Biophysical Determinants of Alveolar Epithelial Plasma Membrane
Wounding Associated with Mechanical Ventilation

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ABSTRACT

Rationale: Mechanical ventilation may cause harm by straining lungs at a time they are particularly prone to injury from deforming stress.

Objective: To define the relative contributions of alveolar overdistension and cyclic recruitment and “collapse” of unstable lung units to membrane wounding of alveolar epithelial cells.

Methods: We measured the interactive effects of tidal volume (VT), transpulmonary pressure (PtP) and of airspace liquid on the number of alveolar epithelial cells with plasma membrane wounds in ex vivo mechanically ventilated rat lungs. Plasma membrane integrity was assessed by Propidium Iodide (PI) exclusion in confocal images of subpleural alveoli.

Main Results: Cyclic inflations of normal lungs from zero end-expiratory pressure (ZEEP) to 40 cm H2O produced VT’s of 56.9±3.1cc/kg and were associated with 0.12±0.12 PI positive cells per alveolus. A preceding tracheal instillation of normal saline (3ml) reduced VT to 49.1±6cc/kg, but was associated with a significantly greater number of wounded alveolar epithelial cells (0.52±0.16 cells per alveolus; p<0.01). Mechanical ventilation of completely saline filled lungs with saline (VT=52cc/kg) to pressures between 10 and 15 cm H2O was associated with the least number of wounded epithelial cells (0.02±0.02 cells per alveolus; p<0.01). In mechanically ventilated, partially saline filled lungs the number of wounded cells increased substantially with VT, but once VT was accounted for, wounding was independent of maximal PtP.

Conclusions: Interfacial stress associated with the generation and destruction of liquid bridges in airspaces is the primary biophysical cell injury mechanism in mechanically ventilated lungs.

Key words: Lung Mechanics, Mechanical Ventilation, Injury, Epithelial Wounding

Running Title: Determinants of Plasma Membrane Wounding

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INTRODUCTION

Plasma membrane (PM) wounding of alveolus resident cells contributes to the pathogenesis of acute lung injury (ALI) and ventilator induced lung injury (VILI)(48). While the presence of cytopathologic changes in epithelial and endothelial cells, which decorate the blood-gas barrier, has been appreciated for some time (10, 26), it should be noted that the majority of wounded alveolus resident cells repair and survive deformation induced insults(17). This is important insofar as wounded and repaired cells activate stress response genes (23), release pro-inflammatory mediators and may thereby contribute to injurious deformation responses commonly referred to as biotrauma (44). Although most students of the topic VILI agree that alveolar overdistension and cyclic recruitment and "collapse" of unstable lung units are the most prevalent biophysical lung injury mechanisms(34, 38, 43), there is less certainty how stresses associated with either mechanism are transduced to generate a specific biologic response.

Viewed through the lens of cellular plasma membrane stress failure, alveolar overdistension is generally thought to be associated with matrix strains that effect lytic tensions at cell-cell and cell-matrix contacts. In contrast, "recruitment and collapse" predispose airway and alveolar lining cells to injurious interfacial stresses as liquid bridges are continually formed, displaced and destroyed in the small airways and air spaces of edematous lungs. Experimental models of overdistension injury mechanisms typically employ cell monolayers that are stretched in liquid culture(41, 46), while the injury associated with "recruitment and collapse" is typically mimicked by advancing gas bubbles or by destroying liquid bridges in cell-coated microchannels(6, 25).

While cytopathologic changes in ventilator injured lungs are widespread and involve both epithelial and endothelial lesions consisting of the loss of basement membrane anchorage, as well as loss of cell-cell contact, plasma membrane blebbing and frank basement membrane fracture (10, 16), the short time scale at which injured cells remodel and repair introduces uncertainty about the specific nature of the stresses that cause particular lesions(21).
Motivated by the hypothesis that the maintenance and/or restoration of alveolar epithelial cell integrity may improve outcomes in ARDS, we had identified a variety of candidate compounds and tested their mechanisms of action (5, 21, 36, 39, 50). In doing so, we learned that hypertonic media protected alveolar epithelia from injury by interfacial stress and were able to attribute the cytoprotective effect to cell stiffening combined with an increase in the adhesive force between cytoskeleton and the plasma membrane (36). However, the very same mechanism, i.e. cell stiffening, has been shown to promote tight junction stress failure and monolayer disruptions in cultured endothelia and epithelia subjected to stretch (40)(28). Stretch induced epithelial monolayer disruptions in liquid culture are also associated with plasma membrane wounds and have served as model systems of alveolar epithelial injury by overdistension (46). Therefore, experiments were carried out to define the relative contributions of tensile stress (associated with stretch and alveolar overdistension) and interfacial stress (associated with alveolar liquid and surfactant disruption) as principal causes of alveolar epithelial wounding in ex vivo mechanically ventilated rat lungs. To this end, we measured the interactive effects of tidal volume (VT), transpulmonary pressure (PTP), respiratory rate and the biophysical properties of airspace liquid on the number of alveolar epithelial cells with plasma membrane wounds. Our results confirm the importance of surface forces in the genesis of epithelial injury, while revealing a remarkable tolerance of alveolar epithelial cells to alveolar distension.

METHODS

Experimental Preparation

As approved by the Institutional Animal Care and Use Committee of the Mayo Clinic, female Sprague Dawley rats (200-250 g) were euthanized with an overdose of pentobarbital, their trachea intubated, and the lungs harvested through a sternal midline incision. Rats destined for
total liquid ventilation (TLV) experiments were anesthetized with an intraperitoneal injection of Ketamine (90 mg/kg) and Xylazine (10 mg/kg), intubated and mechanically ventilated with 100% O2 at a tidal volume (VT) of 6 ml/kg body weight and a Positive-End-Expired-Pressure (PEEP) setting of 3 cm H2O. Five minutes later the airway was occluded at end-expiration to promote lung collapse by gas absorption. Over the ensuing minutes during which the animals usually died, 8 ml of saline were gently instilled into the trachea. Thereafter, the thorax was opened widely, the lungs harvested, and inflated with additional saline while residual gas and foam were aspirated.

**Ventilation Protocols**

**Dry Lung Ventilation (DLV):** Eight normal rat lungs were mechanically ventilated for 20 minutes with room air between PTP’s of zero (ZEEP) and 40 cm H2O at a rate of 40 cycles/min. (Scirec Corp., Montreal, Canada). An additional six lungs were mechanically ventilated to pressures of 40 cm H2O while Positive End-Expiratory Pressure (PEEP) was set to 3 cm H2O.

**Wet Lung Ventilation (WLV):** A total of 54 rat lungs, in which the airspaces had been flooded at the outset with 3cc (~11-13 cc/kg) of liquid were mechanically ventilated with room air in a bi-level pressure preset mode at settings detailed in Table 1. The initial liquid instillate consisted of either normal saline (NS) or a perfluorocarbon solution (PFC) with low surface tension. Cells with plasma membrane defects were identified by Propidium Iodide (PI) exclusion with label applied either from the outset as part of the initial NS instillate or post hoc, i.e. after cessation of mechanical ventilation.(17)

**Total Liquid Ventilation (TLV):** Eighteen completely saline filled lungs were suspended from the trachea in a custom designed bioreactor and were “liquid-ventilated” for up to one hour with a PI containing saline solution (Applied Motion Products, Inc. Watsonville, CA). Peak PTP and VT were set to 15 cm H2O and 12 cc, respectively, whereby delivered volume was adjusted throughout to meet these targets. Using negative airway pressure during the deflation cycle as
indicator, expiratory flow (time) was adjusted to minimize/avoid expiratory flow limitation. This constrained the frequency and duty cycle to 1.0/min and 0.1, respectively.

**Cell Injury Assessment [Figure 1]**

Alveolar epithelial cells with plasma membrane lesions were labeled by tracheal insufflation of a Fluorescein Dextran (FDx) and Propidium Iodide (PI) containing solution at the end of each experimental run (4, 17). In the presence of a plasma membrane defect PI gains access to the cytosol, intercalates with DNA and in this form can be excited to emit red light. Twelve anatomically distinct lung regions were imaged using a Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss; Thornwood, NY) at a depth of up to 20 μm. Two channel images were collected as follows: autofluorescence, excitation \( \lambda = 405 \) nm, image collected at \( \lambda \) between 420-480 nm; PI, excitation \( \lambda = 543 \) nm, image collected at \( \lambda > 560 \) nm. Images were digitized at an eight-bit resolution and were stored in arrays of 512 x 512 pixels. Green light emission (on account of FDx) assured that PI containing fluid had gained access to the regions of interest. The extent of plasma membrane injury was evaluated in a blinded fashion by two independent observers and expressed as a ratio of the number of injured (PI-positive) cells per total number of alveoli in the 12 image fields (cell injury index, CI).

In some WLV experiments and in all TLV experiments the solution used to initially flood the lungs was also supplemented with PI (1 μg/ml). PI exposure during injurious ventilation labels not only mortally wounded (i.e. necrotic or apoptotic) cells, but also cells with transient (successfully repaired) membrane lesions. In contrast, post hoc labeling identifies only cells with permanent membrane defects, i.e. cells which had failed to repair the plasma membrane wound.

**Absolute Lung Volume at ZEEP**
Lung volume at ZEEP was measured by water immersion/displacement in two groups of 5 partially liquid filled lungs (WLV) after ex vivo mechanical ventilation with VT's of 10 and 30 cc/kg body weight, respectively.

**Pressure/Volume curves of completely saline filled lungs**

The saline filled lungs were suspended from the trachea and liquid was allowed to drain passively from the airways until the liquid pressure at mid lung level reached zero. Subsequently alveolar liquid pressure (Freescale #MPXV7002DP) and lung weight-change were measured during three successive stepwise saline inflations and deflations. To this end, a second pressure transducer (Freescale #MPXV7002DP) was fitted with a custom highly compliant gas tight diaphragm to allow it to function as a force transducer. A cantilever resting on ultralow friction knife edge pivot points held the lung at one end, while the opposing end was held captive by the force transducer. A/D conversion and acquisition was made via National Instruments USB-6215 running in house developed Labview software. Data from the first of 3 inflation and deflation cycles were discarded.
RESULTS

Effect of Interfacial Stress on Epithelial Injury associated with Mechanical Hyperventilation [Figure 2]

Cyclic inflations of normal “dry” lungs with room air from ZEEP to a target PTP of 40 cm H2O required a VT of 12.4 ± 0.1 cc (56.9 ± 3.1 cc/kg). There was no significant change in VT and by inference in lung mechanics over the course of a 20 minute long stress exposure. In contrast to DLV, inflation of partially saline filled lungs (WLV) to comparable peak pressures required a VT of only 9.6 ± 1.1 (44.3 ± 5.0 cc/kg; p < 0.01). Moreover, in each instance VT increased to an average of 10.7 ± 1.5 cc (49.1 ± 6.0 cc/kg) during the 20 minute long stress exposure, indicating atemporal rise in the lungs’ dynamic compliance. Imaging of subpleural regions of air ventilated "dry" lungs identified 0.12 ± 0.12 PI positive cells per alveolus. The application of 3 cm H2O PEEP and associated reduction in VT to 10 ± 0.7 cc (43.1 ± 2.8 cc/kg) was associated with fewer PI positive cells/alveolus (0.06 ± 0.03; p > 0.2), but this difference did not reach statistical significance. In contrast to DLV, partially saline filled lungs, which had been cyclically inflated to the same peak PTP of 40 cm H2O had significantly more PI positive alveolus resident cells (cell injury index = 0.52 ± 0.16; p < 0.01) when assessed by post hoc PI labeling [Figure 2]. In WLV runs, in which cells had been exposed to PI containing liquid throughout, the cell injury estimate was even greater (0.75 ± 0.27, p < 0.01). This difference was expected given the capacity of wounded alveolar epithelial cells to repair plasma membrane lesions within tens of seconds, thus regaining the capacity to exclude PI (17, 21). Compared to saline, the tracheal instillation of a liquid with lower surface tension, i.e. PFC (3 cc or 12.9 ± 1.0 cc/kg), was associated with significantly fewer PI positive cells/alveolus (CII = 0.52 ± 0.16 vs. 0.20 ± 0.07; p < 0.01)

The elimination of surface tension in completely saline filled and saline ventilated lungs of groups TLV, was associated with little demonstrable epithelial plasma membrane injury. There was no significant difference in cell injury estimates between lungs that were liquid ventilated
with tidal volumes between 47 and 55 cc/kg for 20 minutes and those ventilated for 60 minutes (CII=0.03±0.03 vs. 0.02±0.02; p=0.67). End-inspiratory lung volumes were consistently greater than the volume at which quasi-static pressure volume curves of completely saline filled rat lungs departed from linear inflation characteristics, i.e. inflation pressures exceeded the curves' upper point of maximum curvature [data not shown]. Six lungs, which had been repeatedly inflated to liquid pressures above 20 cm H2O showed evidence of cell injury (cell injury index=0.34±0.31), typically in the context of profound fluid leaks (data not shown).

Because TLV imposes limits on the rate of lung emptying, TLV associated cell injury estimates were compared to those obtained from a group of partially (3cc) saline filled lungs, which had been ventilated with air at identical frequency (1/min) and duty cycle (0.1) settings. There was a nearly tenfold difference in the number of cells with plasma membrane wounds, which favored TLV (cell injury index = 0.02±0.02 vs. 0.18±0.07; p<0.01).

**Effects of Tidal Volume on Alveolar Epithelial Injury in Flooded Lungs.**[Figure 3]

Cyclic inflations of partially liquid filled lungs from ZEEP to end-inspiratory airway and thus apparent PTP's of 40, 30 and 20 cm H2O generated VT's of 10.0±1.3, 6.6±0.7 and 2.3±0.5 cc's, i.e. 45.2±3.5, 29.0±4.2, and 10.1±2.3 cc/kg, respectively. Only during runs with high VT's and peak pressures did we observe a minor increase in dynamic lung compliance with time. There was a strong correlation between VT and the extent of alveolar epithelial cell injury [Figure 3]. Average cell injury index values increased with VT from 0.09±0.04 to 0.27±0.12 and 0.75±0.27, respectively. Small VT's were less injurious than large ones even at settings that resulted in the same maximal PTP. To demonstrate this, in a group of 8 partially saline filled lungs the increase in PEEP to 24 cm H2O and the concomitant reduction in VT from 12 cc to 2.0 cc(55 to 9 cc/kg) was associated with the fewest number of injured alveolus resident cells (0.02±0.01 PI positive cells per alveolus). In saline flooded lungs the choice of VT had a significant effect on the
absolute lung volume at ZEEP. Mechanical ventilation of “wet” lungs with a VT of 10 cc/kg body weight was associated with a ZEEP volume of 1.01±0.1 cc/g tissue, increasing to 1.26±0.1 cc/g (p<0.01) when ventilated with 30 cc/kg. This observation suggests that the agitation of airspace liquid with large VT’s promotes gas trapping and the formation of foam in airspaces and conducting small airways.

DISCUSSION

Our study underscores the deleterious effects of mechanical hyperventilation on epithelial plasma membrane integrity in lungs with alveolar liquid. While at first glance this observation may hardly seem surprising, the remarkable tolerance of “dry lungs”, i.e. lungs without alveolar liquid, to repeated inflations of similar if not greater magnitude warrants discussion. Our study was motivated by the belief that epithelial plasma membrane wounding contributes to the innate immune and remodeling response of an injured lung and therefore represents a promising target for therapeutic interventions (48). Having identified and tested a number of cytoprotective compounds, it has become clear that achieving the desired effect depends on the specific nature of the injurious stress (28, 36). Although terms like overdistension, hyperinflation, opening/collapse, recruitment/derecruitment, interdependence and tissue shear are commonly used to describe events on the scale of parenchymal networks, the stresses and deformations experienced by individual alveolar epithelial cells are by and large tensile and interfacial in nature. They are tensile insofar as lung inflations to volumes near Total Lung Capacity (TLC) unfold and ultimately stretch the matrix to which alveolar epithelial and endothelial cells adhere (20, 45). Such deformations have the potential of generating shear stresses between focal adhesions and matrix as well as normal stresses at tight inter-epithelial junctions. When similar deformations of sufficient magnitude are examined in cell culture, tight junction failure, loss of cell-matrix adherence and plasma membrane wounds are often observed (41, 46, 49).
Interestingly, we detect relatively little alveolar epithelial injury in intact unperfused “dry” normal lungs, in spite of having imposed huge deformations. This raises questions about the relevance of lung overdistension and tensile stress as prevalent alveolar epithelial injury mechanism and is in stark contrast to the injurious potential of interfacial stress. Before addressing the mechanisms of interfacial stress injury it should be stated that our research focused on only one specific manifestation of injury, namely, the wounding of epithelial plasma membranes. It neither addresses nor discounts other manifestations or mechanisms of ventilator associated lung injury.

The term collapse is often used in the ARDS literature to describe an airless region of the lung (24). When such a region is recruited, i.e. “forced open or reaerated”, cells that line airways and air spaces are exposed to interfacial surface pressure gradients, which may be large enough to wound plasma membranes. Guided by Gaver’s and Charras’ computational models, Oeckler et al proposed that the responsible cell injury mechanism involves a compressive stress gradient and apical cell deformation, which causes a phase separation between the solid actin network and the incompressible cytosol, the formation of a membrane bleb and finally its rupture (7, 19, 36). While the tissue distortion associated with alveolar collapse causes a shear deformation of the interdependent parenchymal network (33), the injurious interfacial stress is oriented normal and not tangential to the apical cell surfaces (19). As predicted by Laplace’s law and confirmed experimentally, this stress varies with surface tension and the geometry of the air/liquid interface. In contrast, apical shear stresses play little to no role as wounding mechanism of small airway and alveolar epithelia (6, 36). Consistent with these predictions and previous observations in ex vivo models, we show that an alveolar liquid with high surface tensions such as normal saline produces significantly more cell injury than one with a lower surface tension such as PFC (Figure 2). When air/liquid interfaces and thus surface tension were eliminated altogether by TLV, the epithelial integrity was more or less
preserved even though the lungs were repeatedly inflated to near maximal volumes for up to one hour. The cytoprotective effect of TLV cannot be attributed to the very low rates of lung inflation, because partially saline filled lungs, when ventilated at same low frequency settings had substantially more injury. In contrast to TLV, in spite of its low surface tension partial liquid ventilation (PLV) of lungs instilled with PFC was associated with moderate interfacial cell injury. Interestingly, a human trial of PLV in patients with ARDS suggested harm of this support modality. (27)

Although cell injury and cell death have long been recognized as integral features of the VILI phenotype, their weight in the histological grading of lung deformation injury is marginal at best (31). It is therefore necessary to interpret our findings in the context of experimental literature, in which cell injury mechanisms were inferred from surrogates, such as hemorrhage, edema and the accumulation of inflammatory cells. In doing so we are struck by the remarkable tolerance of the alveolar epithelial lining of non-edematous, non-perfused lungs to large tidal inflations, which amount to vital capacity maneuvers. This is in clear contrast to the epithelial and endothelial barrier lesions, which can be readily seen following high tidal ventilation in living experimental animals (12, 22, 51). While many investigators of the topic VILI have attributed the cytopathologic changes of the blood gas barrier to “overstretch” (13, 46, 48), the observations of the present study raise the possibility that injurious stresses associated with alveolar distension are in fact interfacial in nature as well.

Lung inflation can produce a protein permeable alveolar epithelium at times allowing alveolar flooding at normal vascular pressures (15). Only at very high lung volumes and vascular pressures does the capillary hoop stress exceed lytic levels, i.e. lead to basement membrane fracture (16, 53). As long as the basement membrane remains intact it is not clear that the epithelial lining itself undergoes stress failure at cell-cell or cell-matrix junctions (8, 14). Morphologic studies in systemic capillaries exposed to high vascular pressures have
demonstrated reversible endothelial openings, providing avenues for transcellular as well as paracellular fluid flux (35). However, such changes are probably manifestations of active remodeling as opposed to repair responses to structural failure. Therefore, we consider plasma membrane stress failure to be a consequence of alveolar flooding and not the primary cause of impaired epithelial barrier properties. The latter is thought to reflect Rho-pathway dependent mechano-sensitive cytoskeletal remodeling events (9) and is associated with changes in the claudin tight junction protein expression profiles (54).

The importance of small tidal volumes in lung protective mechanical ventilation is supported by compelling physiologic mechanisms and by incontrovertible clinical evidence (1). Given the reduced vital capacity of injured lungs (18), the imposition of large tidal volumes risks damaging the lungs by both overdistension as well as underrecruitment (“opening and collapse”). In addition, large parenchymal deformations (strains) will over time impair surfactant kinetics and function, irrespective of the absolute lung volume range over which the lungs are being “stretched” (22, 30, 47). The results of our studies in an experimental model with a narrowly focused injury endpoint, namely epithelial plasma membrane wounding, demonstrate a strain (i.e. VT) dependence of interfacial cell injury mechanisms, in agreement with a recent report in mechanically ventilated anesthetized rats (37). The effect cannot be attributed to peak parenchymal stress (PTP MAX), because neither conventional mechanical ventilation of “dry” normal lungs, nor total liquid ventilation to comparable maximal lung volumes was associated with significant cell damage. We therefore reason that large tidal oscillations promote the generation and destruction of foam in airways and airspaces, thereby injuring the epithelial lining during liquid bridge rupture (25). To test this hypothesis we compared the volumes of the lung at ZEEP in partially liquid filled lungs following 20 minutes of ex vivo mechanical ventilation with tidal volumes of 10 ml/kg and 30 ml/kg body weight, respectively. The reason why lung volume at ZEEP (i.e. zero apparent PTP) is influenced by the amount of foam in airspaces is
because foam formation promotes gas trapping. Consistent with proposed mechanism, the end-expiratory (ZEEP) volume of lungs containing alveolar liquid was 25% greater following mechanical ventilation with 30 ml/kg than it was following mechanical ventilation with a VT of 10 ml/kg.

The application of high levels of PEEP to partially liquid filled lungs prevented epithelial wounding even when lungs were inflated to very large end-inspiratory volumes and pressures. This finding is in line with seminal observations on PEEP related lung protection in mechanically ventilated anesthetized rats (11, 51) and provides the rationale for the so-called “open lung mechanical ventilation” strategy (3). While our observations with respect to PEEP mediated lung protection may be viewed as merely confirmatory, they do offer heretofore underappreciated mechanistic insights. The many degrees of freedom in ventilator settings pose a challenge insofar as PEEP mediated lung protection could be attributed to an increase in mean lung volumes or to the concomitant reduction in VT. We suggest that both variables contribute, insofar as both mediate reductions in interfacial stress by distinct mechanisms. An increase in end-expiratory lung volume reduces the probability of liquid bridge formation by virtue of increasing the dimensions of small airways and alveolar ducts. Moreover, an increase in mean lung volume promotes the translocation of alveolar edema to the interstitial compartment across a “leaky” epithelial barrier by interdependence mechanisms (29). Reductions of VT, in turn, not only preserve surfactant function (30, 47), but also, as already pointed out, limit foam formation in edema fluid.

Conclusions derived from reduced experimental models such as ex vivo ventilated unperfused rat lungs, warrant scrutiny. As already mentioned the probability of pulmonary capillary stress failure and by inference stress failure of the cells that decorate the capillary basement membrane depends on both lung volume and vascular pressure (16). It is therefore conceivable that in the intact organism epithelial stresses and microstrains could be greater
than the ones we have examined in vitro. However, we consider this possibility remote, because in the absence of severe pulmonary venous hypertension, intra-alveolar capillaries are likely to collapse at alveolar pressures of 40 cm H2O and operate under zone 1 perfusion conditions(52). We can only speculate if associated cyclic “opening and closure” of intra-alveolar capillaries stresses and/or wounds endothelial cells. While in VILI models pulmonary capillary endothelial cells account for up to 50% of alveolus-resident cells with structural lesions (16), the airspace labeling method we used in this study does not assure PI access to endothelial cells. For that reason we can also not comment on putative effects of alveolar epithelial wounding on structure or function of adjacent endothelial cells. Finally, our conclusions are based on observations from subpleural alveoli, the deformations of which could theoretically differ from those of more centrally located lung units.

We have shown that normal “dry” lungs suffer only minor epithelial injury when exposed to high tidal ventilation. Since ex vivo mechanical ventilation from ZEEP is associated with cyclic alveolar collapse generating shear stresses between wetted epithelial surfaces during subsequent recruitment, we attribute epithelial wounding in “dry lungs” (group DLV) to interfacial stresses as well. The added cytoprotection observed during total liquid ventilation (group TLV) supports this conclusion. To the extent to which epithelial injury is a significant transducer of biotrauma (44), our findings draw attention to the importance of alveolar edema in the pathogenesis of VILI. While they do not discount parenchymal remodeling and the release of matrix metalloproteases as a consequence of mechanical ventilation with high volumes and pressures, they emphasize interfacial injury mechanisms over stress failure at cell-cell and cell-matrix junctions. By inference they support PEEP management algorithms directed at reestablishing positive end-expiratory PTP(42), suggest that surface tension and fluidity of the alveolar space filling material (e.g. edema fluid vs. hyaline membranes) are critical determinants of mechanical ventilation associated epithelial injury (32) and are consistent with the idea that
ventilation-induced surfactant dysfunction and atelectasis are causal mechanisms in the pathogenesis of the acute respiratory distress syndrome (2).

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### Table 1

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<tr>
<th>Group</th>
<th>n</th>
<th>Fluid Type</th>
<th>Pressure (cm H₂O)</th>
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<th>TI (sec)</th>
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**Abbreviations used in Table 1:**
- **WLV** = Wet Lung Ventilation; **n** = number of observations;
- **NS** = Normal Saline; **PFC** = Perfluorocarbon; **Rate** refers to respiratory frequency; **Duration** refers to the length of the experimental exposure; **TI** = Inspiratory Time, **PI** = Propidium Iodide; **Timing of PI label application** identifies groups in which the initial tracheal instillate did (from the outset) or did not (post hoc) contained PI.
FIGURE LEGENDS

FIGURE 1.: Representative optical section of an injured rat lung labeled by airspace instillation of a solution containing Propidium Iodide (PI) and green fluorescent Dextran. The blue auto-fluorescence arises from the connective tissue of the lung parenchyma. The nuclei of alveolar epithelial cells with plasma membrane defects can be identified by their red nuclear fluorescence.

FIGURE 2.: A comparison of hyperventilation induced epithelial injury estimates between “dry” lungs (DLV), “wet” lungs, i.e. partially liquid flooded lungs (WLV), and completely saline filled lungs subjected to Total Liquid Ventilation (TLV) is shown. The two DLV groups (open symbols) differ with respect to PEEP and tidal volume settings. Open circles represent data from lungs ventilated between ZEEP and 40 cm H2O pressure. The open squares represent data from lungs ventilated between 3 and 40 cm H2O pressure. The two WLV groups differ with respect to the biophysical properties of the alveolar liquid. Closed circles represent data from partially saline filled lungs. Closed squares represent data from partially PFC filled lungs. The two TLV groups differ with respect to the duration of mechanical ventilation. Crosses represent data from lungs that were ventilated for 20 minutes. Bars represent data from lungs that were ventilated for 60 minutes. Cell Injury Index refers to the number of wounded epithelial cells per alveolus in post hoc labeled lungs. Individual as well as group mean values and their standard deviations are shown. Dashed and solid lines identify the groups to which statistical comparisons of data means apply. NS stands for not significant at a p value <0.05.

FIGURE 3.: The effect of tidal volume on epithelial injury estimates in 4 groups of 8 “wet” lungs, i.e. saline flooded lungs (WLV), is shown. The numbers associated with each group refer to the airway pressure limits (equal to apparent transpulmonary pressure) between which the lungs
were “cycled”. Cell Injury Index refers to the number of wounded epithelial cells per alveolus in lungs, in which the initial saline instillate contained Propidium Iodide (PI).
FIGURE 2

Cell Injury Index

DLV  WLV  TLV

p < 0.001  p < 0.001  p < 0.001

p < 0.01

NS
FIGURE 3

Cell Injury Index vs. Tidal Volume (cc/kg) for different conditions:
- WLV0-40
- WLV0-30
- WLV0-20
- WLV24-40