

**Plasma membrane disruptions with different modes of injurious
mechanical ventilation in normal rat lungs**

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Abstract

Objectives: Plasma membrane disruptions (PMD) are caused by excessive mechanical stress and thought to be involved in inflammatory mediator upregulation. Presently, PMD formation has been studied only during mechanical ventilation (MV) with large tidal volumes (V_T), and limitedly to subpleural alveoli. No information is available concerning the distribution of PMD within the lung or the development of PMD during another modality of injurious MV, i.e. MV with eupneic V_T ($7 \text{ ml}\cdot\text{kg}^{-1}$) at low end-expiratory lung volume (EELV). The aim of this study is to assess whether a) MV with eupneic V_T at low EELV causes PMD; and b) the distribution of PMD differs from that of MV with large V_T at normal EELV.

Design: Experimental animal model.

Subjects: Sprague Dawley rats.

Interventions: PMD have been detected as red spots in gelatin included slices of rat lungs stained with ethidium homodimer-1 shortly after anesthesia (control), after prolonged MV with eupneic V_T at low EELV followed or not by the restoration of physiological EELV, and after prolonged MV with large tidal volumes and normal EELV.

Measurements and Main Results: PMD increased during MV at low EELV, mainly at bronchiolar level. Resealing of most PMD occurred on restoration of normal EELV. MV with large V_T caused the appearance of PMD both bronchiolar and parenchymal, the latter to a much greater extent than with MV at low EELV. The increase of PMD correlated with the concomitant increase of airway resistance with both modes of MV.

Conclusions: Amount and distribution of PMD between small airways and lung parenchyma depends on the type of injurious MV. This could be relevant to the release of inflammatory mediators.

Introduction

Ventilator-induced lung injury (VILI) is an important contributor to morbidity and mortality in acute lung injury and acute respiratory distress syndrome (1), and can be induced even in normal lungs, provided large tidal volumes (VT) are used (2). Indeed, the main mechanism which is thought to explain VILI is “volutrauma”, basically the consequence of an excessive strain of lung parenchyma resulting in damage of the alveolar-capillary barrier and edema formation (2). Among the ultrastructural defects revealed by electron microscopy in animal models of volutrauma (3), plasma membrane disruption, i.e. cell membrane wounding due to excessive strain, has received particular attention (4). Instead of using electron microscopy, a technique limited by finite sampling and thus unsuited to quantify the overall damage, Gajic (5) and Doerr (6) assessed plasma membrane disruption by perfusing isolated lungs with propidium iodide, a substance which enters the cells only in presence of membrane discontinuities, binds to nucleic acids, and undergoes red fluorescence enhancement. Using this technique, these investigators demonstrated that mechanical ventilation with large VT leads to an increase of plasma membrane disruption in subpleural alveoli (5, 6).

In addition of being an indicator of excessive strain due to mechanical ventilation, plasma membrane disruption could contribute to the upregulation of inflammatory mediators, leading eventually to “biotrauma” (1, 4). Indeed, plasma membrane disruption can induce the synthesis of Fos (7), a transcription factor whose mRNA is increased by injurious ventilatory strategies in isolated rat lungs (8), and nuclear translocation of NF- κ B (7), a transcription factor essential for the expression of several inflammatory cytokines (9).

Apart from mechanical ventilation with large VT, lung injury can be produced by mechanical ventilation at low-expiratory lung volumes (EELV) in spite of eupneic VT, i.e. that of room air breathing at rest. Indeed, this mode of mechanical ventilation causes epithelial damage of the small airways and rupture of alveolar-bronchiolar attachments in

normal rabbits (10) and rats (11), alterations attributed to abnormal stresses related to cyclic opening and closing of the small airways. Although theoretically plasma membrane disruption could occur during low EELV ventilation, this has not been demonstrated *in vivo*.

The aims of the present study are to assess in the normal animal whether *a)* plasma membrane disruption of bronchiolar and/or parenchymal structures occur during mechanical ventilation at low EELV with eupneic VT; and *b)* amount, distribution, and reversibility of plasma membrane disruption differ from those caused by volutrauma. For these purposes, however, the technique described by Gajic (5) is unsuitable, because *a)* it requires the isolation of the lungs, thus preventing an *in vivo* investigation; and *b)* only subpleural alveoli can be observed, thus preventing the observation of the small airways. The present study has, therefore, required the development of an alternative technique, that overcomes the limitations above.

Material and Methods

Animal preparation

Thirty-one, male Sprague-Dawley rats (weight range 0.38-0.47 kg), premedicated with diazepam ($4 \text{ mg}\cdot\text{kg}^{-1}$), were anesthetized with intraperitoneal injection of pentobarbital sodium ($40 \text{ mg}\cdot\text{kg}^{-1}$) and chloral hydrate ($170 \text{ mg}\cdot\text{kg}^{-1}$). A metal cannula and a polyethylene catheter were inserted into the trachea and carotid artery, respectively.

Airflow (V') was measured with a heated Fleisch pneumotachograph (no.0000; HS Electronics, March-Hugstetten, Germany) connected to the tracheal cannula and a differential pressure transducer (Validyne MP45, $\pm 2 \text{ cmH}_2\text{O}$; Northridge, CA). Tracheal pressure (P_{tr}) and esophageal pressure (P_{es}) were measured with pressure transducers (8507C-2 Endevco, San Juan Capistrano, CA; Statham P23Gb, HS Electronics, March-Hugstetten, Germany) connected to the side arm of the tracheal cannula, and to a balloon-tipped polyethylene

catheter positioned in the esophagus. Transpulmonary pressure (PL) was obtained as Ptr-Pes. The signals from the transducers were amplified (RS3800; Gould Electronics, Valley View, OH), sampled at 200 Hz by a 12-bit A/D converter (AT MIO 16L-9; National Instruments, Austin, TX), and stored on a desk computer. Volume changes were obtained by numerical integration of the airflow signal. Arterial blood PO₂, PCO₂ and pH were measured by means of a blood gas analyzer (Gem Premier 3000; Instrumentation Laboratory, Milan, Italy) on samples drawn at the end of each test session.

The animals were paralyzed with pancuronium bromide (2 mg·kg⁻¹) and ventilated with a specially designed, computer-controlled ventilator (11), delivering water-saturated air or oxygen from a high pressure source (4 atm) at constant flow of different selected magnitudes and durations. A three way stopcock allowed the connection of the expiratory valve of the ventilator to a drum in which the pressure was set at 1.1 (PEEP) or -6 cmH₂O (NEEP) by means of a flow-through system. Baseline ventilation consisted of eupneic VT (7 ml·kg⁻¹), fixed inspiratory (0.22 s) and expiratory duration (0.40 s; I/E=0.55), and frequency (97 min⁻¹).

Procedure and data analysis

Ethidium homodimer-1 (E1903, Sigma-Aldrich, St. Louis, MO), which exhibits the same basic properties as propidium iodide (12), and calcein AM (C1359, Sigma-Aldrich), were used as marker of plasma membrane disruption and indicator of cell viability, respectively, having been repeatedly validated in vitro (13, 14, 15, 16).

Fig. 1 provides a time line representation of the main procedures. The ethidium homodimer-1 (0.5 μM) and calcein AM (2.0 μM) saline solution (32 ml·kg⁻¹) was instilled intratracheally *a*) in 6 rats after 5 min of baseline ventilation on PEEP (CT group); *b*) in 8 rats after baseline mechanical ventilation on PEEP for 12 min and NEEP for 120 min (NP group); *c*) in 6 rats after mechanical ventilation with baseline settings on PEEP for 12 min, NEEP for

120 min, and PEEP again for 30 min (NP-PP group); and *d*) in 6 rats after mechanical ventilation on PEEP for 100 min with large V_T ($\sim 39 \text{ ml}\cdot\text{kg}^{-1}$), high peak P_{tr} (40 cmH₂O), and low frequency ($\sim 18 \text{ min}^{-1}$) (LV-PP group). These settings were chosen to reproduce the operational lung volumes of a previous study (11), in which respiratory mechanics, histology and inflammatory response were assessed both during low volume ventilation with eupneic V_T and ventilation with large V_T at physiological EELV. On NEEP or with large V_T on PEEP, the animals were ventilated with water-saturated oxygen, except during the assessment of lung mechanics and arterial blood gasses and pH, when they were ventilated with water-saturated air (Fig. 1).

In order to assess the effect of prolonged ventilation on the formation of plasma membrane disruption, in an additional group of 5 animals ethidium homodimer-1 and calcein AM saline solution was instilled intratracheally after 120 min of baseline ventilation with water-saturated oxygen on PEEP, water-saturated air being administered during the assessment of lung mechanics, and arterial blood gasses and pH.

After withdrawal of most of the label solution, mechanical ventilation was continued for 5 min. The animals were heparinized and killed by exanguination, mechanical ventilation was stopped, the label solution re-instilled, left for 10 min, and then withdrawn. The chest was widely opened, and $32 \text{ ml}\cdot\text{kg}^{-1}$ of a 12% gelatin (G2500, Sigma-Aldrich) solution were instilled intratracheally, thus expanding the isolated lungs to almost total capacity (17). Thereafter, the lungs were stored at 4°C for further processing.

The animals were handled according to the guiding principles published by the National Institutes of Health and the study was approved by Ministero della Salute, Rome, Italy.

Microscopy

Two blocks were obtained from the left lower lobe, and one from each of the other four lobes. From each block, kept immersed in cold saline ($\sim 5^{\circ}\text{C}$), a 150 μm thick slice was cut with a vibratome (752 Vibroslice, Campden Instruments Ltd, Loughborough, UK). Slices were examined at a magnification of 100X with an Axioskop 40 FL (Carl Zeiss, Germany), equipped with a camera (AxioCam MRc 5, Carl Zeiss) and two filter sets (00 and 09; Carl Zeiss) suited for ethidium homodimer-1 (excitation 495 nm, emission 635 nm) and calcein AM (excitation 495 nm, emission 515 nm). Images, digitized at 8-bit resolution, were 870 x 652 μm , subtending a volume of 0.085 mm^3 . Representative images from the NP and NP-PP groups are shown in Fig. 2.

To avoid any arbitrary choice of the operator, the following procedure was adopted. In bright field, the slice was inspected until a bronchiolus, cut nearly normal to its axis, was found, and photographs were taken in bright field, with the filter block for calcein and ethidium homodimer-1. Hence, each image was chosen independent of ethidium homodimer-1 and calcein labeling. At least 5 bronchioli were photographed per slice, giving more than 30 bronchioli per animal.

On each field a 3 step analysis was performed in a blind fashion using a custom made LabView program (LabView and IMAQ Vision for LabView; National Instruments, Austin, TX): a) on the image obtained with the filter block for ethidium homodimer-1, all the red spots, i.e. labeled nuclei, were marked; b) the marked red spots, superimposed on the same image in bright field, were classified either bronchiolar or non-bronchiolar; and c) after elimination of the markers and superimposition of a lattice, the relative surface occupied by bronchiolar and non-bronchiolar structures, and hence the density of bronchiolar, non-bronchiolar, and total red spots was assessed by point counting. Finally, the overall viability of lung parenchyma was qualitatively assessed by inspecting the image obtained with the filter block for calcein AM.

To quantify the precision and the reproducibility of bronchiolar and non-bronchiolar red spot counts, 10 images were analyzed by six operators two times. The results are shown in Table 1.

Respiratory system mechanics

Lung mechanics and blood gases were assessed while breathing room air during the initial (PEEP1) and final period (PEEP2) of baseline ventilation on PEEP (NP-PP and LV-PP groups) and at the beginning (NEEP1) and end (NEEP2) of the prolonged period of baseline ventilation on NEEP (NP and NP-PP groups) (Fig. 1). On PEEP, the lungs were inflated 3-4 times to a Ptr of ~ 25 cmH₂O before all measurements. Pulmonary quasi static elastance (Est), interrupter resistance (Rint), which reflects airway resistance, viscoelastic resistance (R_{visc}) and time constant (τ_{visc}) were assessed according to the rapid airway occlusion method, as previously described (11). On completion of the measures, the expiratory valve was switched to -10 cmH₂O in order to assess the difference between end-expiratory and residual volume (EELV-RV).

Statistics

Analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL). Results are presented as mean \pm SEM. Comparisons among experimental conditions were performed using mixed between within groups factorial ANOVA for repeated measurements. When an interaction was present, the Bonferroni post-hoc test was performed. Linear regressions were assessed using the least square method. The level for statistical significance was taken at $P < 0.05$.

Results

Microscopy

Tissue viability, as evaluated from calcein staining, was preserved in all groups. No difference in the representation of bronchiolar and non-bronchiolar structures occurred among groups, the bronchiolar surface averaging ~30% of the field area in all groups, nor in the distribution of red spots among lobes.

Relative to the CT group, mechanical ventilation with eupneic V_T at low EELV increased the density of both bronchiolar and non-bronchiolar red spots (Fig. 3). The increase of the density of non-bronchiolar red spots, though substantial (608%), was markedly lower than that of bronchiolar red spots (1796%); as a consequence, the ratio between the density of bronchiolar and non-bronchiolar red spots increased from 1.8 ± 0.24 in the CT group to 5.4 ± 0.7 in the NP group. In contrast, the densities of both bronchiolar and non-bronchiolar red spots of the NP-PP group, though increased, were not significantly different from corresponding CT values (Fig. 3).

Mechanical ventilation with large V_T on PEEP increased the density of both bronchiolar and non-bronchiolar red spots, which became significantly greater than that of the CT and NP-PP group (Fig. 3). The ratio between the density of bronchiolar and non-bronchiolar red spots (2.6 ± 0.37) was, however, not statistically different from that of the CT and NP-PP groups. While the density of non-bronchiolar red spots was significantly larger in the LV-PP than NP group, that of bronchiolar red spots was similar in both groups (Fig. 3); the ratio between the density of bronchiolar and non-bronchiolar red spots was therefore significantly higher in the NP group.

Respiratory gases

The behavior of PaO_2 , $PaCO_2$, and pH_a is shown in Fig. 4. No significant differences occurred among groups at PEEP₁, nor between NP and NP-PP group at NEEP₁ and NEEP₂.

Mechanical ventilation with eupneic VT at low EELV decreased PaO₂ and pH_a, and increased PaCO₂. On restoration of PEEP, PaO₂ and PaCO₂ of the NP-PP group returned to the initial (PEEP₁) values, while pH_a remained low.

Mechanical ventilation with large VT on PEEP decreased PaO₂ and pH_a, and increased PaCO₂; all changes being significantly larger than those observed with mechanical ventilation at low EELV.

Mechanics

No significant differences in mechanical parameters occurred among groups at PEEP₁, nor between NP and NP-PP group at NEEP₁ and NEEP₂.

Relative to PEEP₁, EELV-RV at NEEP₁ decreased by 85±2%, while Est (208±25%), Rint (62±8%), R_{visc} (152±28%) and τ_{visc} (37±14%) increased (Fig. 5). At NEEP₂, EELV-RV decreased moderately (-8±1%), while Est (100±20%), Rint (86±8%), and R_{visc} (189±36%) increased markedly. In the NP-PP group, restoration of the EELV-RV with PEEP was incomplete (-16±2%), Est (21±5%) and Rint (29±5%) remained elevated, while R_{visc} and τ_{visc} resumed their initial values.

Mechanical ventilation with large VT on PEEP caused pulmonary edema in all rats. Relative to PEEP₁, EELV-RV decreased (-77±3%), and Est (315±25%), Rint (72±10%), R_{visc} (310±44%) and τ_{visc} (25±10%) increased (Fig. 5).

A significant linear relationship was found between the density of bronchiolar red spots and the increase of Rint in the NP group from PEEP₁ to NEEP₂. This occurred also in the LV-PP group from PEEP₁ to PEEP₂ in spite of an unfavorable grouping of the data (Fig. 6).

Effect of prolonged baseline ventilation

While pH_a decreased significantly (Δ pH_a = -0.07±0.02; P<0.001), neither PaO₂ (Δ PaO₂ = -3.6±6 mmHg) nor PaCO₂ (Δ PaCO₂ = 2.6±3.4 mmHg) changed significantly after 120

min of mechanical ventilation with eupneic VT at normal EELV. Relative to the CT group, no significant changes occurred in the density of both bronchiolar ($60.8 \pm 10.1 \text{ mm}^{-3}$ vs $49.7 \pm 9.9 \text{ mm}^{-3}$; $P=0.239$) and non-bronchiolar red spots ($33.4 \pm 5.4 \text{ mm}^{-3}$ vs $26.5 \pm 3.4 \text{ mm}^{-3}$; $P=0.46$). Furthermore, the changes in EELV-RV ($-1 \pm 3\%$), Est ($0 \pm 3\%$), Rint ($-15 \pm 8\%$), Rvisc ($-2 \pm 6\%$) and τ visc ($-10 \pm 6\%$) between PEEP1 and PEEP2 were not significant ($P > 0.2$ in all cases).

Discussion

The procedure described herein has proven effective in assessing the distribution of plasma membrane disruptions throughout the lung in vivo. In the attempt to reach this goal, Gajic et al. (5) cut propidium iodide perfused lungs, but most of the cells were damaged and the nuclei stained with propidium iodide. This artifact should have, however, affected our technique marginally. Firstly, the density of both bronchiolar and non-bronchiolar red spots was markedly higher in the NP and LV-PP groups than in the CT and NP-PP groups, in spite of the same procedure being used in all groups. Furthermore, because of dilution with gelatin solution and circulating saline used to keep the gelatin solid, the concentration of ethidium homodimer-1 at the time of cutting should have been substantially lower than the lowermost value needed to stain the cells damaged by the blade (Fig. 7). Finally, confocal microscopy performed on a limited number of specimens showed that the red spots were very rarely positioned on the cutting surfaces.

A limitation of the technique is that staining gives no information about the cell type affected by plasma membrane disruption. During mechanical ventilation with eupneic VT, alveolar granulocytes or macrophages are absent at normal EELV and uncommon at low EELV (11). Thus, in CT, NP and NP-PP groups, parenchymal red spots can be nuclei of epithelial, mesenchymal, and/or endothelial cells, whereas, given the lack of a dense

peribronchiolar capillary network, bronchiolar red spots should be nuclei of epithelial cells. In contrast, some red spots in LV-PP group could have been inflammatory cells (2, 11).

In the CT group, the label solution was instilled shortly after anesthesia and surgical preparation, while the fall of EELV was prevented by the application of PEEP (18). Hence the density of bronchiolar and non-bronchiolar red spots of the CT group (Fig. 3) should be representative of the amount of pulmonary cells with plasma membrane disruption present under normal conditions, or it could be an overestimate, if labeling and inclusion procedures were causing additional lesions. It has been reported that plasma membrane disruption occurs in ~20% of rat cardiac cells, a percentage that increases about three fold with isoproterenol administration (19). A large percentage of plasma membrane disruptions is also found in skeletal muscles, especially during eccentric contractions (20). In contrast, the results of the CT group indicate that plasma membrane disruption is uncommon in normal lungs if EELV and VT are kept in the eupneic range, in line with Gajic et al. suggestion (5).

Relative to control animals, ventilated for a few minutes with water saturated room air, mechanical ventilation both at low EELV with eupneic VT and at physiological EELV with large VT increased the density of bronchiolar and non-bronchiolar red spots markedly (Fig. 3). This cannot be attributed to the administration of water saturated oxygen in the NP and LV-PP group, nor to the prolonged period of mechanical ventilation, because in animals ventilated for two hours with water saturated oxygen and eupneic VT at normal EELV, the density of both bronchiolar and non-bronchiolar red spots was similar to that of the CT group, while R_{int} did not differ between PEEP1 and PEEP2, as observed in rats ventilated with water saturated room air and eupneic VT on PEEP (11).

The increased density of bronchiolar red spots of the NP group can be attributed to cyclic opening and closing of peripheral airways, as supported by in vitro studies (15, 16). Opening and closing of peripheral airways can occur because of *a*) a substantial increase of

the thickness of the fluid lining the airways; *b*) a loss of the tethering action of the surrounding parenchyma with the fall of EELV; and *c*) an increase of surface tension beyond critical values. Although the lung wet-to-dry ratio could not be assessed in the present rats, condition *a*) seems an unlikely occurrence in NP and NP-PP groups, because no edema fluid nor secretions were seen in the trachea and the normal relation between EELV-RV and PL was maintained upon PEEP application. Moreover, only a slight elevation of the wet-to-dry ratio has been previously observed in closed-chest rabbits during mechanical ventilation at low EELV with eupneic V_T (21). In contrast, condition *b*) and *c*) were met during mechanical ventilation at low EELV, because NEEP application caused both an immediate (from PEEP₁ to NEEP₁) and a progressive (from NEEP₁ to NEEP₂) reduction of EELV-RV (Fig. 5). While the immediate increase of pulmonary E_{st} , R_{int} , and R_{visc} was due to the fall of EELV with NEEP (10, 11, 21), the further, substantial increase mainly reflected the increase of surface tension due to surfactant depletion or inactivation (22, 23, 24), because the concomitant decrease of EELV-RV was small (Fig. 5). Surfactant activity is of paramount importance to minimize the shear stress related damage of epithelial cells *in vitro* (16); indeed, during mechanical ventilation at low EELV *in vivo*, instillation of exogenous surfactant decreases whilst inactivation of endogenous surfactant enhances bronchiolar epithelial injury (10). Direct mechanical damage to small airway epithelium is likely the main cause of the progressive increase of R_{int} during this mode of mechanical ventilation, because in the NP group there was a significant relationship between the increase of R_{int} and density of bronchiolar red spots (Fig.6).

In the NP group, the density of non-bronchiolar red spots was also increased relative to CT values, though less than that of bronchiolar red spots (Fig. 3). This is relevant in connection with pulmonary interdependence (25). Because of cyclic opening and closing or steady collapse of the small airways, during volume changes abnormal stresses affect the

alveoli surrounding non-ventilated parenchyma, thus causing plasma membrane disruption and eventually ruptures of alveolar septa. The increase of the density of non-bronchiolar red spots in the NP group is therefore consistent with the finding of an increased percentage of broken bronchiolar-alveolar attachments in animals ventilated at low versus normal EELV (10, 11).

The absence of a significant difference in the density of both bronchiolar and non-bronchiolar red spots between the NP-PP and CT group (Fig. 3) suggests that the great majority of the cells which are damaged during mechanical ventilation at low EELV reseals once the normal EELV is restored. This is in line with the concept of resealing via a calcium-dependent lipid trafficking mechanism (4), and agrees with the finding in isolated rat lungs of a markedly decreased plasma membrane disruption if a period of mechanical ventilation with eupneic VT is made to follow that with injurious VT (5).

The largest increase in the density of non-bronchiolar red spots occurred in the LV-PP group (Fig. 3), in line with the notion that during mechanical ventilation with large VT on PEEP, the primary injury is due to overstrain of lung parenchyma. Assuming a spherical alveolar shape and a mean alveolar diameter of 80 μm (26), the mean number of red spots per alveolus computed from the individual values of non-bronchiolar red spot density amounts to 0.013 ± 0.002 . In spite of major differences in the experimental settings, this value is close to that (0.018) found in subpleural alveoli of isolated lungs ventilated with large VT (40 $\text{ml} \cdot \text{kg}^{-1}$) on PEEP (5). This, together with the absence of significant differences in the density of the red spots among lobes, suggests that distribution of strain related plasma membrane disruption is grossly uniform throughout the lungs, although many more slices per lobe should have been analyzed in order to reach this conclusion.

In the LV-PP group, the density of bronchiolar red spots, higher than that of the CT group, was similar to that of the NP group (Fig. 3), and significantly related to the increase of

Rint between PEEP1 and PEEP2, in spite of an unfavorable distribution of data points along the x-axis (Fig. 6). This suggests that cyclic opening and closing of small airways was occurring also during mechanical ventilation with large V_T on PEEP. Indeed, EELV-RV, though still higher than that of the NP group, was markedly decreased ($-77\pm 3\%$) during mechanical ventilation with large V_T (Fig. 5). Moreover, marked parenchymal heterogeneity was occurring in the LV-PP group, as suggested by the increased τ_{visc} and the presence of lung edema, that favors local airway closure and parenchymal inhomogeneity by flooding the airways and the alveoli and increasing the surface tension (27). Indeed, edema fluid was present in the trachea of all animals ventilated with large V_T .

At present, no studies exist that directly relate plasma membrane disruption to cytokine release *in vivo*. Although the study was not aimed to investigate the role of plasma membrane disruption in the upregulation of inflammatory mediators, the results could be relevant to this topic. In normal rabbits and rats the release of inflammatory mediators during mechanical ventilation with eupneic V_T at low EELV is marginal, if any (11, 21), whereas a substantial increase of cytokines concentration in serum and/or broncho-alveolar lavage fluid during mechanical ventilation with sufficiently large V_T is usually found in normal animals (8, 11, 28). This would fit with the suggestion of a pro-inflammatory role for plasma membrane disruption (1, 4, 7), because the density of the red spots was markedly higher (62%) in the LV-PP than NP group (Fig. 3). One could also hypothesize that parenchymal lesions are more important in inducing inflammatory upregulation, because the difference in density of the red spots between the LV-PP and NP group was accounted for by the density of non-bronchiolar red spots alone (Fig. 3). Further studies are needed to support these suggestions.

Conclusions

The technique presented herein has allowed in vivo detection and quantification of plasma membrane disruption that affect lung tissues. Using this technique, it has been assessed that mechanical ventilation at low EELV with eupneic VT or at normal EELV with large VT induces plasma membrane disruption in normal lungs, the former type of mechanical ventilation mainly at bronchiolar level, the latter at both bronchiolar and parenchymal level, and to a greater cumulative extent. This may be relevant to the different inflammatory response that occurs with these two types of mechanical ventilation.

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Table 1. Precision and reproducibility of red spot counts

		SD	CV (%)
<i>Precision</i>	NBRS	2.6	11.5
	BRS	2.3	8.8
	NBRS+BRS	1.5	3.1
<i>Reproducibility</i>	NBRS	2.1	9.5
	BRS	3.5	13.8
	NBRS+BRS	3.6	7.4

SD and CV: standard deviation and variation coefficient expressing intra-observer (precision) or inter-observer (reproducibility) variability. NBRS and BRS: non-bronchiolar and bronchiolar red spots.

Figure Legends

Figure 1. Time line representation of the main procedures used in rats mechanically ventilated with eupneic VT ($7 \text{ ml}\cdot\text{kg}^{-1}$) on PEEP for 5 min (CT group), on NEEP (NP group), on NEEP and then on PEEP (NP-PP group), and with large VT ($39 \text{ ml}\cdot\text{kg}^{-1}$). Lines indicate the period of mechanical ventilation: thin and thick line correspond to VT of 7 and $39 \text{ ml}\cdot\text{kg}^{-1}$, respectively. Hatched bars indicate when lung mechanics, arterial blood gasses and pH were assessed. Crossed bars correspond to the periods in which water saturated air was replaced by water saturated oxygen. Arrows indicate timing of intratracheal injection of ethidium homodimer-1 and calcein AM solution (EC) and gelatin (G).

Figure 2. Bronchioli and surrounding lung parenchyma from a rat of the NP (*A and C*) and NP-PP (*B and D*) group. The red spots are ethidium homodimer-1 stained nuclei of cells with plasma membrane disruptions. Diffuse calcein AM staining (green) indicates tissue viability.

Figure 3. Mean density of non-bronchiolar [NBRS] and bronchiolar red spots [BRS] in the CT, NP, NP-PP and LV-PP groups. Bars: SEM. Significantly different from CT group: * $P<0.05$, ** $P<0.01$; significant difference between [NBRS] and [BRS] in the same group: ° $P<0.05$, °° $P<0.01$; significantly different from corresponding value of NP group: †† $P<0.01$.

Figure 4. Mean values of arterial PO_2 , PCO_2 , and pH in NP, NP-PP and LV-PP groups at various times during the experimental procedure. Bars: SEM. Significantly different from corresponding values on PEEP1: * $P<0.05$, ** $P<0.01$; significant difference between NEEP1 and NEEP2 in the same group: ° $P<0.05$, °° $P<0.01$; significantly different from corresponding value of NP-PP group: † $P<0.05$, †† $P<0.01$.

Figure 5. Mean values of the difference between end-expiratory and residual volume (EELV-RV), lung interrupter resistance (Rint), quasi-static elastance (Est), viscoelastic

resistance (R_{visc}), and time constant (τ_{visc}) in NP, NP-PP and LV-PP groups at various times during the experimental procedure. Bars: SEM. Values significantly different from corresponding ones on PEEP1: * $P < 0.05$, ** $P < 0.01$; significant difference between NEEP1 and NEEP2 in the same group: ° $P < 0.05$, °° $P < 0.01$; significantly different from corresponding value of NP-PP group: † $P < 0.05$, †† $P < 0.01$.

Figure 6. Relationship between density of bronchiolar red spots [BRS] and increase of interrupter resistance (ΔR_{int}) from PEEP1 to NEEP2 in the NP group (squares), and from PEEP1 to PEEP2 in the LV-PP group (triangles). Numbers are slope (\pm SEM) of the relationship.

Figure 7. Relation between density of red spots in the lung tissue ([RS]) and ethidium homodimer-1 concentration in the solution in which unstained gelatin-included slices obtained from normal lungs were incubated for 15 minutes at room temperature immediately after cutting. The dashed line indicates the ethidium homodimer-1 concentration that should have occurred in the lungs during inclusion, on the assumption of complete mixing between label solution and gelatin. Dilution with the circulating saline, used to keep the gelatin solid during the cut, should have caused a further decrease of that concentration.











