manufacturer's instructions (BD Biosciences, San Diego, CA). Total protein was analysed using a colorimetric Bio-Rad protein assay (Bio-Rad, Regents Park, NSW, Australia).

2.5. Analysis of lung tissues by morphology and morphometry

Lungs were fixed with 10% phosphate buffered formalin (PBF) instilled via the endotracheal tube at a pressure of 10 cmH_2O. Two hours later the lungs and heart were removed en bloc from the thoracic cavity and stored in a PBF filled container overnight. At the time of processing the heart was dissected free and the remaining tissues were processed whole in paraffin, and embedded with the caudoventral aspect down. Sections were cut at 5 μm from the caudoventral aspect to include as many lung lobes as possible, and stained routinely with haematoxylin and eosin. Inflammatory cells in the histological sections of lung were counted by blindly selecting ten fields at ×100 (oil immersion) from each section. In each of these ten fields, the number of erythrocytes, alveolar macrophages, alveolar neutrophils, and septal neutrophils was counted. To determine the degree of lung inflation by morphometry, ten fields from each lung section were blindly selected and digitally captured under the ×40 objective. A 100-point grid was superimposed over each image, and the number of grid intersection points that coincided with an alveolar wall was determined. For each animal, the sum of the grid counts over the ten digitized images was taken as the relative inflation score. The pathologist was blinded to the study groups. Five lungs in each of the following groups were rated: PEEP 2—no IM (Group 7a), PEEP 6—no IM (Group 7b), PEEP 2—inflation with 40 mL/kg every 5 min (Group 3a) or every 75 min (Group 4a), and PEEP 6—inflation with 40 mL/kg every 5 min (Group 3b) and every 75 min (Group 4b). The selection of these groups was based on the assumption that comparison between control groups (7a and 7b) and groups undergoing large

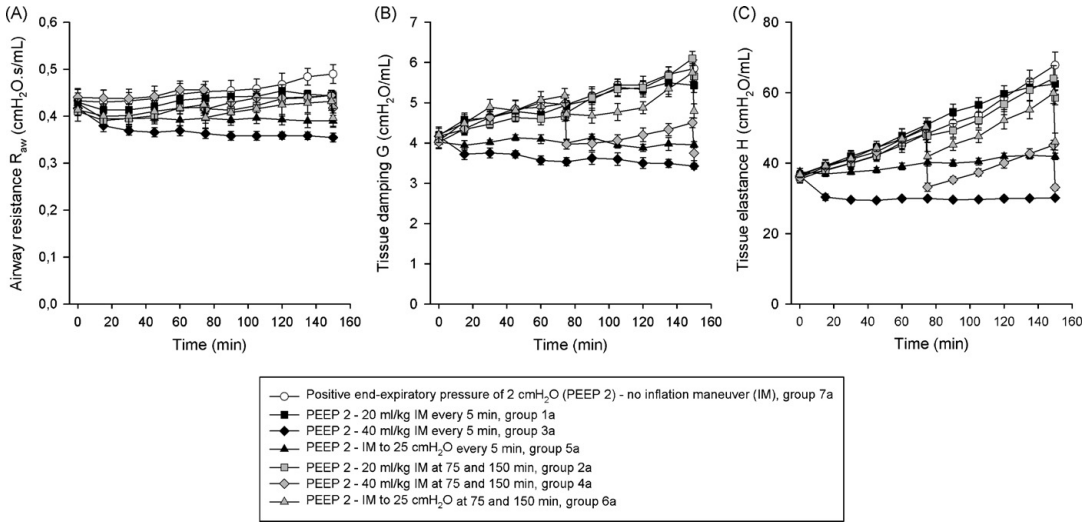

Fig. 1. Impact of inflation maneuvers on lung mechanics. Airway resistance (R_{aw}, Panel A) and coefficients of tissue damping (G) and tissue elastance (H) (Panels B and C, respectively) are displayed as a function of time on the ventilator. PEEP was left unchanged at 2 cmH_2O. Data are expressed as group means ± standard error of the mean. Differences between groups were significant (see Section 3 for details).

inflations would be adequate to demonstrate lung injury in tissue samples.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) with Holm–Sidak post hoc tests was used for histology score and baseline comparisons of Z_{rs} data, peak airway opening pressure levels, and BALF outcome parameters. For repeated physiological measurements repeated measures ANOVA with Holm–Sidak post hoc tests were used (Z_{rs} values were only compared at equivalent PEEP levels). Data were transformed where appropriate to ensure the assumptions of normality and equal variance were satisfied. Where this was not possible equivalent non-parametric comparisons were used. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Z_{rs} values and peak airway opening pressure levels at baseline

After standardization of lung volume history and before allocation to study groups no differences were found for R_{aw}, G, H, and

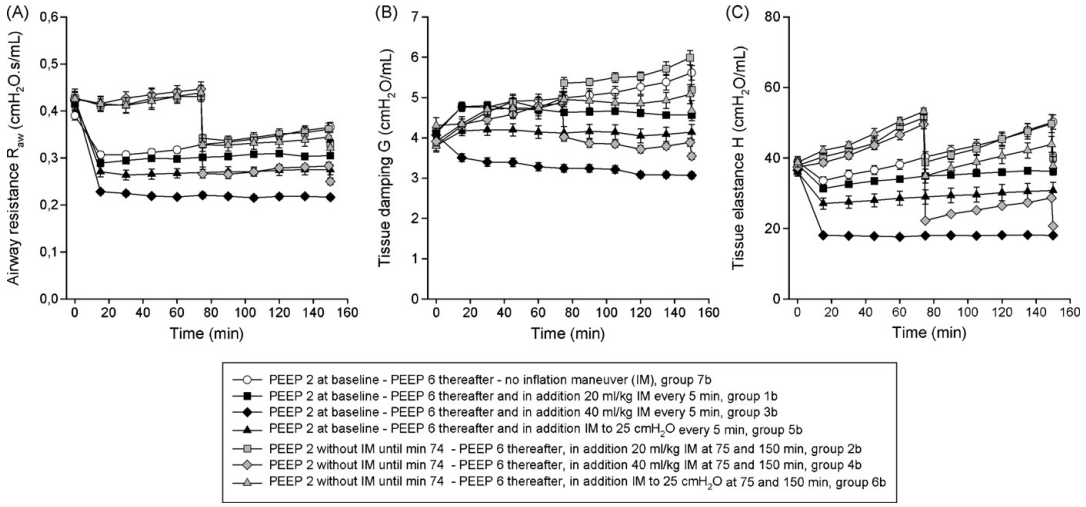

Fig. 2. Impact of inflation maneuvers and PEEP elevation on lung mechanics. Airway resistance (R_{aw}, Panel A) and coefficients of tissue damping (G) and tissue elastance (H) (Panels B and C, respectively) are illustrated as a function of time on the ventilator. Data are expressed as group means ± standard error of the mean. Differences between groups were significant (see Section 3 for details).

Table 1
Peak airway opening pressure (P_{ao}) in cmH_2O before, during, and after lung volume recruitment maneuvers (RMs) at selected time points (mean, SD).

Study group	P_{ao} at baseline	P_{ao} at 74 min (before RM)	P_{ao} during RM	P_{ao} at 75 min (after RM)	P_{ao} at 149 min (before RM)	P_{ao} during RM	P_{ao} at 150 min (after RM)
1a	7.9 (0.5)	9.7 (0.8)	17.4 (2.7)	9.6 (0.8)	11.0 (0.9)	22.3 (1.8)	10.8 (0.8)[a,*]
2a	7.8 (0.2)	9.4 (0.6)	16.6 (1.6)	9.3 (0.5)	11.2 (1.0)	22.0 (1.7)	10.5 (0.6)[a,*]
3a	7.9 (0.5)	7.3 (0.4)	30.6 (0.9)	7.2 (0.4)	7.3 (0.4)	30.8 (0.8)	7.2 (0.4)[b,*]
4a	8.0 (0.3)	9.8 (0.9)	33.0 (1.6)	7.8 (0.3)	9.2 (0.5)	33.5 (1.5)	7.7 (0.3)[b,*]
5a	8.0 (0.3)	8.5 (0.4)	25	8.3 (0.3)	8.7 (0.4)	25	8.5 (0.4)[c,*]
6a	7.8 (0.2)	9.5 (0.4)	25	8.4 (0.5)	10.5 (0.9)	25	8.8 (0.7)[a,*]
7a	7.8 (0.3)			9.5 (0.5)			11.6 (1.0)[a,*]
1b	7.9 (0.3)	12.6 (0.3)	24.8 (1.1)	12.5 (0.3)	12.8 (0.3)	25.8 (1.1)	12.6 (0.4)[c,*]
2b	8.1 (0.4)	9.9 (0.7)	24.3 (0.5)	13.3 (0.6)	15.0 (0.9)	26.7 (0.6)	14.0 (0.6)[a,*]
3b	8.0 (0.5)	10.5 (0.2)	41.7 (0.6)	10.3 (0.2)	10.3 (0.2)	41.4 (0.8)	10.2 (0.2)[c,*]
4b	8.0 (0.2)	9.7 (0.4)	35.9 (1.6)	11.0 (0.1)	11.9 (0.3)	40.7 (1.5)	10.7 (0.2)[a,*]
5b	7.7 (0.4)	11.9 (0.9)	25	11.8 (0.9)	12.1 (1.1)	25	12.0 (1.0)[a,*]
6b	8.2 (0.3)	9.9 (0.3)	25	12.7 (0.9)	14.1 (1.1)	25	13.2 (1.2)[a,*]
7b	8.0 (0.2)			13.3 (0.3)			14.6 (0.2)[a,*]

In groups 1a–7a PEEP of 2 cmH_2O was applied throughout the protocol. In groups 1b–6b PEEP was elevated from 2 to 6 cmH_2O during application of the first inflation maneuver (IM) at 5 or 75 min and then left unchanged at 6 cmH_2O until the end of the study. In Group 7b PEEP was elevated from 2 to 6 cmH_2O after baseline measurements. Definition of abbreviations: 1a/b and 2a/b: IM with 20 mL/kg every 5 min or 75 min, respectively; 3a/b and 4a/b: IM 40 mL/kg every 5 or 75 min, respectively; 5a/b and 6a/b: IM to 25 cmH_2O every 5 or 75 min, respectively; 7a/b: PEEP 2 and PEEP 6 with no IM, respectively. "a" indicates statistically significant differences between selected time points at baseline, 75, and 150 min; "b" denotes a statistically significant P_{ao} decrease between baseline and both 75 and 150 min, but no significant difference between 75 and 150 min, and "c" indicates a statistically significant P_{ao} increase between baseline and both 75 and 150 min with no difference between 75 and 150 min ($p > 0.06$ in all cases).

[*] Denotes a statistically significant P_{ao} decrease when comparing the time points 149 min and 150 min, i.e. before and after a RM ($p \leq 0.03$, paired t-test).

peak airway opening pressure (P_{ao}) levels between study groups ($p > 0.49$ in all cases) (Figs. 1 and 2, Table 1).

3.2. R_{aw} after lung volume recruitment at PEEP level of 2 cmH_2O

Compared with baseline values R_{aw} statistically significantly increased at 150 min in the control group (Fig. 1A). IMs with 20 mL/kg resulted in steady R_{aw} values ($p > 0.17$ in both cases) while frequent (i.e. every 5 min) application of IMs to 25 cmH_2O or with 40 mL/kg produced a statistically significant but physiologically unimportant decrease in R_{aw} over time. When comparing study groups at the end of the protocol we found small but statistically significantly higher R_{aw} in controls when compared to groups receiving pressure controlled IMs or IMs of 40 mL/kg.

3.3. G and H after lung volume recruitment at PEEP level of 2 cmH_2O

G steadily and significantly increased from baseline to the time points 75 and 150 min in controls and study groups receiving IMs of 20 mL/kg (Fig. 1B). Application of more substantial and frequent IMs (25 cmH_2O and 40 mL/kg) resulted in stable *G* values. Similarly, to significantly decrease the steady rise of *G* in groups receiving intermittent IMs, large IMs were necessary. A general pattern of progressive increase in *H* over time can be seen in all groups apart from those given large IMs (40 mL/kg or 25 cmH_2O) every 5 min (Fig. 1C). Where a single IM was given every 75 min, an abrupt decrease in *H* was seen; however *H* subsequently increased along the same trajectory.

3.4. R_{aw} after lung volume recruitment with PEEP level of 6 cmH_2O

In the study groups 1b, 3b, 5b, and 7b PEEP was increased to 6 cmH_2O after baseline measurement, whereas in study groups 2b, 4b, and 6b PEEP was only elevated to 6 cmH_2O during application of the first IM at 75 min. When PEEP was increased from 2 to 6 cmH_2O R_{aw} decreased in all groups, regardless of the timing of the increase in PEEP (Fig. 2A). From the time point 15 min on, R_{aw} statistically significantly rose over time in the control group with 6 cmH_2O PEEP. Frequent IMs with 20 mL/kg and 40 mL/kg produced a statistically significant but physiologically unimportant rise and fall, respectively, in R_{aw}. After 150 min R_{aw} values of controls were significantly higher when compared to all other groups, except for infrequent IMs with 20 mL/kg ($p = 0.08$).

3.5. G and H after lung volume recruitment at PEEP level of 6 cmH_2O

In the control group *G* steadily and significantly increased until the end of the protocol (Fig. 2B). After the time point 15 min, irrespective of the magnitude, frequent application of IMs produced stable *G* values. When a sporadic IM was given every 75 min, large IMs (40 mL/kg) were required to significantly decrease *G* values. IMs given every 5 min on top of a PEEP of 6 produced a stable *H* over the ventilation period (Fig. 2C), regardless of the type of magnitude of the IM. Less frequent IMs were associated with a progressive increase in *H* up until the time the IM was applied; with a subsequent increase in *H* following a similar trajectory (Fig. 2C).

3.6. Pressure–volume relation during the 3-s ramp inflation

In Fig. 3A–D typical examples of the pressure–volume relation during the first 3-s of the ramp inflation maneuver are given for one animal per study group (1a–6a and 1b–6b). Delayed application of the first RM at the time point 75 min (Fig. 3B and D) resulted in lower values of inflation volume at a given P_{ao} when compared with early application of RMs. Irrespective of the time point of first RM application and the PEEP level, inflation above ~25 cmH_2O was followed by another steep rise of the inflation limb without signs of flattening up to a peak P_{ao} of 35 cmH_2O.

3.7. Heart rate and transcutaneous oxygen saturation (Table 2)

No differences were found between study groups at baseline and at the time points 75 and 150 min ($p > 0.13$ in all cases). Over time heart rate significantly decreased when compared to baseline values.

3.8. Cell counts, total protein and cytokines concentrations in BALF

BALF analysis for total ($p = 0.14$) and differential cell counts (macrophages $p = 0.10$, neutrophils $p = 0.73$) produced no difference

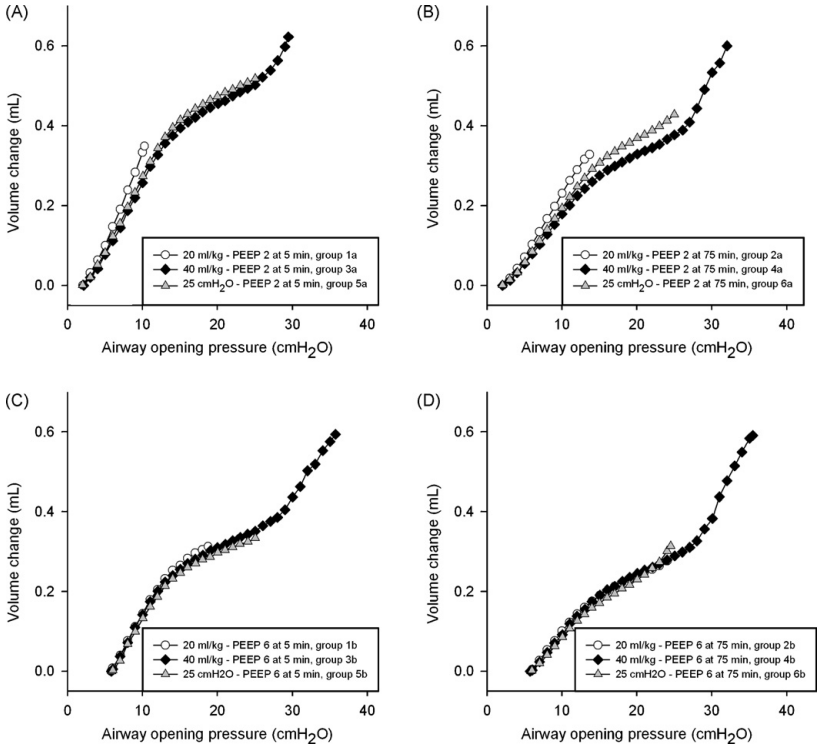

Fig. 3. Inflation volume versus airway opening pressure recorded during the first part of the inflation maneuver (3-s ramp). Panels A and B show inflation limbs during the first recruitment maneuver (RM) applied on top of PEEP 2 cmH_2O at the time points 5 or 75 min, respectively. Panels C and D illustrate inflation limbs during the first RM at the time points 5 or 75 min, respectively, obtained shortly after PEEP elevation from 2 to 6 cmH_2O. One typical example for each group is presented.

between groups. Results from measurement of TNF-α, MIP-2, IL-6, and total protein are displayed in Table 3.

3.9. Analysis of lung tissues by morphology and morphometry

Numbers of alveolar macrophages and erythrocytes, and alveolar and septal neutrophils, as well as lung inflation scores, determined by morphometry (Fig. 4), are presented in Table 4.

4. Discussion

In the present study we found that frequent application of substantial IMs on top of elevated PEEP levels results in stable respiratory system mechanics without causing lung injury after short-term ventilation with low V_T. We also showed that both an increase in PEEP (without use of IMs) and application of IMs resulting in peak P_{ao} below 25 cmH_2O are

Table 2
Heart rate and transcutaneous oxygen saturation (S_{tcO_2}) at selected time points (mean, SD).

Study group	Heart rate baseline	Heart rate 75 min	Heart rate 150 min	S_{tcO_2} baseline	S_{tcO_2} 75 min	S_{tcO_2} 150 min
1a	241 (38.2)	227 (31.3)	232 (38.6)	96.2 (1.4)	95.2 (2.1)	95.6 (1.1)
2a	238 (47.3)	206 (21.1)	190 (17.5)	96.8 (1.9)	96.9 (0.6)	96.2 (0.7)
3a	278 (13.7)	220 (20.2)	214 (17.8)	94.9 (2.4)	96.9 (0.9)	96.9 (0.8)
4a	236 (45.5)	220 (21.9)	234 (25.2)	95.7 (2.6)	94.8 (2.9)	95.1 (0.8)
5a	261 (39.8)	226 (22.1)	235 (13.4)	94.5 (1.2)	95.2 (1.7)	95.2 (0.8)
6a	221 (44.1)	204 (35.0)	207 (37.0)	97.2 (1.6)	96.4 (1.3)	96.1 (0.9)
7a	251 (37.7)	201 (14.6)	214 (15.5)	97.6 (2.3)	97.2 (1.5)	96.6 (1.2)
1b	249 (20.4)	210 (14.8)	224 (22.8)	95.2 (3.4)	94.5 (1.4)	95.0 (1.2)
2b	247 (36.9)	231 (26.9)	233 (14.5)	95.3 (2.1)	95.9 (0.7)	96.1 (0.8)
3b	263 (20.5)	225 (34.4)	231 (30.8)	96.1 (3.3)	96.9 (1.5)	96.5 (0.7)
4b	253 (34.4)	204 (31.7)	214 (42.6)	97.5 (1.5)	94.8 (1.9)	95.3 (0.9)
5b	218 (16.9)	222 (34.2)	224 (45.0)	96.4 (2.3)	96.4 (0.8)	96.0 (1.2)
6b	253 (47.0)	217 (38.9)	210 (23.2)	96.0 (1.9)	94.8 (2.2)	94.9 (1.6)
7b	233 (19.6)	207 (12.9)	220 (21.2)	97.2 (1.5)	96.5 (2.2)	96.2 (0.8)

In groups 1a–7a PEEP of 2 cmH_2O was applied throughout the protocol. In groups 1b–6b PEEP was elevated from 2 to 6 cmH_2O during application of the first inflation maneuver (IM) at 5 or 75 min and then left unchanged at 6 cmH_2O until the end of the study. In group 7b PEEP was elevated from 2 to 6 cmH_2O after baseline measurements. Definition of abbreviations: 1a/b and 2a/b: IM with 20 mL/kg every 5 or 75 min, respectively; 3a/b and 4a/b: IM 40 mL/kg every 5 or 75 min, respectively; 5a/b and 6a/b: IM to 25 cmH_2O every 5 or 75 min, respectively; 7a/b: PEEP 2 and PEEP 6 with no IM, respectively.

Table 3
Tumor necrosis factor alpha (pg/mL), macrophage inflammatory protein-2 (pg/mL), interleukin-6 (pg/mL), and total protein (mg/mL) in bronchoalveolar lavage fluid.

Study groups	TNF-α	MIP-2	IL-6	Total protein
1a	120 (103–133)	130 (104–153)	263 (215–284)	0.18 (0.17–0.22)
2a	155 (125–174)	149 (135–173)	283 (234–371)	0.19 (0.18–0.20)
3a	189 (161–195)	194 (142–221)	347 (288–459)	0.19 (0.18–0.21)
4a	158 (129–181)	188 (150–200)	425 (262–452)	0.22 (0.20–0.27)
5a	129 (120–142)	165 (154–177)	253 (226–327)	0.17 (0.17–0.22)
6a	152 (140–155)	169 (148–173)	277 (229–354)	0.20 (0.20–0.21)
7a	156 (151–198)	162 (152–211)	229 (217–253)	0.18 (0.18–0.19)
1b	136 (131–156)	150 (147–159)	222 (206–304)	0.17 (0.15–0.20)
2b	129 (118–135)	140 (115–157)	218 (192–232)	0.20 (0.18–0.24)
3b	158 (142–162)	192 (150–224)	264 (248–388)	0.22 (0.20–0.43)
4b	151 (127–169)	160 (136–188)	207 (187–340)	0.21 (0.20–0.24)
5b	159 (130–184)	167 (141–184)	272 (204–316)	0.18 (0.17–0.19)
6b	136 (119–152)	169 (167–174)	309 (255–373)	0.18 (0.18–0.24)
7b	151 (120–176)	124 (110–135)	333 (283–365)	0.21 (0.21–0.25)

In groups 1a–7a PEEP of 2 cmH_2O was applied throughout the protocol. In groups 1b–6b PEEP was elevated from 2 to 6 cmH_2O during application of the first inflation maneuver (IM) at 5 or 75 min and then left unchanged at 6 cmH_2O until the end of the study. In group 7b PEEP was elevated from 2 to 6 cmH_2O after baseline measurements. Definition of abbreviations: 1a/b and 2a/b: IM with 20 mL/kg every 5 or 75 min, respectively; 3a/b and 4a/b: IM 40 mL/kg every 5 or 75 min, respectively; 5a/b and 6a/b: IM to 25 cmH_2O every 5 or 75 min, respectively; 7a/b: PEEP 2 and PEEP 6 with no IM, respectively. Data are displayed as median (interquartile range). Differences between groups were statistically not significant ($p = 0.16$, 0.10, 0.48, and 0.11 for TNF-α, MIP-2, IL-6, and total protein, respectively).

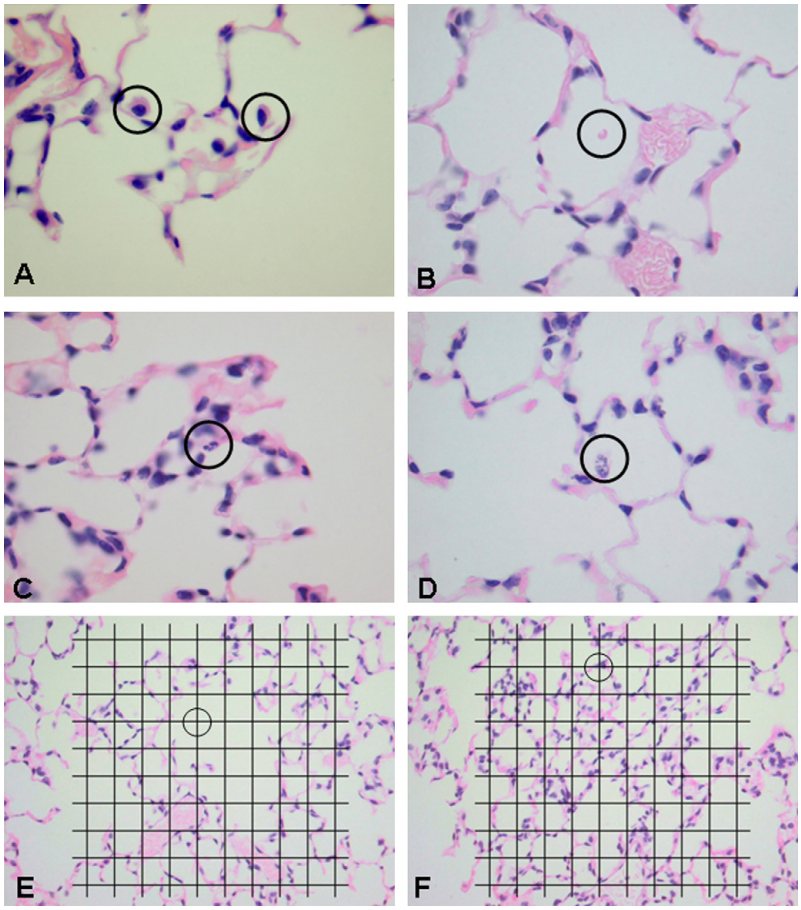

Fig. 4. Photomicrographs of representative lung features. (A) Example of alveolar macrophages, encircled (×100 oil). (B) Alveolar red cell, encircled (×100 oil). (C) Neutrophil, encircled, within alveolar septum (×100 oil). (D) Alveolar neutrophil, encircled (×100 oil). (E) Grid superimposed for counting alveolar septal intersects, with negative intersection encircled (×40). (F) Grid superimposed for counting alveolar septal intersects, with positive intersection encircled (×40).

Table 4
Lung injury and inflation scores in selected histological lung sections.

Study groups	Alveolar macrophages	Alveolar neutrophils	Septal neutrophils	Alveolar erythrocytes	Inflation score
PEEP 2, no IM	15 (3)	1.4 (1.7)	26 (9)	26 (12)	303 (22)
PEEP 2, 40 mL/kg every 5 min	14 (6)	0.6 (0.9)	37 (12)	28 (15)	310 (17)
PEEP 2, 40 mL/kg every 75 min	13 (4)	0.8 (0.8)	35 (7)	27 (21)	326 (25)
PEEP 6, no IM	14 (6)	1.0 (0.7)	37 (6)	26 (14)	324 (13)
PEEP 6, 40 mL/kg every 5 min	14 (5)	1.2 (0.8)	33 (12)	24 (23)	294 (36)
PEEP 6, 40 mL/kg every 75 min	17 (5)	0.5 (0.6)	31 (7)	22 (10)	320 (40)

Data are displayed as mean (standard deviation). Differences between groups were statistically not significant ($p = 0.92$, 0.72, 0.69, 0.99, and 0.40 for alveolar macrophages, alveolar neutrophils, septal neutrophils, alveolar erythrocytes, and inflation score, respectively). Definition of abbreviations: PEEP: positive end-expiratory pressure; IM: inflation maneuver.

insufficient to prevent or reverse increases in R_{aw}, G, and H.

In murine models, absence of IMs or application of mild IMs that do not result in an increase in peak airway pressure > 25 cmH_2O throughout mechanical ventilation, results in the gradual development of atelectasis, particularly during low V_T ventilation with low PEEP (Allen et al., 2006; Thammanomai et al., 2007). Progressive atelectasis may lead to ventilation–perfusion mismatch, ventilation inhomogeneity, shear stress, and eventually lung injury (Duggan et al., 2003; Lapinsky and Mehta, 2005). When using a volume-controlled ventilation mode, reduction in lung volume not only results in a fall in compliance and higher peak pressure levels, but also causes lung injury by overdistension of the remaining open lung units (Tsuchida et al., 2006). Benefits of RMs include prevention and re-opening of atelectasis, improvement of compliance and gas exchange, and prevention of derecruitment-associated lung injury (Koh et al., 2005). However, RMs have also been reported to be transient, ineffective, and injurious (Musch et al., 2004); the harmful effects include overdistension of open lung units, low ventilation-to-perfusion ratio, and reduced venous return via increased intrathoracic pressure.

Mechanical ventilation with low V_T and low PEEP in the present study resulted in a physiologically unimportant increase in R_{aw} (~10%) and in a significant increase in G and H (~45% and ~90%, respectively) over time. Increases in G reflect energy dissipation and are associated with a rise in lung tissue resistance and/or regional heterogeneity as a result of peripheral airway constriction (Bates and Lutchen, 2005). H reflects energy storage in lung tissues, represents lung stiffness, and correlates with lung volume (Allen et al., 2007; Cannizzaro et al., 2008). Hence, the alterations of estimates of respiratory system mechanics after low V_T–low PEEP ventilation are consistent with a progressive loss of lung volume secondary to atelectasis. This is supported by the almost linear increases in G and H reflecting gradual airway closure (Irvin and Bates, 2003). In order to prevent this progressive atelectasis we applied IMs in a volume-controlled manner without pressure limit or in a pressure-controlled mode. Volume-controlled IMs delivered a fixed V_T and resulted in peak P_{ao} determined by lung compliance. In contrast, pressure-controlled IMs ensured that a selected peak P_{ao} was not exceeded, but provided different V_T depending on respiratory mechanics.

PEEP alone was not able to prevent the progressive rise in R_{aw}, G, and H in the present study, although the rate of apparent loss of lung volume appeared to be slower when a PEEP of 6 cmH_2O was employed. This finding is in line with results from recent studies (Farias et al., 2005; Ko et al., 2008). Although the PEEP level of 6 cmH_2O was chosen arbitrarily, we anticipated that this level was likely to be adequate to prevent derecruitment in mice with healthy lungs ventilated for a short period. However, it is conceivable that higher PEEP values may have prevented loss of lung volume. Comparison of pressure-controlled RMs showed a greater decrease in H after IMs with PEEP 6 when compared with PEEP 2. However, when comparing the course of R_{aw} and H after the time point 15 min, we found similar rates of relative changes at both PEEP levels. These results suggest that during pressure-controlled RMs a PEEP of 6 cmH_2O is more effective than a PEEP of 2 cmH_2O at reversing estimates of respiratory mechanics initially, but equally inadequate to impede alterations thereafter.

The stability of H after application of frequent and large RMs indicates that full recruitment of lung units was probably achieved after the first RM. Infrequent application of large RMs had transient effects on H; however the pattern of progressive increase in H after the IM followed a similar trajectory to that seen before the IM. This finding is in line with those from other studies in animals with healthy and pre-injured lungs (Mead and Collier, 1959; Allen et al., 2002, 2006; Allen and Bates, 2004; Frank et al., 2005; Ko et al., 2008) and may reflect inadequate PEEP during low V_T ventilation.

We assessed the inflammatory response to the various ventilation patterns using counts of inflammatory cells and measurements of TNF-α, MIP-2, and IL-6 in BALF. These chemoattractants have been shown to be important mediators in the development of VILI (Frank et al., 2006) and to be elevated (IL-6) after injurious short-term ventilation in healthy mice (Allen et al., 2006). In line with results from others (Frank et al., 2005; Allen et al., 2006) application of RMs producing transient elevations in peak P_{ao} did not increase cytokine concentrations in BALF. However, it is possible that the duration of our protocol was too short to induce cytokine production. Similarly, total protein concentration did not differ between study groups, indicating that RM-induced stretch of airways and alveoli did not adversely affect epithelial–endothelial barrier function (Parker and Townsley, 2004). Also, absence or application of RMs had no impact on transcutaneous oxygen saturation; however, it should be noted that healthy lungs were ventilated over a short period of time and FIO_2 of 0.5 was delivered in order to avoid survival issues.

In the present study only large IMs resulting in peak P_{ao} above 30 cmH_2O produced overall improvements in respiratory mechanics, whereas IMs reaching peak P_{ao} values at or below 25 cmH_2O did not reverse the ventilation-induced increases in R_{aw}, G, and H. By contrast, repetitive IMs with 40 mL/kg (especially superimposed on a higher PEEP), producing peak P_{ao} values above 35 cmH_2O, provided the most significant improvement in lung function and stable respiratory system mechanics with little intra-group variability. This finding is closely linked to the development of a "secondary" pressure–volume sigmoid with lung inflation beyond 20 cmH_2O (Zosky et al., 2008), which makes total lung capacity difficult to define in mice (Soutiere and Mitzner, 2004). This bimodal pressure–volume behavior was demonstrated in some small species long time ago (Leith, 1976); however, its importance was

not recognised until recently (Soutiere and Mitzner, 2004; Zosky et al., 2008). The findings in the present study therefore support the view that the improvement of estimates of respiratory system mechanics after large RMs is due to fundamental changes in quasi-static and dynamic lung compliance, as substantiated by the inflation PV curve and the values of R_{aw}, G, and H, respectively. Though the mechanisms responsible for the increased lung compliance above P_{ao} of 25 cmH_2O are not clear, alveolar unfolding and surfactant redistribution have been proposed as possible explanations (Soutiere and Mitzner, 2004; Escolar and Escolar, 2004), while others suggest that alveolar mouths, previously closed by a surfactant-lined liquid film, open during recruitment of peripheral lung units at high transpulmonary pressures providing a new population of available alveoli (Scarpelli, 1998; Kitaoka et al., 2007; Namati et al., 2008). A structural reorganization of peripheral lung units resulting in an increased number of alveoli and homogeneous ventilation may explain why our initial hypothesis, i.e. frequent and large RMs would induce lung injury via overdistension, could not be confirmed in this study.

The following limitations of the present study have to be acknowledged. Firstly, given that there is no consensus on how to recruit non-aerated lungs we arbitrarily chose different types of RMs. It is likely that the time interval between RMs, duration of RMs, ramp to plateau ratio, and degree of PEEP and IMs result in different findings. Nonetheless, application of higher PEEP levels or longer duration of RMs (e.g. over 30 s) usually compromises survival due to decreased cardiac output. Secondly, though it is true that development of derecruitment and response to PEEP and IMs depend on the type and the degree of a pre-existing lung injury (Allen and Bates, 2004), our study aim was to investigate RMs in healthy lungs since lung function measurements are often performed in healthy mice. Lastly, since the phenomenon of a second sigmoidal inflation limb has not been reported in humans, conclusions from our study are not applicable to clinical practice.

In conclusion, infrequent application of large RMs is sufficient to reverse increases in bronchial resistance and lung elastance in mice with healthy lungs. To maximize lung volume recruitment throughout mechanical ventilation protocols using ventilation strategies of low V_T and "adequate" PEEP, repetitive IMs reaching peak P_{ao} values above 25 cmH_2O are required to provide stable respiratory mechanics. This is particularly useful in animal model studies where similar baseline conditions after standardized procedures are desirable. Furthermore, frequent application of substantial RMs resulting in peak P_{ao} beyond 35 cmH_2O provides stable respiratory mechanics without histological signs of lung injury and without elevation of TNF-α, IL-6, MIP-2, and total protein concentrations in BALF during short-term mechanical ventilation in mice. Finally, given the impact of RMs on respiratory system mechanics documented by the present study requires that the specifications of PEEP and IMs used are reported in all studies investigating the effects of mechanical ventilation in mice.

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