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Ventilation with ‘clinically-relevant’ high tidal volumes does not promote stretch-induced injury in the lungs of healthy mice

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Abstract

Objective—Ventilator-induced lung injury is a crucial determinant of the outcome of mechanically ventilated patients. Increasing numbers of mouse studies have identified numerous pathways and mediators that are modulated by ventilation, but it is conceptually difficult to reconcile these into a single paradigm. There is substantial variability in tidal volumes used in these studies, and no certainty about the pathophysiology that such varied models actually represent. This study was designed to investigate whether ventilation strategies ranging from ‘very high’ to more ‘clinically-relevant’ tidal volumes induce similar pathophysiologies in healthy mice, or represent distinct entities.

Design—In vivo study.

Setting—University research laboratory.

Subjects—C57/Bl6 mice.

Interventions—Anesthetised mice were ventilated with various tidal volumes up to 40ml/kg.

Measurements and Main Results—Respiratory system compliance and arterial blood gases were used to evaluate physiological parameters of injury. Lung wet:dry weight ratio, lavage fluid protein and cytokines were used to assess pulmonary edema and inflammation. All ventilation strategies induced changes in respiratory system compliance, although the pattern of change was unique for each strategy. 10ml/kg and 40ml/kg ventilation also induced decreases in arterial pO₂ and blood pressure. Any physiological changes induced during the 10, 20 and 30ml/kg strategies were largely reversed by recruitment maneuvers at the end of the protocol. Markers of pulmonary edema and inflammation indicated that only 40ml/kg induced substantial increases in both, consistent with development of lung injury.

Conclusions—Tidal volumes up to 20ml/kg are unlikely to induce substantial lung over-stretch in models using healthy, young mice. Signs of injury/inflammation using such models are likely to result from other factors, particularly alveolar derecruitment and atelectasis. The results of such studies may need to be re-evaluated before clinical relevance can be accurately determined.

Keywords

acute respiratory distress syndrome; cytokines; inflammation; lung compliance; pulmonary edema; tidal volume

INTRODUCTION

The exacerbation of lung injury by mechanical ventilation, known as ventilator-induced lung injury (VILI), is a crucial consideration in the treatment of critically ill patients.

Inappropriately high tidal volume (V_T) has been clearly identified as a primary contributor to mortality among patients with acute respiratory distress syndrome (ARDS) [1], and many studies have investigated the mechanisms by which high lung stretch induces inflammation and respiratory dysfunction. In particular the number of mouse studies has increased dramatically since the first publications in 2002-2003 [2, 3], taking advantage of genetically modified animals and the similarity between the mouse and human immune systems. These have identified a plethora of pathways and mediators apparently modulated by mechanical ventilation, but it remains difficult to reconcile these into a coherent paradigm. Importantly, there has been huge variability regarding experimental protocols in these mouse studies, and whether they are investigating similar or distinct phenomena remains unclear. Specifically, *in vivo* VILI models unavoidably incorporate numerous insults apart from lung stretch, including shear forces resulting from repetitive alveolar collapse and reopening, surgical stress, anesthesia and others, which are likely to impact upon the outcomes.

Regarding the modelling of lung overstretch in mice, two general schools of thought persist - use of very high V_T , and use of moderately high, more 'clinically relevant' V_T . The rationale behind very high V_T strategies (i.e. 30-40 ml/kg) is that these are likely to induce similar magnitudes of stretch in healthy mouse lungs to those which occur in the 'Baby Lung' of ARDS patients [3-13], although these protocols are frequently criticized as being too extreme to have any relevance to humans. Attempts to mimic more clinically comparable injurious V_T (i.e. 10-20ml/kg) in mice have also been widely used [14-22], with authors claiming that this is more relevant in light of the ARDSNet trial [1] which identified ~12ml/kg as being injurious (compared to ~6ml/kg). However, as mice have entirely different respiratory mechanics from humans [23, 24], these assumptions may not be physiologically valid, and such studies could be misleading.

This study therefore aimed to compare the impact of different V_T on the development of lung injury in mice. Overall, our data indicate that so-called 'clinically-relevant' V_T in mice are indeed unlikely to induce high lung stretch, and that other factors within the models such as alveolar derecruitment dominate the pathophysiology. Careful evaluation/re-evaluation of mouse-based VILI studies will be important to clarify what pathophysiological processes dominate within particular models, and thus how to properly interpret the results of such studies and their translation towards clinical situations.

MATERIALS AND METHODS

Animal preparation

All experimental protocols were approved by the Ethical Review Board of Imperial College London, and carried out under the authority of the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986. Male C57Bl/6 mice (Charles River, Margate, UK) aged 8-12 weeks were randomly allocated to receive either no treatment (non-ventilated controls) or one of the ventilation protocols as described below. Non-ventilated controls (NVC) received no surgery and were sacrificed by anesthetic overdose. Ventilated animals were anesthetised with ketamine (80 mg/kg) and xylazine (8 mg/kg), an endotracheal tube was inserted via tracheotomy, and mice were ventilated using a custom-made, volume-controlled mouse ventilator system [3, 25]. Airway flow was determined using a miniature pneumotachograph connected to a differential pressure transducer, which was calibrated at the start of every experiment. V_T was calculated by integrating airway flow during inspiration. Airway pressure was determined by a transducer connected to the

ventilator circuit. Inspiratory:expiratory ratio was kept constant at 1:2 throughout the experiment. A catheter was introduced into the left carotid artery for monitoring of arterial blood pressure (BP), blood gas analysis, and infusion of fluids (0.9% NaCl containing 10U/ml heparin at 0.3ml/hr). Anesthesia was maintained by regular intraperitoneal administrations of ketamine (40mg/kg) and xylazine (4mg/kg) every 25-30 minutes.

Protocols and physiological measurements

All animals were ventilated with a respiratory rate of 80 breaths/minute and positive-end expiratory pressure (PEEP) of 3cmH₂O throughout the procedures. Following baseline physiological measurements to ensure stability of the preparation, mice were allocated to receive one of the following strategies: V_T 40ml/kg, using air/4% CO₂; V_T 30ml/kg using air/4% CO₂; V_T 20ml/kg using air/2% CO₂; V_T 10ml/kg using air (no supplemental CO₂). Additionally, a group of animals were ventilated using this lowest V_T but with the addition of recruitment maneuvers (RM, 35cmH₂O, 5 seconds) every 30 minutes throughout. CO₂ was added to inspired gases for groups with V_T > 10ml/kg to avoid respiratory alkalosis.

Ventilation was continued for 3 hours, or until BP dropped below 50mmHg. Preliminary experiments determined that this was the maximum period over which mice ventilated with either of the 'extremes', i.e. 10ml/kg (without RM) and 40ml/kg remained hemodynamically stable. At the end of each protocol, 2 RM were carried out to assess reversibility of changes in physiological parameters. Respiratory system compliance (C_{rs}) was determined by the end-inflation occlusion technique [3, 25] at the start of the ventilatory protocol and every 15 minutes until the end. Blood gas analyses were performed at baseline, 15 and 120 minutes after the start of the protocol, and at the end of the protocol. In order to prevent hypovolemia blood sampling was limited to 70μl, and the volume replaced with saline each time.

Sample collection

Following termination, the right lung was tied off and used to determine wet:dry weight ratio. The left lung was lavaged using 400μl sterile saline (recovering ~200-300μl, depending on the presence of edema fluid within the lungs). Lavage fluid was centrifuged (5 minutes, 1500 rpm, 4°C), and supernatants stored at -80°C. Cell pellets were resuspended, counted by hemacytometer, and differential cytology carried out on Diff-Quik stained slides prepared by cytopsin. Protein concentration in lavage fluid was determined using a protein assay kit (Bio-Rad Laboratories, Hemel Hempstead, UK). Lavage fluid levels of IL-6, Keratinocyte-derived chemokine (KC) and soluble Receptor for Advanced Glycation End-products (sRAGE) were determined by ELISA (R&D Systems, Abingdon, UK).

Histology

In some animals, at the end of the protocol the heart and lungs were removed *en bloc*. Lungs were instilled with 4% paraformaldehyde via a fine cannula passed through an extended endotracheal tube, to produce a pressure of ~15cmH₂O. Lungs were paraffin embedded and processed for histological evaluation by H&E staining.

Pressure-volume curves

In separate experiments, pressure-volume curves of the respiratory system were created by ventilating anaesthetised mice with 3cmH₂O PEEP and respiratory rate 80 breaths/minute, with gradually increasing inspiratory flow. Each breath incorporated an end-inspiratory pause, and curves were created by plotting plateau pressure (P_{plat}) against delivered V_T.

Data Analysis

Data were analysed by one-way ANOVA with Tukey's post test using SPSS 19.0. A p value < 0.05 was considered significant. The model assumption of normality of residuals was assessed by QQ plot and Shapiro-Wilk test, and log-transformed where necessary. Data are presented as mean with error bars representing upper and/or lower limit of 95% confidence interval [26].

RESULTS

Cardiorespiratory variables

Arterial blood pressure was well maintained (80-100mmHg) in all groups up to 2 hours of ventilation, whereupon it fell substantially in both the 40ml/kg and 10ml/kg (without RM) groups, but not others (Fig 1A). In most groups all animals were ventilated for the full 3 hours, the exception being 40ml/kg V_T , within which animals reached the 50mmHg 'cut-off for hemodynamic stability' at different times as injury developed. The average length of ventilation in this group was 153 ± 20 minutes. Kaplan-Meier analysis (using 50mmHg blood pressure as a surrogate for death) indicated significantly worse survival with 40ml/kg compared to all other groups (Fig 1B).

Each ventilatory strategy induced an overall increase in peak inspiratory pressure (PIP), but the patterns of change were qualitatively different (Fig 1C). 10ml/kg V_T (without RM) induced a gradual, continuous increase in PIP over 3 hours. 20ml/kg and 30ml/kg V_T both induced initial increases followed by stable PIP for the remainder of the protocol. In contrast, mice ventilated with 40ml/kg showed an initial *decrease* in PIP, followed by a substantial, rapidly progressing increase. These changes were mirrored by Crs, in which 10ml/kg, 20ml/kg and 30ml/kg all induced immediate decreases of varying magnitude, whereas 40ml/kg only induced a decrease in Crs towards the end of the protocol, following an initial increase (Fig 1D). Use of RM at the end of the protocols reversed any changes in respiratory mechanics close to their initial values, in all groups except 40ml/kg. Furthermore, frequent use of RM every 30 minutes throughout the 10ml/kg protocol (10ml/kg + RM group) prevented the on-going deterioration with this V_T (Fig 1C/D).

Arterial oxygenation was well maintained over 3 hours ($pO_2 > 90$ torr (12kPa)) in most of the ventilated groups (Fig 2A), except for those animals ventilated using 10ml/kg (without RM) and 40ml/kg V_T . However, most of the deterioration of pO_2 in the 10ml/kg (without RM) group was found to be reversible by RM at the end of the protocol. pCO_2 (Fig 2B) increased between 15 to 120 minutes in all animals ventilated with V_T 20ml/kg, most noticeably those using 10ml/kg without RM, and this was again to some degree reversible by RM at the end. In contrast, animals ventilated using 30ml/kg showed no pCO_2 increase over the entire 180 minutes, and animals ventilated with 40ml/kg only showed an increase during the final 60 minutes (coinciding with the decrease in pO_2). Arterial pH was similar (range 7.34-7.39) between all of the groups at 15 minutes after the beginning of ventilation, indicating that the combinations of V_T and inspired CO_2 were appropriate to avoid respiratory alkalosis. Subsequently, pH decreased in all animals, although remained above 7.25 in all groups except animals ventilated with 10ml/kg (without RM) or 40ml/kg (Fig 2C).

Pulmonary edema/permeability

To evaluate alveolar-capillary barrier permeability and pulmonary edema, lavage fluid protein content and lung wet:dry ratios were determined in all ventilated groups, and compared with data from non-ventilated animals. In the group ventilated using 10ml/kg + RM throughout, lung wet:dry ratio was only marginally increased (~8%, non-significant ($p=0.733$)) compared to untreated NVC mice (Fig 3A). The increase in wet:dry ratio was

enhanced to a similar degree (up to 20-30%) in animals ventilated using 10ml/kg without RM ($p < 0.01$ vs. NVC), 20 ml/kg ($p < 0.001$ vs. NVC) and 30ml/kg ($p < 0.05$ vs. NVC). In contrast, wet:dry ratio was considerably greater following 40ml/kg ventilation compared to all other strategies (~70% increase vs. NVC). Lavage fluid protein showed a very similar pattern (Fig 3B), with levels in the 10ml/kg + RM group similar to NVC, a moderate increase in animals ventilated with 10-30 ml/kg V_T , but much higher with 40 ml/kg. It is important to note that wet:dry ratio and lavage fluid protein did not increase when V_T was raised from 10 to 20 to 30 ml/kg.

Pulmonary Inflammation

Consistent with previous studies [27, 28] there was minimal recruitment of neutrophils into the alveolar space within the 3 hour timeframe of this ventilation protocol (none of the V_T groups induced a neutrophil content of >6% lavage cells – data not shown). There was however clear upregulation of soluble inflammatory markers within the alveolar space. Lavage fluid KC (a functional homologue of IL-8) and IL-6 showed very similar patterns, with significant upregulation only seen following 30 and 40ml/kg ventilation (Fig 4A/B). In contrast, the pattern of sRAGE expression was somewhat different (Fig 4C), with 10ml/kg (without RM) and 20ml/kg both increasing sRAGE compared to NVC animals, while 30ml/kg did not ($p = 0.08$). Intriguingly the 10ml/kg + RM group had significantly lower levels of sRAGE than the 10ml/kg without RM animals. As with all other markers, 40ml/kg induced the most substantial upregulation.

Histology

Histological examination of the lungs indicated minimal change following ventilation using 10ml/kg (without RM), despite the substantial changes in respiratory mechanics induced by this strategy (Fig 5). In contrast, ventilation with 40ml/kg caused increased infiltration of neutrophils into the lung tissue, as well as signs of alveolar wall thickening and the presence of proteinaceous material within the alveolar space.

Pressure-volume curves

Individual pressure (P_{plat}) - volume (V_T) curves were determined for 5 mice (Fig 6A). In all animals, following an initial steep gradient in the curve there was an inflection point above which the curve started to flatten. This occurred between 13-16 cmH₂O P_{plat} , corresponding to ~24-28ml/kg V_T . Importantly however, after this point the curve did not flatten completely, indicating that the lung continues to expand even when P_{plat} was increased >60 cmH₂O (delivered V_T >50ml/kg). Furthermore, all animals subjected to this protocol began to breath spontaneously within 5 minutes of switching to continuous positive airway pressure (Fig 6B), indicating that application of such high pressures/volumes do not apparently produce life-threatening lung damage immediately in mice.

DISCUSSION

Despite substantial research into the pathophysiology of VILI our understanding remains limited. Of the various factors which may cause injury, high tidal volume is most widely accepted as contributing to mortality among patients [1, 29-31], with tidal volumes of 10-15ml/kg considered injurious in humans. In contrast, the importance of other factors, such as atelectasis promoting alveolar collapse and reopening remain much less clear. For example, recent systematic reviews and meta-analyses have been unable to conclusively show that prevention of atelectasis by increased PEEP or recruitment manoeuvres produces a significant mortality benefit [32-35]. Most animal models of 'pure' VILI are therefore generally designed to incorporate lung stretch as an injurious factor. However, it is far from certain that all animal models actually induce substantial lung stretch. Experimentally,

attempts to investigate this 'high stretch-induced lung injury' in mice have utilised V_T ranging from 12-45ml/kg, under the apparent assumption that these are merely degrees on a 'continuum of stretch'. In fact, such strategies almost certainly represent entirely different pathophysiological processes.

In the current study we found that ventilation with 10ml/kg in the absence of recruitment maneuvers induced deteriorations in respiratory system mechanics and blood pressure to such an extent that 3 hours was the maximum period over which hemodynamic stability could be assured. In addition, lung wet:dry ratio and lavage fluid protein were both moderately, but clearly greater than observed in untreated mice (~20% increase in wet:dry ratio, ~4x increase in BAL protein), which could be interpreted as indicative of stretch-induced injury. Importantly however, in the current study these changes were substantially attenuated by the inclusion of RM throughout ventilation, strongly suggesting that with this V_T the major contributor to lung 'injury' is in fact atelectasis-related, despite use of 3cmH₂O PEEP throughout. In fact, it has been shown recently that even 6cmH₂O PEEP could only slow down, but not prevent derecruitment and atelectasis during 'low' V_T ventilation in mice [36]. Moreover, neither lavage fluid protein nor lung wet:dry ratio were further increased when V_T was raised as high as 30ml/kg. If the alveolar-epithelial permeability observed at 10 or 20ml/kg were due to lung over-stretch, we should expect greater changes at 30ml/kg, which was not the case. These results provide evidence that the apparent injury (compared to untreated NVC animals) observed with V_T <30ml/kg is related to other aspects of the experiment than overstretching the lung. In addition to atelectasis, the potential influence of supine positioning, surgical stress, anesthesia and dry gas ventilation could all be important. Our findings also highlight the dangers inherent in the sole use of non-ventilated animals as controls in studies designed to investigate pathophysiological processes induced by lung stretch.

Previously, it was reported in rats that irrespective of ventilation strategy, equivalent changes in respiratory mechanics equate to equivalent degrees of lung injury [37]. In contrast, the current data demonstrate clearly that the *pattern*, rather than the degree of mechanics changes, reflects the development of alveolar-epithelial barrier dysfunction in mice. The characteristic 'late', rapid decrease in compliance in the 40ml/kg very high V_T group is likely to reflect on-going pulmonary edema development with this strategy, as confirmed by lavage fluid protein and lung wet:dry ratio, whereas early, linear changes observed in lower V_T groups would reflect derecruitment. Indeed, the changes in respiratory system mechanics at 10, 20 and 30ml/kg V_T were largely reversible by RM at the end of experiment, whereas those at 40ml/kg V_T were not. The difference between our study and the previous one in rats may reflect species differences, including fundamental physical properties of the mouse lung [24] and the extraordinarily low elastance of the mouse chest wall [38].

We found no neutrophil influx into the alveolar space within 3 hours, consistent with previous studies showing that even with inhaled lipopolysaccharide, substantial neutrophil influx does not occur until ~4 hours after challenge [39]. Leukocyte accumulation was however apparent on histological examination of lung tissue, presumably within the intravascular or interstitial space, following 40ml/kg V_T . In contrast, proinflammatory cytokines KC and IL-6 were upregulated within lavage fluid by 30ml/kg and, more substantially 40ml/kg, but not the other V_T strategies. The epithelial injury/dysfunction marker sRAGE [40] showed a somewhat different pattern, with 10ml/kg and 20ml/kg both inducing a greater level of sRAGE than was observed with 30ml/kg. We speculate that at V_T 20ml/kg the increases in sRAGE are a consequence of atelectasis-induced epithelial shear stress, rather than lung over-stretch, supported by the fact that animals ventilated with 10ml/kg + RM throughout displayed similar sRAGE levels to untreated mice. To the best of our

knowledge this is the first description of the impact of VILI on sRAGE, and the data suggest that this follows the pattern of permeability/edema markers (lavage fluid protein and wet:dry weight ratio) more closely than the pattern of inflammatory cytokine production.

In the current study, only a V_T of ~40ml/kg induced substantially greater pulmonary edema/epithelial permeability and inflammation compared to the other ventilation strategies used. With a V_T of 30ml/kg there were no substantial physiological changes over 3 hours, but BAL cytokines (KC and IL-6) showed clear increases. This suggests that at this level the V_T is sufficiently high to recruit any lung areas with a tendency to collapse at end-expiration (further supported by the low sRAGE levels in this group), and lung stretch begins to dominate the pathophysiology over atelectasis, which appears to be the primary 'injurious' factor at lower V_T . While such a V_T of 30-40 ml/kg undoubtedly looks very high, it is important to bear in mind a number of considerations. Firstly, this is reflective of the nature of mouse respiratory mechanics which, as demonstrated by the PV curves, are substantially different from that of humans. Although the method used to produce the PV curves in this study was not entirely conventional (as they were dynamically measured during tidal mechanical ventilation), this method allowed us to demonstrate that mice were able to tolerate the apparently extreme pressures/volumes achieved and return to spontaneous breathing. Moreover, the data obtained are consistent with previous studies using alternative static techniques [23]. Secondly, our data should be considered within the context of uninjured lungs. The effects of stretch with such high V_T in healthy mouse lungs are likely to be comparable to that induced regionally by lower V_T in either ventilated ARDS patients (due to heterogeneous injury and 'Baby Lung' [41]) or so-called '2-hit' models of VILI. Both of these situations involve pre-injured or pre-stimulated lungs, which would significantly enhance the sensitivity of lung tissues to stretch. It is also relevant to appreciate that any form of airway manipulation within small animals, e.g. delivery of intratracheal agents, is likely to induce sufficient alteration in respiratory mechanics as to constitute a 'hit'. Therefore, 'clinically comparable' V_T in mice may potentially be useful to produce stretch-induced injury in the case of 2-hit models [42], although as models become more complicated it is harder to dissect the contribution of stretch versus other insults.

Finally, it is worth clarifying that the 'numbers' reported here for tidal volumes should not be considered absolute. The precise V_T that causes substantial stretch to the lung will be influenced to a greater or lesser extent by mouse weight, age, strain, ventilator waveform, anesthetic regimen etc. More important is the appreciation that with in vivo mouse ventilation, V_T should not simply be considered as a continuum of stretch, i.e. with 12ml/kg overstretching lungs and 40 ml/kg overstretching them more, as our data suggest that this is not the case. In our opinion, studies on lung stretch (particularly those reporting interventions to alleviate the consequences of stretch) should demonstrate that the pattern of mechanics change is consistent with late edema formation rather than early derecruitment, and/or that outcomes are not reversible by recruitment.

Somewhat in contradiction to our findings, a number of mouse studies using 1-hit 'pure VILI' models *have* demonstrated differences between 'injurious' 15-20ml/kg and lower 'non-injurious' V_T [14, 16-22]. However, most of these studies [14, 17, 21, 22], and many others in the VILI arena, utilise not only different V_T between groups, but also different respiratory rate, to manage pCO_2 . It has been proposed [43] that higher respiratory rate during VILI may exacerbate epithelial/endothelial damage but attenuate cytokine production. Studies incorporating altered respiratory frequency may therefore introduce unknown variables into the model, greatly complicating their interpretation.

CONCLUSIONS

In summary, all of the data in our study indicate that V_T 20ml/kg is unlikely to induce excessive stretch in the lungs of otherwise healthy ventilated mice; rather, the observed changes in protein permeability, gas exchange, wet:dry ratio, and inflammatory markers appear to be primarily a result of other factors, particularly atelectrauma. This highlights an important point that ‘non-ventilated’, uninjured animals are generally not the most appropriate controls for injurious high V_T ventilation because multiple confounding factors exclude us from being able to interpret changes as due to stretch. Differences in respiratory system mechanics between species mean that the definition of ‘high V_T ’ in a model using healthy mice should not be driven by what represents a high V_T in injured human lungs. We conclude that ‘clinically relevant’ tidal volumes taken from human experience are unlikely to induce a ‘clinically relevant’ injury in healthy mice, and that mediators and pathways identified using such models may need to have their translational importance to stretch-induced lung injury re-evaluated.

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Figure 1.

A) Time course of change in arterial blood pressure during ventilation. B) Kaplan-Meier analysis, using 50mmHg as a surrogate for death, indicated that 40ml/kg V_T induced significantly higher 'mortality' than all other ventilation strategies (* $p < 0.05$ by Log-rank test). C) Change in peak inspiratory pressure, and D) change in respiratory system compliance during ventilation. Each V_T strategy induced distinct patterns of changes in peak inspiratory pressure (PIP) and compliance (Crs). 10ml/kg, 20ml/kg and 30ml/kg all induced increased PIP and decreased Crs which started immediately, but were largely reversible by recruitment maneuvers (RM) carried out at the end. The changes observed during 10ml/kg V_T were effectively prevented by RM throughout the protocol (10ml/kg + RM group). In contrast, 40ml/kg induced an initial *decrease* in PIP and *increase* in Crs (presumably a result of continuous alveolar recruitment), which only deteriorated towards the end of the protocol, and were not reversible by RM. The variability in PIP and Crs apparent towards the end of the 40ml/kg protocol was primarily due to loss of animals as they became hemodynamically unstable at different times. Data are presented as mean with error bars representing upper or lower limit of 95% confidence interval. $N = 4-6$ / group (at the start of experiments).

Figure 2.

Time course of change in arterial oxygenation (A), carbon dioxide (B) and pH (C) during ventilation. Blood gases were well maintained in most of the groups throughout 3 hours. The exceptions were 10ml/kg (without RM) and 40ml/kg, which showed substantial deteriorations. Changes in parameters were substantially reversed by recruitment maneuvers at the end of the 10ml/kg but not the 40ml/kg strategy, although it should be noted that post-RM blood gases could only be evaluated in a very small number of 40ml/kg animals due to their extremely low blood pressure at this point. Data are presented as mean with error bars representing upper or lower limit of 95% confidence interval. N=4-6 / group (at the start of experiments).

Figure 3.

Alveolar-epithelial barrier permeability assessed by lung wet:dry weight ratio (A) and lavage fluid protein (B) following ventilation. Both 10 and 20ml/kg V_T marginally increased wet:dry ratio and lavage fluid protein compared to non-ventilated control (NVC) mice. The addition of RM throughout (10ml/kg + RM) attenuated any changes induced by 10ml/kg ventilation, while 30ml/kg V_T did not lead to any further increases in markers of permeability. In contrast, 40ml/kg V_T induced substantial changes in both markers compared to all other groups. For wet:dry ratio (A), data were log-transformed for analysis and back-transformed for display. Confidence intervals are therefore not symmetrical around the mean, so data are presented as (geometric) mean with error bars representing upper and lower limit of 95% confidence interval. For lavage protein (B), data are presented as (arithmetic) mean with error bars representing upper and lower limit of 95% confidence interval. N=4-5 / group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by ANOVA with Tukey's post test.

Figure 4.

Intra-alveolar inflammation evaluated by lavage fluid levels of KC (A), IL-6 (B) and soluble RAGE (C). KC and IL-6 levels were only increased following 30 or 40ml/kg V_T compared to untreated NVC animals. In contrast, both 10ml/kg (without RM) and 20ml/kg somewhat increased sRAGE, whilst 30ml/kg did not. The addition of RM throughout the 10ml/kg protocol (10ml/kg + RM) attenuated sRAGE levels virtually back to those observed in NVC. 40ml/kg led to significantly increased sRAGE, compared to all other groups. For KC (A) and sRAGE (C), data were log-transformed for analysis and back-transformed for display. Data are presented as (geometric) mean with error bars representing upper and lower limit of 95% confidence interval. For IL-6 (B), data are presented as (arithmetic) mean with error

bars representing upper and lower limit of 95% confidence interval. N=4-5 / group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by ANOVA with Tukey's post test.

Figure 5. Histological examination indicated minimal changes between samples from untreated non-ventilated control (NVC) mice and ventilation with 10ml/kg (without RM). In contrast, ventilation with 40ml/kg promoted increased infiltration of neutrophils into the lung tissue (closed arrows), as well as alveolar wall thickening and the presence of proteinaceous material within the alveolar space (open arrows). Magnification x400.

Figure 6.

Pressure-volume curve of 5 individual mice (A). Mice were ventilated using positive-pressure ventilation with an increasing inspiratory flow. V_T was plotted against plateau pressure (P_{plat}). There was no flattening of the curves after reaching an inflection point at ~24-28ml/kg, indicating that the lungs continued to expand even up to 50-60ml/kg. Panel B shows real-time recordings of airway flow and peak inspiratory pressure against time during pressure-volume determination experiments. Within 5 minutes of switching from positive pressure ventilation to continuous positive airway pressure (CPAP), all mice began spontaneous breathing, indicating that even inflation up to V_T 60ml/kg / ~65 cmH₂O P_{plat} does not cause overt, immediate lung damage.