Choosing the frequency of deep inflation in mice: balancing recruitment against ventilator-induced lung injury

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Allen, Gilman B., Benjamin T. Suratt, Lisa Rinaldi, Joseph M. Petty, and Jason H. T. Bates. Choosing the frequency of deep inflation in mice: balancing recruitment against ventilator-induced lung injury. Am J Physiol Lung Cell Mol Physiol 291: L710–L717, 2006.—Low tidal volume (Vt) ventilation is protective against ventilator-induced lung injury but can promote development of atelectasis. Periodic deep inflation (DI) can open the lung, but if delivered too frequently may cause damage via repeated overdistention. We therefore examined the effects of varying DI frequency on lung mechanics, gas exchange, and biomarkers of injury in mice. C57BL/6 males were mechanically ventilated with positive end-expiratory pressure (PEEP) of 2 cmH2O for 2 h. One high Vt group received a DI with each breath (HV). Low Vt groups received 2 DIs after each hour of ventilation (LV) or 2 DIs every minute (LVDI). Control groups included a nonventilated surgical sham and a group receiving high Vt with zero PEEP (HVZP). Respiratory impedance was measured every 4 min, from which tissue elastance (E) and damping (G) were derived. E and G rose progressively during LV and HVZP, but returned to baseline after hourly DI during LV. During LVDI and HV, G and H remained low and gas exchange was superior to that of LV. Bronchoalveolar lavage fluid protein was elevated in HV and HVZP but was not different between LV and LVDI. Lung tissue IL-6 and IL-1β levels were elevated in HVZP and lower in LVDI compared with LV. We conclude that frequent DI can safely improve gas exchange and lung mechanics and may confer protection from biotrauma. Differences between LVDI and HV suggest that an optimal frequency range of DI exists, within which the benefits of maintaining an open lung outweigh injury incurred from overdistention.

METHODS

Male C57BL/6 mice (24–30 g) were anesthetized with intraperitoneal (IP) ketamine (90 mg/kg) and xylazine (10 mg/kg), their tracheas surgically intubated with an 18-gauge metal cannula and then connected to a flexiVent (SCIREQ, Montreal, PQ) small animal ventilator. Mice were paralyzed with pancuronium (0.8 mg/kg ip) and ventilated at 180 breaths/min with humidified room air, a Vt of 0.25 ml and PEEP of 2 cmH2O. Body temperature was continuously measured with a rectally placed probe thermometer (Physitemp Instruments, Clifton, NJ) and kept at 37°C using a circulating warm water heating pad. Mice were hydrated with IP-normal saline at the onset of the experiment (0.7 ml, 37°C) and after the first hour (0.3 ml).
To ensure adequate anesthesia, heart rate was monitored with a continuous electrocardiogram (Silicog International) tracing. An additional dose of ketamine (45 mg/kg) and xylazine (5 mg/kg) was administered every 40 min for maintenance of deep anesthesia. No mice required any additional anesthesia during the protocol to keep heart rate below 450 beats/min (46). The entire protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Vermont.

**Experimental protocol.** After a 5-min initial stabilization period, volume history was standardized with two 0.75-ml DIs, delivered over 4 s. The DIs were delivered in a quasisinusoidal, nonsustained, and nonpressure-limited fashion in order to mimic the same tidal inflations delivered during high Vt ventilation. A volume of 0.75 ml was chosen because it is the approximate volume needed to inflate the lung to total lung capacity in a normal 25-g mouse when delivered over resting residual lung volume (35). Following volume history standardization, mice were assigned to receive one of five different ventilation protocols (matched in total minute ventilation) over 2 h: 1) low Vt (0.25 ml), 180 breaths/min, 2 cmH2O PEEP, with two DIs (0.75 ml) every minute (LV; n = 8); 2) low Vt (0.25 ml), 180 breaths/min, 2 cmH2O PEEP, with two DIs (0.75 ml) every hour (HV; n = 8); 3) high Vt (0.75 ml), 60 breaths/min, 2 cmH2O PEEP (HVZP; n = 7) as a positive injurious control; when accounting for gas compression, actual delivered Vt averaged 0.20 ml in the LV and LVDI groups (8 ml/kg) and 0.61 ml in the HV and HVZP groups (25 ml/kg); 5) a sham surgical control group (Sham; n = 6) of nonmechanically ventilated mice was anesthetized with Avertin [tribromo-ethyl alcohol (Aldrich, Milwaukee, WI), for determination of arterial pH, oxygen tension (PaO2), and phragmatic left ventricular puncture, using a heparinized needle and 20 mg/ml at a dose of 400 mg/kg, underwent surgical exposure of the trachea, large airways, and vessels were dissected away; and the alveolar-arterial oxygen tension (PAO2), calculated using the alveolar gas equation as previously described (3). Immediately before the final DIs to demonstrate the effects of the LV strategy on gas exchange once G and H had reached their peak over the entire hour following the previous DIs. The alveolar-arterial oxygen gradient (A-aDO2) was calculated by subtracting PaO2 from the alveolar oxygen tension (PAO2), calculated using the alveolar gas equation as previously described (3). Immediately after thoracotomy, BAL was obtained by instilling 1.0 ml of PBS into the lungs via tracheal cannula and slowly suctioned back for a return of ~0.8 ml. The left atrium was then cut, and 5 ml of PBS were slowly perfused through the right ventricle outflow tract to Blanch the lungs of intravascular blood. Blanchled lungs were then surgically removed; the trachea, large airways, and vessels were dissected away; and the lung tissue was flash-frozen in liquid nitrogen and stored at −80°C.

**BAL fluid analysis.** Protein content was calculated using a colorimetric assay (Bio-Rad Laboratories, Hercules, CA) standardized tograded concentrations of bovine serum albumin. cDNA was synthesized from total RNA using TRIzol and then DNase treated using RNEasy columns (Qiagen), and 1.0 μg of total RNA was used as a template to synthesize the first-strand cDNA using random primers and SuperscriptIII reverse transcriptase mix, according to instructions by the manufacturer (GIBCO-BRL). Real-time semiquantitative RT-PCR was performed using the Taqman Universal PCR Master Mix and the ABI PRISM 7700 Sequence Detection System. The Assay-On-Demand primers and probes used were mouse hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1) (Assay Mm00446948_m1), mouse IL-β (Assay Mm00434228_m1), and mouse IL-6 (Assay Mm00446190_m1), all purchased from Applied Biosystems (Foster City, CA). cDNA levels were measured using the ΔΔCt method and normalized to Hprt, and the data are presented as mean expression relative to the housekeeping gene, Hprt, and then calculated as a quotient relative to a randomly chosen sham control specimen.

**Impedance data analysis.** Respiratory impedance (Zrs) was determined by measuring piston volume displacement and pressure in the ventilator cylinder while delivering 2-s oscillatory volume perturbations to the airway opening. These perturbations were composed of 13 superimposed sine waves having frequencies, ranging from 1.0 to 20.5 Hz, chosen to be mutually prime to reduce the harmonic distortion that can occur in nonlinear systems (26). Before beginning the protocol, dynamic calibration signals were obtained to correct for the physical characteristics of the ventilator and ventilator tubing in subsequent measurements of Zrs (28, 40). Zrs itself was determined via Fourier transform from the signals of ventilator piston volume and cylinder pressure as described previously (23, 28). Zrs was interpreted by being fit with the model

\[
Z_{rs} = Z_m + i2\pi f l a w + \frac{G - iH}{(2\pi f)^2}
\]

where

\[
\alpha = \frac{2}{\pi} \arctan\left(\frac{H}{G}\right)
\]

and i represents the square root of −1, and f represents frequency.

The parameters airways resistance Rn and airways inertance (Iaw) largely characterize the resistive and inertive properties, respectively, of the airways, whereas G and H characterize the dissipative and elastic properties of the lung tissues (26). In particular, the parameter H is equal to respiratory elastance at an oscillation frequency of 1/2π Hz. Hysteresis (η) is the quotient G/Iaw. Increases in η are believed to reflect changes in intrinsic tissue properties and/or increased regional heterogeneity in lung function (32, 36). We invoked the normalization scheme of Ito et al. (30) to express G and H in the same units as Rn (cm H2O s ml−1) without changing their numerical values.

All graphing and statistical analyses were performed using Origin software (version 7.5, Northampton, MA). ANOVA was used to compare values for all groups, followed by post hoc Bonferroni tests for means comparison between groups. Values for BALF protein and tissue cytokines were logarithmically transformed before performing ANOVA to reduce differences in variation between the HVZP and other groups to nonsignificant levels. Differences were considered significant when P was <0.05.
RESULTS

$Z_{rs}$ measures demonstrated a progressive rise in $H$ over each hour immediately following DIs in the LV group when two DIs were administered only once per hour (ANOVA, $P < 0.05$). By contrast, $H$ remained relatively unchanged over the entire two h in the HV and LVDI groups (Fig. 1A). Although $H$ did not significantly change over the first hour in the HVZP group, it increased significantly by the end of 2 h when compared with 0 and 60 min (ANOVA, $P < 0.05$). Values for $G$ followed a similar progressive rise following DIs in the LV group, becoming significantly higher by the end of each hour (Fig. 1B). Unlike values for $G$ and $H$, $\eta$ remained essentially unchanged throughout the protocol during LV with DIs every hour, a pattern similar to that observed in the other groups (ANOVA, $P < 0.05$), but there were no significant differences between the other groups, all of which were more alkalotic than LV by the end of the protocol (Table 1). This is likely the result of initial pilot experiments that targeted minute ventilation to normal pH and $P_{aCO_2}$ during the LV protocol. The other protocols were subsequently matched to the same minute ventilation. The resulting higher pH in the other groups was thus most likely due to a higher dead space-to-Vt ratio in the LV group, as arterial $P_{aCO_2}$ trended higher in this group compared with the other groups having identical minute ventilation (Table 1). Oxygenation and ventilation were superior after DIs every minute (LVDI) compared to the other groups (ANOVA, $P < 0.05$), but there were no significant differences between the other groups, all of which were more alkalotic than LV by the end of the protocol (Table 1).

![Graphs of impedance values](http://ajplung.physiology.org/)

**Fig. 1.** Mean impedance values ± SE bars, plotted against time with elastance ($H$) plotted in A, tissue damping ($G$) in B, hysteresivity ($G/H$) in C, and Newtonian airways resistance ($R_N$) plotted in D. Closed circles represent the LV group [low tidal volume ($V_t$), deep inflation (DI) every hour]; open circles represent the LVDI group (low $V_t$, DI every minute); open triangles represent the HV group (high $V_t$, DI every breath); and gray triangles represent the HVZP (high $V_t$, zero PEEP) group.

### Table 1. Arterial blood gas analysis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>LV</th>
<th>LVDI</th>
<th>HV</th>
<th>HVZP</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td></td>
<td>7.38±0.02*</td>
<td>7.48±0.03</td>
<td>7.48±0.02</td>
<td>7.47±0.02</td>
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<tr>
<td>$P_{aCO_2}$</td>
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<td>41.7±5.8</td>
<td>29.2±1.9</td>
<td>27.3±2.3†</td>
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<td>$P_{aO_2}$</td>
<td></td>
<td>64.3±4.0</td>
<td>91.7±5.7†</td>
<td>92.2±4.3†</td>
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<tr>
<td>$S_aO_2$</td>
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<td>89.6±2.0%</td>
<td>97.2±0.5%</td>
<td>94.0±2.0%</td>
<td>92.7±4.1%</td>
</tr>
<tr>
<td>A-A$DO_2$</td>
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<td>33.9±4.2</td>
<td>21.4±7.2</td>
<td>23.3±3.5</td>
<td>26.8±6.6</td>
</tr>
</tbody>
</table>

Mean values for pH, arterial carbon dioxide tension ($P_{aCO_2}$), arterial oxygen tension ($P_{aO_2}$), percent arterial hemoglobin saturation ($S_aO_2$), and the arterial-alveolar oxygen gradient (A-A$DO_2$) are listed ± SE for LV [low tidal volume ($V_t$), deep inflation (DI) every hour], LVDI (low $V_t$, DI every minute), HV (high $V_t$, DI every breath), and HVZP (high $V_t$, zero PEEP). $P_{aCO_2}$, $P_{aO_2}$, and A-A$DO_2$ are in mmHg. *Statistically different from all other groups (ANOVA, $P < 0.05$), and †statistically different from that of the LV group (ANOVA, $P < 0.05$).
G-CSF levels were not significantly different compared with one another (data not shown).

Differences between groups for RT-PCR-relative mRNA expression of cytokines IL-6 and IL-1β mirrored the same patterns observed for their corresponding tissue cytokine levels, but did not reach the same statistical significance (Fig. 5). Mean relative mRNA expression of IL-6 was significantly higher in the HV and HVZP groups compared with sham, but differences between HV, LV, and LVDI groups did not reach statistical significance (Fig. 5A). Although mean relative mRNA expression of IL-1β was highest in HVZP and appeared greater in LV when compared with sham and LVDI, these differences were not significant.

DISCUSSION

This study demonstrates that periodic recruitment with relatively frequent delivery of DIs (every minute) during ventilation with a low Vt can improve oxygenation, ventilation, and lung mechanical function with no evidence of lung injury by 2 h. Despite the potential for overdistention injury with such frequent DIs, there was no increase in BALF protein or inflammatory cytokine levels or mRNA expression in the LVDI group relative to the LV group by the end of the protocol. In fact, this study suggests that frequent DI during low Vt ventilation may be protective from the injury that would otherwise be incurred through a progressive increase in mean tissue stress due to atelectasis. The assertion that the lung is becoming stiffer in the LV group due to progressive collapse of lung regions (as opposed to changes in rheological properties of the parenchyma) is supported by the simultaneous rises in $G$ and $H$, immediately reversed by DI, with no corresponding rise in $\eta$. This explanation is in accordance with prior studies demonstrating a decline in end-expiratory lung volumes during low Vt ventilation (39). This account is also fully supported by our previous in vivo microscopic analysis of subpleural alveoli, demonstrating that proportionate rises in $G$ and $H$ reflect the development of atelectasis that is readily reversed (or recruited) by DI (3). Keeping the lung open with frequent DI avoids driving each tidal inflation into an effectively smaller and stiffer lung and hence limits overdistention of the remaining open lung regions (14, 42). This is the most plausible mechanism to account for the lower tissue levels of IL-6 and IL-1β in the LVDI group, both of which are “early phase” proinflammatory cytokines shown to be elaborated by ex vivo rodent lungs subjected to overinflation (27, 43) and by cultured alveolar epithelial cells subjected to repeated stretch (31). The pattern observed among
BALF G-CSF levels is less easily explained. G-CSF is a potent neutrophil regulatory cytokine expressed in both humans and mice, and it has been shown to worsen experimental ALI (8) and amplify the development of ALI during neutropenic recovery in rats (7). The role of G-CSF in VILI, however, is less well understood. In our model, the most injurious mode of ventilation, HVZP, led to a significant increase in BALF G-CSF, suggesting that this cytokine may play a role in the neutrophil infiltration seen during the pathogenesis of VILI (11). Because HVZP ventilation can theoretically result in both global overdistention at peak inflation and cyclic alveolar closure/reopening at end exhalation due to zero PEEP, we do not feel our data support making any assertion regarding the mechanism of G-CSF release in this model. However, we do feel this represents a novel and important finding regarding the release of this potent neutrophil regulatory cytokine during a known injurious ventilation strategy.

Our results thus suggest that frequent DI during LV ventilation is safe and potentially protective. On the other hand, keeping the lung open by delivering DI with every breath, as in the case of the HV group, leads to evidence of injury in the form of increased BALF protein levels, as well as elevated tissue levels of IL-6 and IL-1β. This effect is amplified in the setting of zero PEEP (HVZP) and is likely the result of the combined effects of repeated overdistention and cyclic alveolar closure and reopening. This is in accordance with previous studies that have demonstrated the ability of PEEP to prevent atelectasis and alveolar recruitment/derecruitment (22, 25) and to attenuate the injury incurred from high Vt ventilation (43, 45).

Comparing results from the HV and LVDI groups allows us to address the central postulate of our study: although DI with every breath (60/min in HV) is injurious, lower Vt ventilation with DI every minute is safe and potentially protective. This demonstrates that an optimal frequency range of DI delivery exists, at which point the potentially injurious effects of overdistention are outweighed by the protective benefits of maintaining a predominantly open lung. These findings are particularly interesting when considering that disrupted epithelial plasma membranes in overdistended rat lungs have been shown to spontaneously repair within minutes of returning to non-injurious ventilation (21). This suggests that even if periodic overinflation damages epithelial plasma membranes, provided overinflation occurs infrequently enough, repair is possible during the interim and injury does not accumulate. On the other hand, if DI is delivered with every breath, the intrinsic reparative processes of the lung may be overwhelmed, allowing injury to progress over time. Our findings are also consistent with other animal studies that demonstrate periodic recruitment with DI to be safe and potentially protective. Cakar and colleagues (10) demonstrated in rats that although continuous high-pressure ventilation over 2 h causes translocation of bacteria from the air space into the blood, delivery of periodic sustained high pressure recruitment maneuvers during low pressure ventilation does not. Frank and colleagues (19) also recently demonstrated that periodic DI in acid-injured rats yields no evidence of added epithelial injury, and in fact may confer protection to the endothelium.

DIs were delivered every minute during low Vt ventilation in mice and thus appear safe and potentially protective from...
VILI compared with DI given either with every breath or at two DIs every hour. Delivery of DIs every hour, as in the case of the LV group, although not clearly injurious in terms of BALF protein levels, leads to significantly higher stress within the lung (increased G and H) and signs of potential biotrauma to the lung tissues (Fig. 4). Conversely, delivery of DI every second, as in the HV group, leads to increased BALF protein, likely due to repeated disruption of the epithelium without sufficient time for repair. The optimal frequency range for DI in our model thus lies somewhere between once per hour and once per second. Clearly, the optimal frequency range of DI delivery might be very different in the setting of preexistent injury and is likely to vary among countless different scenarios. However, testing our hypothesis in uninjured mice is a necessary step in proof of concept that an optimal frequency range exists.

Our findings have obvious implications for the recruitment of the injured human lung during low Vt ventilation (i.e., 6 ml/kg). However, extrapolating our results to the clinical situation must be done guardedly. In the present study we employed uninjured mice, whereas it is known that the injured lung is more prone to atelectasis than a normal lung (2, 3). The optimal recruitment frequency could thus be quite different in an injury model, although previous animal studies have demonstrated that recruitment maneuvers can have near equivalent effects on markers of injury in both injured and uninjured lungs (17). Another issue is that we employed a standard lower Vt in our mice that allowed for effective ventilation at a commonly utilized respiratory rate of 180 breaths/min. It is unclear, however, how this Vt relates to the arbitrarily defined “low Vt” in humans of 6 ml/kg (6). It is also likely that PEEP may influence the optimal frequency of recruitment maneuvers since others have demonstrated that the effects of recruitment are better sustained when followed by PEEP (2, 39), and in some scenarios high PEEP may even obviate the need for frequent recruitment maneuvers (18, 34). Nevertheless there are documented scenarios in which high PEEP is insufficient to adequately recruit the lung without the delivery of periodic high-pressure DI recruitment maneuvers (20, 37). Thus understanding the effects of DI frequency in the context of a given level of PEEP is a worthy objective for this study. With respect to markers of injury, others have shown that when DI is followed by zero PEEP, markers of remodeling (i.e., procollagen III expression) are upregulated compared with when PEEP is followed by modest PEEP (17). Similarly in the present study, the injury incurred by DI delivered every second without PEEP (HVZP) was shown to be markedly attenuated by the addition of PEEP during HV (Figs. 2–5). In fact, the biggest protection benefit demonstrated in this study, in terms of BALF protein, BALF and tissue cytokine levels, and cytokine mRNA expression was bestowed upon the high Vt strategy when supplemented with moderate PEEP.

Despite all groups being matched to the same total minute ventilation, infrequent DI with a resultant rise in G and H ultimately led to less effective ventilation in the LV group. Although this is an important finding regarding the effects of DI frequency on gas exchange, it also resulted in a potentially relevant difference in PaCO2 for the LV group. Of relevance to this study, hypercapnea has been shown to influence the development of ALI and VILI (9, 13, 41). However, these studies employed significantly higher than normal physiological levels of hypercapnea. The mean value for PCO2 in the LV group in this study, although relatively higher than that of the other groups, was not above normal physiological range, and the differences between groups in this study were significantly less profound than in previous cited studies. In spite of this difference, the majority of in vivo work has shown hypercapnea to confer protection from VILI (9, 41). Thus if the difference in Pco2 in the LV group were to be of any significance, we postulate it would be more likely to have blunted any elevation in markers of injury for the LV group.

Another significant, and unavoidable, limitation of our study was the short duration of the experiment. Patients with ALI typically receive mechanical ventilation for days, whereas we studied our mice for only 2 h. Virtually all experimental models of VILI are limited in duration because of the numerous technical obstacles to keeping animals alive during prolonged injurious ventilation. This is one reason many investigators have employed ex vivo perfused lung models of VILI (27, 44). However, we wanted to investigate the effect of DI in vivo and thus chose a protocol end point to which we were confident the majority of mice would survive. Even so, the duration of our protocol did not differ greatly from that of numerous other published VILI studies (12, 43), and even though it was relatively short, we were still able to demonstrate that an optimal recruitment frequency range exists.

Although the relative expression of mRNA for IL-6 and IL-1β did not demonstrate the same statistically significant differences between groups as observed for tissue cytokine levels, they mirrored the same patterns (Figs. 4 and 5). One potential explanation is that unanticipated degradation of mRNA could have reduced the signal-to-noise ratio in the mRNA measurements relative to their more stable respective protein products. It is also important to bear in mind that quantifications of cytokine protein by ELISA and mRNA by RT-PCR are examining two separate, albeit not mutually exclusive, end points. In fact, the 2-h duration of this experiment and the differences observed between tissue antigen and mRNA levels suggest that preformed release of cytokine likely made up a significant fraction of the tissue cytokines measured. Thus, in the short term, release of IL-6 and IL-1β into the tissues appears differentially affected by DI frequency. Over a longer term, through transcriptional upregulation, HVZP ventilation is still by far the most injurious strategy, and added PEEP once more confers protection from this signal. Nevertheless, the observed mRNA expression of these cytokines in the LV and LVDI groups supports the notion that frequent DI to potentially injurious volumes is not only safe in the short term but potentially protective beyond the time frame studied in this experiment.

We conclude that frequent DI can reduce the mean tissue stress level during low Vt ventilation and optimize gas exchange with no clear evidence of injury from overdistention after 2 h. Furthermore, periodic DI may also confer protection from biotrauma by keeping the lung more open and thus limiting the mean stresses incurred within the tissue during each tidal inflation. The measured biomarkers of injury further suggest that there exists an optimal frequency range of DI delivery, within which the benefits of maintaining a predominantly open lung outweigh the potential injury incurred from periodic overinflation. In light of previously published work (21), we speculate this may be due to a threshold tolerance of
DI frequency, beyond which the intrinsic reparative properties of the lung epithelium are overwhelmed. Finding the optimal frequency range of DI, which is likely to vary with the pathological state, degree of lung injury, and level of PEEP, could be vital to the management of patients requiring mechanical ventilation for ALL.

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