POSITIVE END-EXPIRATORY PRESSURE PREVENTS LUNG MECHANICAL STRESS CAUSED BY RECRUITMENT/DERECRUITMENT

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Running head: Effects of PEEP on recruitment maneuvers.

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Abstract

This study tests the hypotheses that a recruitment maneuver per se yields and/or intensifies lung mechanical stress. Recruitment maneuver was applied to a model of paraquat-induced acute lung injury (ALI) and to healthy rats with (ATEL) or without (CTRL) previous atelectasis. Recruitment was done by using 40 cmH2O CPAP for 40 s. Rats were, then, ventilated for 1 h at ZEEP or PEEP (5 cmH2O). Atelectasis was generated by inflating a sphygmomanometer around the thorax. Additional groups did not undergo recruitment, but were ventilated for 1 h under ZEEP. Lung resistive and viscoelastic (ΔP2) pressures, and static elastance (Est) were computed before and immediately after recruitment, and at the end of 1-h ventilation. Lungs were prepared for histology. Type III procollagen (PCIII) mRNA expression in lung tissue was analyzed by RT-PCR. Lung mechanics improved after recruitment in the CTRL and ALI groups. One-hour ventilation at ZEEP increased alveolar collapse, Est and ΔP2. Alveolar collapse was similar in ATEL and ALI, and recruitment opened the alveoli in both groups. ALI showed higher PCIII expression than ATEL or CTRL groups. One-hour ventilation at ZEEP did not increase PCIII expression, but augmented it significantly in the three groups when applied after recruitment. However, PEEP ventilation after recruitment avoided any increment in PCIII expression in all groups. In conclusion, recruitment followed by ZEEP was more deleterious in ALI than in mechanical atelectasis, although ZEEP alone did not elevate PCIII expression. Ventilation with 5 cmH2O PEEP prevented derecruitment and aborted the increase in type III procollagen expression.

Keywords: acute lung injury; protective ventilation-strategy; pulmonary mechanics; procollagen expression.
Introduction

Mechanical ventilation can worsen preexisting lung disease because of regional overdistention or shear forces generated by repeated opening and closing of collapsed alveoli (atelectrauma) (20, 49, 50). These processes are associated with increased activation of inflammatory mediators and histological lesions indistinguishable from acute respiratory distress syndrome (ARDS) (41, 50-52). In the last 10 years avoidance of ventilator-induced lung injury (VILI) has dominated the literature on the management of ARDS (27, 29, 52).

VILI is the result of a complex interplay among various mechanical forces acting on lung structures during mechanical ventilation. Increased mechanical load in parenchyma cells and connective tissues can initiate an adaptative process to high stress, with collagen deposition and vascular cell proliferation (38). The mechanical factors responsible for VILI are thought to be related to recruitment/derecruitment of previously collapsed alveoli and/or pulmonary overdistension, thereby applying tension to lung parenchyma, especially in the context of ARDS (10).

The lung-protective mechanical ventilatory strategy proposed for ARDS emphasize the need to "open the lung and keep it open" while avoiding alveolar overdistension (32). The reduction of tidal volume to limit plateau pressure is currently recommended for the ventilatory management of ARDS. However, excessive reduction in tidal volume may result in harmful alveolar derecruitment depending on the level at which positive end-expiratory pressure (PEEP) is set. (1, 35, 42, 50, 53). Conversely, high-PEEP strategies can also lead to detrimental consequences, such as the development of air leaks (44).

The use of recruitment maneuvers has been proposed as an adjunctive lung-protective strategy to reverse low tidal volume-related derecruitment. (1, 21, 28, 42). In experimental studies, recruitment maneuvers have been able to minimize lung injury,
allowing the use of lower PEEP and peak airway pressures, thus reducing the potential VILI (44). Since the deleterious effects of high airway pressures over normal or injured lungs have been well established (19, 20, 54), and since the recruitment maneuver itself requires sustained pressures high enough to reach total lung capacity (28), the recruitment maneuver may cause some damage. Given the scanty data about the effects of the recruitment maneuver \textit{per se} on lung remodeling, we envisaged this study to address the following questions: a) if recruitment \textit{per se} could yield lung mechanical stress even in normal animals, b) if lung recruitment could intensify the lesion in animals with previous lung injury, c) if the amount of collapse prior to the maneuver could modulate this response, and d) if the use of PEEP ventilation after recruitment could attenuate the lung injury induced by recruitment and/or derecruitment. For such purposes, respiratory mechanics, pulmonary histology, and type III procollagen (PCIII) mRNA expression were analyzed in control animals and in those with acute lung injury or mechanical atelectasis. Rats were ventilated with a lung protective strategy with PEEP or zero-PEEP (ZEEP).

\textbf{Materials and Methods}

\textit{Animal Preparation}

A total of fifty-seven Wistar rats [250 ± 10 (SD) g] were used. They were randomly assigned to three main groups. In the control group (CTRL, \(n = 19\)), saline [0.9% NaCl, 5 mL/kg body weight (BW)] was injected intraperitoneally. In ATEL group (\(n=19\)), a model of reproducible atelectasis previously developed was used (16). Briefly, atelectasis was generated by a pediatric sphygmomanometer wrapped around the thorax of the animal from the axilla to the subcostal plane. The cuff was inflated to a transpulmonary pressure of \(-8 \text{ cmH}_2\text{O}\), which was maintained for 5 seconds. Then the sphygmomanometer was taken off to allow further measurements (16). In ALI group (\(n = 19\)), paraquat was injected intraperitoneally (10 mg/kg
BW) 24 h prior to the measurements (45). The animals were sedated (diazepam 5 mg, intraperitoneally), anesthetized (pentobarbital sodium 20 mg/kg BW, intraperitoneally), paralyzed (gallamine triethyliodide 2 mg/kg BW, intravenously) and mechanically ventilated (Samay VR15, Universidad de la Republica, Montevideo, Uruguay).

An adequate pneumotachograph (1.5 mm ID, length = 4.2 cm, distance between side ports = 2.1 cm) (34) was connected to the tracheal cannula for the measurements of airflow ($V'$). Tidal volume ($V_T$) was calculated by digital integration of flow signal. The tracheal pressure ($P_{tr}$) was determined (Validyne MP45-2 differential pressure transducer, Engineering Corp, Northridge, CA, USA). The flow resistance of the equipment ($R_{eq}$) amounted to 0.12 cmH$_2$O.s/mL, and was subtracted from respiratory system and pulmonary viscous resistances (12). Changes in esophageal pressure ($P_{es}$), which reflect chest wall pressure ($P_w$), were measured with a 30-cm-long water-filled catheter (PE205) with side holes at the tip connected to a PR23-2D-300 Statham differential pressure transducer (Hato Rey, Puerto Rico). The catheter was passed into the stomach and then slowly returned into the esophagus; its proper positioning was assessed using the "occlusion test" (5). This test consisted in comparing $\Delta P_{es}$ and $\Delta P_{tr}$ during spontaneous inspiratory efforts subsequent to airway occlusion at end expiration. In all instances $\Delta P_{es}$ was close to $\Delta P_{tr}$, the difference not exceeding 3%. The frequency responses of $P_{tr}$ and $P_{es}$ measurement systems were flat up to 20 Hz, without appreciable phase shift between the signals. All signals were conditioned and amplified (Beckman type R Dynograph, Beckman Instruments, Schiller Park, IL, USA). Flow and pressure signals were also passed through 8-pole Bessel filters (902LPF, Frequency Devices, Haverhill, MA, USA) with the corner frequency set at 100 Hz, sampled at 200 Hz with a 12-bit analog-to-digital converter (DT2801A, Data Translation, Marlboro, MA, 33.6 on September 22, 2017 http://jap.physiology.org/ Downloaded from
USA), and stored on a personal computer. All data were collected using LABDAT software (RHT-InfoData Inc., Montreal, Quebec, Canada).

An arterial cannula was inserted into one of the femoral arteries to sample blood for the determination of oxygen saturation (AVL Biomedical Instruments, Roswell, GA, USA).

Experimental protocol

The experimental protocol is depicted in Figure 1. Volume control ventilation with a tidal volume of 5 mL/kg BW, a frequency of 80 breaths/min, and inspiratory/expiratory ratio of 1:2 was defined as baseline ventilation. The animals were ventilated with an inspired oxygen fraction (FiO₂) of 0.21.

A recruitment maneuver consisting of a single continuous positive airway pressure of 40 cmH₂O for 40 seconds was then performed. After recruitment, baseline tidal volume, respiratory rate, and inspiratory/expiratory ratio were resumed.

Just after the recruitment maneuver, 5 animals from each group were ventilated for one hour under ZEEP, while other 5 rats were ventilated with 5 cmH₂O of PEEP, to avoid derecruitment. To analyze the effects of one hour ZEEP ventilation by itself without recruitment maneuver, the animals of the CTRL, ALI, and ATEL groups were ventilated during 1-hour at ZEEP, without a previous recruitment maneuver, constituting the CTRL-ZEEP, ALI-ZEEP, and ATEL-ZEEP groups, respectively (n=4, each). To understand the aspects related to each lung preparation per se, animals from the three major groups (CTRL, ALI, and ATEL) were not submitted to recruitment maneuver or mechanical ventilation, i.e., rats were killed and the lungs were removed at end-expiratory volume (Non-vent CTRL, Non-vent ALI, and Non-vent ATEL groups, n = 5, each). In ATEL group, lungs were removed immediately after the induction of atelectasis.

Respiratory Mechanics
Respiratory mechanical data were obtained in the CTRL and ALI groups at three occasions: before the recruitment maneuver (PRE), immediately after recruitment (RM), and at the end of the 1-hour ventilation period ensuing recruitment (POST).

Respiratory mechanics were measured by end-inflation occlusion method (3, 4, 31). Briefly, constant tidal volume ($V_T = 5 \text{ mL/kg}$) and flow ($V' = 6 \text{ mL/s}$) were applied to all animals (31, 48). After end-inspiratory occlusion, there is an initial fast drop in tracheal pressure ($\Delta P_{1,rs}$) from the preocclusion value down to an inflection point ($P_{i,rs}$), followed by a slow pressure decay ($\Delta P_{2,rs}$) until a plateau is reached. This plateau pressure corresponds to the elastic recoil pressure of the respiratory system ($P_{\text{plat},rs}$). $\Delta P_{1,rs}$ selectively reflects the pressure spent to overcome the combination of airways, pulmonary, and chest wall Newtonian resistances (3, 5), and $\Delta P_{2,rs}$ reflects the pressure dissipated by stress relaxation, or viscoelastic properties, of the lung and chest wall tissues, together with a small contribution of pendelluft in normal situations (48). The same procedures apply to the chest wall pressure ($P_w$) yielding the values of $\Delta P_{1,w}$, $P_{i,w}$, $\Delta P_{2,w}$, and $P_{\text{plat},w}$, respectively. Transpulmonary pressures ($\Delta P_{1,L}$, $P_{i,L}$, $\Delta P_{2,L}$) were calculated by subtracting the chest wall pressures ($\Delta P_{1,w}$, $P_{i,w}$ $\Delta P_{2,w}$) from the corresponding values pertaining to the respiratory system ($\Delta P_{1,rs}$, $P_{i,rs}$, $\Delta P_{2,rs}$). Total pressure drop ($\Delta P_{\text{tot}}$) is equal to the sum of $\Delta P_1$ and $\Delta P_2$, yielding the values of $\Delta P_{\text{tot},rs}$, $\Delta P_{\text{tot},L}$, and $\Delta P_{\text{tot},w}$. Respiratory system, lung, and chest wall static elastances ($E_{\text{st},rs}$, $E_{\text{st},L}$, and $E_{\text{st},w}$, respectively) were calculated by dividing $P_{\text{plat},rs}$, $P_{\text{plat},L}$, and $P_{\text{plat},w}$, respectively, by $V_T$. All data were analyzed using ANADAT data analysis software (RHT-InfoData Inc., Montreal, Quebec, Canada).

Histological Study
At the end of the experiments, the trachea was clamped at end-expiratory volume, and the abdominal aorta and vena cava were sectioned, quickly killing the animals. The chest wall was opened and the lungs removed *en bloc*.

A 3x3x10 mm strip of subpleural parenchyma was cut from the periphery of the right lung. Pleural tissue was removed and the strip was stored in liquid nitrogen for analysis of type III procollagen mRNA expression.

In order to perform the morphological study, the left lung was quick-frozen by rapid immersion in liquid nitrogen (36). Fixation was made with Carnoy’s solution (ethanol-chloroform-acetic acid, 70:20:10) at -70°C. After 24 h, ethanol concentration was progressively increased (70%, 80%, 90%, 100%, respectively, 1 h each solution, at -20°C). The lungs were then kept in 100% ethanol for 24 h at 4°C. After fixation tissue blocks were embedded in paraffin, and cut 4-µm-thick. Slides were stained with hematoxylin-eosin. Morphological analysis was performed with an integrating eyepiece with a coherent system made of a 100-point grid and 50 lines, coupled to a conventional light microscope (Axioplan, Zeiss, Germany). The volume fraction of collapsed and normal pulmonary areas and the fraction of the lung occupied by large-volume gas-exchanging air spaces (hyperinflated structures with morphology distinct from that of alveoli and wider than 120 µm) were determined by the point-counting technique (56) at a magnification of x40 across 10 random, noncoincident microscopic fields.

A semiquantitative system was used to account for the severity of alveolar collapse. A 5-point semiquantitative severity-based scoring system was used. The pathologic findings were graded as negative=0, slight=1, moderate=2, high=3 and severe=4 in 10 noncoincident microscopic fields (100x magnification). A median score for each of the
variables (0=normal lung parenchyma; 1=0–25%; 2=25–50%; 3=50–75%; 4=75–100% of areas with alveolar collapse) was then calculated.

*Type III Procollagen mRNA expression*

The relative expression of type III procollagen mRNA (PCIII) was obtained by semi-quantitative Reverse-Transcription and Polymerase Chain Reaction (RT-PCR) of rat lung tissue in all groups.

Total RNA was isolated from the frozen lung tissue by the method of Chomczynski and Sacchi (14). RNA samples were quantitated by absorbance at 260/280 nm.

To make the first DNA strand, total RNA isolated from rat lung was reverse-transcribed with SuperScript (Gibco BRL, Grand Island, NY, USA) at 37°C for 60 min. First-strand complementary DNA (cDNA) synthesis was performed in a 20-µl reaction containing 1µg of total RNA, 50 U murine leukemia virus reverse transcriptase, 20 U RNase inhibitor, 2.5 µM oligo(dT)_{16}, 2µl of 5X first strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂), 1 mM dNTP and diethyl pyrocarbonate (DEPC)-treated water. The reaction was performed in a water bath at 37°C for 80 min and 99°C for 5 min. The negative control [reverse transcriptase replaced with DEPC-treated water - RT(-)] was included with all reverse transcription reactions. The resultant cDNA was diluted in 10 µl of DEPC-treated water and stored at -20°C. PCR technique was used to amplify the synthesized cDNA. The following solution was employed in PCR reaction: 0.2 µmol/l of each dNTP, 50 mmol/l of KCl, 10 mmol/l of Tris-Cl (pH 8.3) and 1.5 mmol/l of MgCl₂ plus 2.5 U of thermostable DNA polimerase (Taq polimerase, Gibco BRL, Grand Island, NY, USA) and 0.2 µmol/l of each sense and antisense primers.
For rat PCIII mRNA, one pair of oligonucleotides (5’CTGCCATTGCTGGAGTTG3’ and 5’GCAGCCATCCTCTAGAAC3’), corresponding to nucleotides 903 to 920 and 1529 to 1546, respectively, was synthesized (Gene-bank accession no. AJ005395). PCR was performed with 36 cycles of denaturation (94°C, 1 min), annealing (54°C, 1 min) and extension (72°C, 1 min). The final elongation was at 72°C for 10 min. In the PCIII mRNA detection by RT-PCR, the rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) primers were added into the same RT-PCR reaction tube and GAPDH products were used as internal positive control. The rat GAPDH primers predicted to amplify the 211-bp PCR product used were: sense: 5’-GTCTTCACCACCATGGAG-3’; antisense: 5’-CGATGCCAAAGTTGTCATG-3’ (corresponding, respectively, to nucleotides 325-342 and 517-535 of rat GAPDH gene, Gene-bank accession no. M17701). The semi-quantitative method of RT-PCR, used to quantify the PCIII mRNA expression in the experimental rat lung, was validated in preliminary experiments as previously described (37). All reactions included a negative control RT(-). The identity of the amplification was confirmed by determination of the molecular size on agarose gel electrophoresis with 100 bp DNA molecular marker (Gibco BRL, Grand Island, NY, USA).

**Statistical Analysis**

The normality of the data (Kolmogorov-Smirnov test with Lilliefors’ correction) and the homogeneity of variances (Levene median test) were tested. Both conditions were satisfied in all instances, and thus one-way ANOVA for repeated measures was used to determine the effect of recruitment maneuver on respiratory mechanics along time in each group. One-way ANOVA was used to compare morphological and mRNA data among all groups. In both
cases, if multiple comparisons were required, Tukey test was applied. The significance level was set at 5%.

**Results**

The respiratory system parameters followed the lung mechanical behavior, because chest wall mechanics did not change among groups. Lung mechanics improved immediately after recruitment maneuver both in CTRL and ALI groups (Figs. 2 and 3). After recruitment, one-hour ventilation under ZEEP increased lung static elastance and total and viscoelastic pressure variations ($\Delta P_{tot}$ and $\Delta P_2$). These values were even higher than those observed before the maneuver (Figs. 2 and 3, upper panels). Ventilation with 5 cmH$_2$O PEEP avoided the worsening of respiratory mechanics observed after one-hour ventilation ensuing recruitment (Figs. 2 and 3, lower panels).

Table 1 shows the respiratory system plateau pressures achieved in CTRL and ALI groups in all situations. Plateau pressure ($P_{plat,rs}$) was higher in ALI than in CTRL group, fell in both groups after recruitment maneuver, and returned to values similar to or higher than those obtained before RM (PRE) in ZEEP. One-hour ventilation with 5 cmH$_2$O PEEP after RM (POST) led to $P_{plat,rs}$ lower than the values PRE in CTRL group, but similar to PRE in ALI group.

Histological changes in non-ventilated ALI group included interstitial edema, atelectasis, inflammation with increased amount of polymorphonuclear cells, and hyaline membrane. ATEL group depicted only atelectasis without cellular infiltration (Fig. 4). Recruitment maneuver reduced alveolar collapse in ALI and ATEL groups to the same extent, but 1-hour ventilation in ZEEP led to atelectasis even in CTRL group (Table 2).
Interestingly, recruitment maneuver re-expanded collapsed alveoli more homogeneously in ATEL than in ALI group, which still presented areas of patchy atelectasis after recruitment (Fig. 4, Table 3). Ventilation with PEEP reduced alveolar collapse in ALI and ATEL groups. (Fig. 4 and Table 2).

Figure 5 shows procollagen type III mRNA expression among Non-vent, CTRL-RM-ZEEP, CTRL-ZEEP, ATEL-ZEEP, ATEL-RM-ZEEP, ALI-ZEEP and ALI-RM-ZEEP groups. In this set of experiments, data were related to the values obtained in the Non-vent group. ALI showed higher PCIII expression than ATEL or CTRL groups. Recruitment maneuver followed by ZEEP ventilation increased PCIII mRNA expression similarly in all groups (Fig. 5). The results cannot be attributed to a possible effect of mechanical ventilation alone since there were no changes in non-recruited control animals after 1 h ventilation in ZEEP (Fig. 5). To clarify the beneficial effects of PEEP in each group (CTRL, ATEL and ALI), all values were related to ZEEP ventilation without RM. PEEP ventilation after RM prevented the increase in PCIII mRNA expression in all three main conditions (Fig. 6).

**Discussion**

Recruitment maneuver improved lung mechanics, but its beneficial effects disappeared as soon as one hour of ventilation on ZEEP had elapsed. On the other hand, the recruitment maneuver itself increased type III procollagen mRNA expression, even in healthy lungs (Fig. 5). By contrast, ventilation with 5 cmH₂O PEEP after recruitment avoided the worsening of mechanical and histological parameters, as well as PCIII mRNA production, observed in all lung preparations (Fig. 6). Interestingly, although the plateau pressures achieved during PEEP after recruitment were higher than those observed during
ZEEP (Table 2), the PCIII mRNA expression remained much lower under PEEP than under ZEEP. It has been shown that type III collagen increases early in the evolution of lung fibrotic process (40), thus PCIII mRNA expression was used as a marker of lung parenchyma remodeling. It is noteworthy that derecruitment has also to be considered as an additional possible mechanism of damage in this model, since a relatively low recruitment pressure was used. However, we decided to use similar pressures that have been applied in clinical studies. The increase in PCIII mRNA expression in CTRL animals submitted to RM together with the absence of a rise in PCIII mRNA expression in ATEL group without RM suggest that recruitment itself is playing a role in lung damage.

The ALI model used in the present study leads to a well reproducible moderate lung injury, characterized by alveolar collapse, interstitial edema, and hyaline membrane, without alveolar edema (45). The ALI reproducibility was of great importance since lung injury severity as well as ventilation settings could be standardized among all animals, thus avoiding the usual limitations observed in clinical trials. The ATEL model (16) was used to discriminate whether the deleterious effects of recruitment maneuvers were related to the previous degree of atelectasis present in our ALI model (45), or, alternatively, to parenchymal inflammation.

Recruitment maneuvers are characterized by sustained increase in airway pressure (26). The use of recruitment maneuvers has been suggested as adjunct to lung-protective ventilation strategies in ARDS patients (1). It is also of use during general anesthesia to improve arterial oxygenation, which is mainly thwarted by atelectasis (25, 46, 47, 55). However, controversy exists over the possibility of harm being caused by recruitment because of excessively high intrathoracic pressure and volume (8, 11, 19, 54). The literature has a paucity of data on various methods of performing an RM in animal or human subjects (30). In the present study,
we chose to perform a single recruitment maneuver with 40 cmH\textsubscript{2}O CPAP for 40 s (1), because most investigators use a single RM consisting of a CPAP of approximately 30-40 cmH\textsubscript{2}O (9, 18).

Acute lung injury increased lung static elastance, resistive and viscoelastic/inhomogeneous pressures. The increase in $E_{st}$ could be attributed to an increase in stiffness of lung tissue due to larger surface forces, and airway closure and/or alveolar collapse (17). The mechanisms that account for the increase of $\Delta P_2$ are related to lung inhomogeneities due to alveolar collapse/overdistension, edema, and surfactant functional changes. Furthermore, alveolar collapse could pull open alveolar ducts and might distort the parenchyma, thereby affecting local tissue mechanics (45). $P_1$ increased in ALI because of the reduction of central airway caliber caused by edema, fluid accumulation, reflex bronchoconstriction, and/or reduced lung volume. The immediate improve in lung mechanics after RM was transitory (Figs. 2 and 3), as previously described by other authors (23, 39).

Interestingly enough, mechanical parameters were even worse after 1-hour ventilation under ZEEP (33). The decrease in $E_{st}$ indicates recruitment of previously closed alveolar space, while the reduction in $\Delta P_2$ suggests a decrease in mechanical inhomogeneities and tissue viscoelasticity. Additionally, $P_1$ diminished after recruitment probably because of the parenchymal tethering that distends the airways. Many studies suggest that the high potential for recruitment is related to chest wall mechanical behavior (22, 39). However, in the present study we observed no change in chest wall mechanical parameters, probably because of the lack of abdominal distension or pleural effusions. Thus, our findings suggest that the beneficial effects of recruitment in extrapulmonary ARDS are not fundamentally linked to chest wall behavior.
We demonstrated that recruitment maneuver increases PCIII mRNA expression in pulmonary tissue in three different conditions (Fig. 5). The relative high pressure necessary to reexpand and open collapsed lung units with a single RM may expose the alveoli to shear forces, increasing PCIII mRNA expression similarly in the three groups. Our results are in accordance with previous reports demonstrating increased procollagen mRNA expression in lungs submitted to high airway pressures (38), high inflation (6), or cyclic mechanical strain (10). Propeptides of collagen are released during fibril formation as a result of cleavage by specific extracellular N- and C-terminal proteinases (2). The N-terminal peptide of type III procollagen has been used as a biologic marker of collagen synthesis (13). Many cell types in lung, e.g., fibroblasts and alveolar macrophages, may contribute to the increase in lung parenchyma mRNA for PCIII. However, few studies have focused on cells of peripheral lung parenchyma.

D’Angelo et al. (17) showed that prolonged low volume ventilation on ZEEP induces peripheral airway injury, even in normal lungs. Although the viscoelastic deterioration observed after 1 hour of ZEEP (Fig. 3) supported their findings, it is interesting to note that this was not enough to increase PCIII mRNA expression in lung tissue. PCIII mRNA expression was only triggered when 1-h ZEEP ventilation was preceded by a recruiting maneuver (Fig. 5).

Acute lung injury animals, not submitted to RM, showed increased PCIII mRNA expression in the pulmonary tissue (Fig. 5). These results are in accordance to what observed in ARDS, where type III collagen predominates early at the early phase of the disease (15, 40).

Kloot et al. (30) demonstrated that responses to PEEP, tidal volume, and recruitment differ among models of ALI induced by lavage, oleic acid, and intratracheal instillation of Escherichia coli. Ours results showed that although the recruitment maneuver
re-expanded collapsed alveoli in both ATEL and ALI groups (Table 2), this beneficial effect was more significant in atelectatic lungs without inflammation (Table 3). Bilek et al. (7) reported that pulmonary surfactant protects lung epithelium from mechanical stresses associated with airway reopening injury. However, RM is deleterious both in the models of mechanical atelectasis and ALI, and control group. Thus, the tissue stress induced by RM is probably the major component that account for the increment in PCIII mRNA.

Ventilation with 5 cmH\textsubscript{2}O PEEP after recruitment avoided the stimulation of PCIII mRNA expression induced by the maneuver (Fig. 6). Recruiting the lung and preventing derecruitment decreases the potential for lung injury by avoiding the repetitive shear stress associated with opening and closing unstable lung units (24, 29). Thereby, we can not discard that the increase in PCIII mRNA expression was avoided in PEEP ventilation also by preventing derecruitment.

It has been already demonstrated that hypoxia leads to increased collagen synthesis in rat pulmonary artery (6). However, the beneficial effect of 5 cmH\textsubscript{2}O PEEP ventilation after RM can not be attributed to an improve in oxygenation, since there was no statistically significant difference in oxygen saturation between ZEEP and 5 cmH\textsubscript{2}O PEEP ventilation groups (Mean ± SD: ALI-RM-ZEEP: 93.8 ± 3.9%, ALI-RM-PEEP: 92.4 ± 3.8%). Although low tidal volume ventilation has been established as an essential element in lung protective strategy, controversy still exists over the approach used to set PEEP (53). PEEP levels as high as 15-20 cmH\textsubscript{2}O have been applied to avoid alveolar derecruitment (53). Recently, Halter et al. (24) demonstrated that recruitment followed by inadequate PEEP results in unstable alveoli and may induce VILI despite improved oxygenation. In this line, we observed that optimal compliance was achieved with 5 cmH\textsubscript{2}O in this model of moderate lung injury in rats. In
accordance with our findings, Rimensberger et al. (44) demonstrated that lungs could be
ventilated at optimal compliance when using recruitment maneuvers followed by a PEEP
below the lower inflection point, but above the closing pressure. It is possible, therefore, that a
significant portion of the lung was kept open in our animals, even at these low PEEP levels,
足够的 to decrease damage. Accordingly, the extent of end-expiratory collapse was much
smaller in animals ventilated for 1 hour after recruitment under PEEP when compared to those
under ZEEP (Table 2, Fig. 4).

It is interesting to note that in animals with either acute lung injury or atelectasis,
ventilation with 5 cmH₂O PEEP avoided the increase in PCIII mRNA expression, in spite
of the higher plateau pressure achieved in these groups (Table 1). These findings suggest
that the protective effect of an adequate PEEP level overcomes the expected deleterious
effect of high tonic tissue stretching. Our results showed a key role of PEEP after
recruitment. This finding is supported by the recent demonstration that once plateau
pressure is maintained below 30-35 cmH₂O by reducing Vₜ, increasing PEEP markedly
augments recruitment (43). Richard et al. suggested that in this situation PEEP might play a
fundamental role in the lung protective strategy aiming to minimize lung collapse (43).

In clinical scenario patients undergo high levels of PEEP to achieve optimal recruitment,
and during these interventions they may be exposed to sudden derecruitment of previously
recruited lung by a sudden withdrawal of PEEP. Sudden loss of recruitment may accentuate
ventilation inhomogeneity and cause further closing of small airways, rendering the lung more
vulnerable to injury. Recently, Suh et al. (50) showed that derecruitment of initially recruited
lung may increase lung injury associated with mechanical ventilation. In our study,
recruitment maneuver followed by ZEEP led to derecruitment, showing an increased
expression of PCIII mRNA (Fig. 5). Conversely, a PEEP level as low as 5 cmH₂O after RM
avoided derecruitment in this model of moderate ALI. Thus, not only recruitment maneuver per se but also the derecruitment after previous reopening of collapsed alveoli can be the triggering factor for procollagen expression.

In conclusion, recruitment/derecruitment triggered type III procollagen expression in healthy lungs with atelectasis and with previous lung inflammation. In addition, the use of low PEEP levels right after the maneuvers aborted the increase in type III procollagen expression, being an effective strategy to minimize the potential harm associated to cellular mechanical stress.
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References


24. Halter JM, Steinberg JM, Schiller HJ, DaSilva M, Gatto LA, Landas S, and Nieman GF. Positive end-expiratory pressure after a recruitment maneuver


FIGURE LEGENDS

Figure 1. A schematic flow chart of the design of the study. CTRL: control groups, ALI: rats with acute lung injury induced by paraquat, ATEL: rats with mechanical atelectasis. Respiratory mechanics were measured before (PRE) and after recruitment maneuvers (RM), and 1 hour after RM (POST). Lung histology and molecular biology (type III procollagen mRNA) were analyzed only after 1-h ventilation. Rats were ventilated for 1 hour after recruitment in ZEEP (zero end-expiratory pressure) or PEEP (positive end-expiratory pressure).

Figure 2. Lung static elastance measured immediately before (PRE) and after recruitment maneuvers (RM), and 1 hour after RM (POST) in control (CTRL) and paraquat injected rats (ALI). Upper panel: rats ventilated for 1 hour after RM under ZEEP; lower panel: rats ventilated for 1 hour after RM under PEEP. Values are means + SEM of 5 animals (15 determinations/rat). * Significantly different from values PRE ($p < 0.05$). ** Significantly different from values immediately after RM ($p < 0.05$).

Figure 3. Lung resistive ($\Delta P1$) and viscoelastic/inhomogeneous ($\Delta P2$) pressure variations immediately before (PRE) and after recruitment maneuvers (RM), and 1 hour after RM (POST) in control (CTRL) and paraquat injected rats (ALI). The total column represents the total pressure variation in each group. Upper panel: rats ventilated for 1 hour after recruitment under ZEEP; Lower panel: rats ventilated for 1 hour after recruitment under PEEP. Values are means + SEM of 5 animals (15 determinations/rat). *Significantly
different from values PRE ($p < 0.05$). **Significantly different from values immediately after RM ($p < 0.05$).

**Figure 4.** Photomicrographs of lung parenchyma stained with hematoxylin-eosin in control groups (CTRL), in rats with acute lung injury induced by paraquat (ALI), and in rats with mechanical atelectasis (ATEL). The animals from each group were non-recruited and non-ventilated (Non-vent) or ventilated for 1 hour in ZEEP or PEEP after RM (RM-ZEEP and RM-PEEP, respectively). Photographs were taken at an original magnification of 100x.

**Figure 5.** Relative expression of type III procollagen mRNA (PCIII) obtained by amplification of PCIII and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) by semi-quantitative Reverse-Transcription and Polymerase Chain Reaction (RT-PCR) of rat lung tissue in different situations. Non-vent = non-recruited and non-ventilated normal rats; control rats ventilated for 1 hour in ZEEP without recruitment maneuvers (RM) (CTRL-ZEEP) and after recruitment maneuvers (CTRL-RM-ZEEP), rats submitted to mechanical atelectasis and ventilated for 1 hour in ZEEP without RM (ATEL-ZEEP) and after RM (ATEL-RM-ZEEP), rats with paraquat induced ALI and ventilated for 1 hour in ZEEP without RM (ALI-ZEEP) and after RM (ALI-RM-ZEEP). M.W.= Molecular weight. Values are means + SEM ($n = 4$) of the ratio between the densitometric values of PCIII and GAPDH bands obtained in RT-PCR experiments. *Significantly different from Non-vent group ($p < 0.05$). #Significantly different from all other groups ($p < 0.05$).
Figure 6. Relative expression of type III procollagen mRNA (PCIII) obtained by amplification of PCIII and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) by semi-quantitative Reverse-Transcription and Polymerase Chain Reaction in control groups (CTRL), rats with mechanical atelectasis (ATEL), and in rats with acute lung injury induced by paraquat (ALI). The animals from each group were non-recruited but ventilated for 1 hour in ZEEP (ZEEP), or submitted to recruitment maneuvers and ventilated for 1 hour in ZEEP (RM-ZEEP) or PEEP (RM-PEEP). All values were related to ZEEP ventilation without RM. M.W= Molecular Weight; RT(-) = Reverse transcription in the absence of reverse transcriptase. Values are mean + SEM (n = 4) for the ratio between the densitometric values of PCIII and GAPDH bands obtained in RT-PCR experiments. *Significantly different from the ventilated non-recruited rats in each situation (p < 0.05). Note the different ordinate values.
Table 1. Plateau pressures over the course of the experiments in control and acute lung injury groups

<table>
<thead>
<tr>
<th></th>
<th>ZEEP</th>
<th>PEEP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CTRL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE</td>
<td>2.75 ± 0.04</td>
<td>8.22 ± 0.28</td>
</tr>
<tr>
<td>RM</td>
<td>1.97 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.17 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>POST</td>
<td>3.85 ± 0.13&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7.77 ± 0.16&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>ALI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRE</td>
<td>3.79 ± 0.37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.17 ± 0.31</td>
</tr>
<tr>
<td>RM</td>
<td>2.32 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.15 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>POST</td>
<td>4.00 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.90 ± 0.38&lt;sup&gt;b,d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± SEM of 5 animals in each group (15 determinations in each situation/rat). Plateau pressure (Pplat) was determined before recruitment maneuver (PRE), just after recruitment (RM), and after 1-hour ventilation (POST) in zero end-expiratory pressure (ZEEP) or positive end-expiratory pressure (PEEP=5 cmH₂O). CTRL = control rats, and ALI = rats with paraquat induced acute lung injury. <sup>a</sup>Significantly different from PRE (p < 0.05); <sup>b</sup>Significantly different from group RM (p < 0.05); <sup>c</sup>significantly different from CTRL-PRE (p < 0.05); and, <sup>d</sup>significantly different from group CTRL-POST (p < 0.05).
Table 2. Morphometrical parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal (%)</th>
<th>Alveolar collapse (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-vent</td>
<td>94.00 ± 0.80</td>
<td>6.00 ± 1.20</td>
</tr>
<tr>
<td>CTRL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM-ZEEP</td>
<td>78.28 ± 1.27 (^a)</td>
<td>21.72 ± 1.27 (^a)</td>
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<tr>
<td>RM-PEEP</td>
<td>91.11 ± 0.32 (^b)</td>
<td>8.89 ± 0.32 (^b)</td>
</tr>
<tr>
<td>Non-vent</td>
<td>56.67 ± 2.56</td>
<td>43.33 ± 0.99</td>
</tr>
<tr>
<td>ALI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM-ZEEP</td>
<td>65.67 ± 0.81 (^a)</td>
<td>34.33 ± 0.81 (^a)</td>
</tr>
<tr>
<td>RM-PEEP</td>
<td>85.63 ± 0.26 (^a) (^b)</td>
<td>14.37 ± 0.26 (^a) (^b)</td>
</tr>
<tr>
<td>Non-vent</td>
<td>57.73 ± 0.63</td>
<td>42.27 ± 0.73</td>
</tr>
<tr>
<td>ATEL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RM-ZEEP</td>
<td>67.10 ± 0.90 (^a) (^b)</td>
<td>32.98 ± 0.90 (^a)</td>
</tr>
<tr>
<td>RM-PEEP</td>
<td>87.78 ± 0.25 (^a) (^b)</td>
<td>12.22 ± 0.25 (^a) (^b)</td>
</tr>
</tbody>
</table>

Values are means ± SEM of 5 animals in each group. All values are percentage of normal, collapsed areas in 10 random, non-coincident fields per rat. CTRL = control rats; ALI = rats with paraquat induced acute lung injury; ATEL = rats submitted to mechanical atelectasis, Non-vent = non-recruited and non-ventilated rats; RM-ZEEP and RM-PEEP = rats ventilated for 1 hour after recruitment in ZEEP or PEEP (5 cmH\(_2\)O), respectively. \(^a\)Significantly different from non-vent group (\(p < 0.05\)). \(^b\)Significantly different from group RM-ZEEP (\(p < 0.05\)).
Table 3. Lung histology score of alveolar collapse

<table>
<thead>
<tr>
<th>Group</th>
<th>Alveolar collapse (score)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-vent</td>
<td>0 (0-0.3)</td>
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<tr>
<td>CTRL</td>
<td>RM-ZEEP 1 (1-2)</td>
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<tr>
<td></td>
<td>RM-PEEP 0 (0-1) b</td>
</tr>
<tr>
<td>Non-vent</td>
<td>4 (3-4)</td>
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<tr>
<td>ALI</td>
<td>RM-ZEEP 3 (2-3) a</td>
</tr>
<tr>
<td></td>
<td>RM-PEEP 1 (1-2) a,b</td>
</tr>
<tr>
<td>Non-vent</td>
<td>4 (2.8-4)</td>
</tr>
<tr>
<td>ATEL</td>
<td>RM-ZEEP 2 (1-2) a</td>
</tr>
<tr>
<td></td>
<td>RM-PEEP 1 (0-1) a,b,c</td>
</tr>
</tbody>
</table>

Values are expressed as median (25th percentile, 75th percentile) of 5 animals in each group. Lung tissue injury was scored by two investigators who were blinded with regard to the group analyzed. CTRL = control rats; ALI = rats with paraquat induced acute lung injury; ATEL = rats submitted to mechanical atelectasis, Non-vent = non-recruited and non-ventilated rats; RM-ZEEP and RM-PEEP = rats ventilated for 1 hour after recruitment in ZEEP or PEEP (5 cmH₂O), respectively. \(^a\)Significantly different from non-vent group \((p < 0.05)\). \(^b\)Significantly different from group RM-ZEEP \((p < 0.05)\). \(^c\)Significantly different from group RM-PEEP in ALI group \((p < 0.05)\).
Figure 1.
Figure 2

**Lung Elastance (cmH₂O/ml)**

**ZEEP**

- POST: *
- PRE: *
- RM: **
- ZEEP: *

**PEEP**

- POST: **
- PRE: *
- RM: *
- ZEEP: **

<table>
<thead>
<tr>
<th>Condition</th>
<th>CTRL</th>
<th>ALI</th>
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<tbody>
<tr>
<td><strong>ZEEP</strong></td>
<td></td>
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</tr>
<tr>
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<tr>
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<td>POST</td>
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</table>
Figure 3

**ZEESP**

![Graph showing ΔP (cmH₂O) for ZEEP condition across different time points (PRE, RM, POST) for CTRL and ALI groups.](image)

**PEEP**

![Graph showing ΔP (cmH₂O) for PEEP condition across different time points (PRE, RM, POST) for CTRL and ALI groups.](image)
Figure 4
Figure 5

![Graph showing PCIII/GAPDH ratios for different groups: Non-vent, CTRL-ZEEP, CTRL-RM-ZEEP, ATEL-ZEEP, ATEL-RM-ZEEP, ALI-ZEEP, ALI-RM-ZEEP. Bars are labeled with asterisks (*) indicating statistical significance.](image)
Figure 6

CTRL

PCIII / GAPDH

M.W.  RT(-)  ZEEP  RM-ZEEP  RM-PEEP

ATEL

PCIII / GAPDH

M.W.  RT(-)  ZEEP  RM-ZEEP  RM-PEEP

ALI

PCIII / GAPDH

M.W.  RT(-)  ZEEP  RM-ZEEP  RM-PEEP