**Supplemental Digital Content 2**

***Additional Experiments***

*Animal preparation and experimental protocol*

All procedures on rats were approved by the Ethics Committee of the Carlos Chagas Filho Institute of Biophysics, Health Sciences Centre, Federal University of Rio de Janeiro (CEUA-019). Ten healthy Wistar rats (weight 433±32.5 g) were sedated (diazepam 10 mg/kg intraperitoneally), anesthetized (ketamine 75 mg/kg and midazolam 2 mg/kg intraperitoneally), and tracheotomised. A catheter was introduced into the right carotid artery for blood sampling and monitoring of mean arterial pressure (MAP). Body temperature was maintained at 37.5±1°C using a heating plate. Gelafundin® (B. Braun, São Gonçalo, RJ, Brazil) was administered in 0.5-mL increments to maintain MAP >60 mmHg. Animals were then paralyzed (pancuronium 0.4 mg intramuscularly, followed by a 0.4-mg intravenous bolus diluted in 1 mL Ringer’s lactate) and mechanically ventilated (Servo-I, MAQUET, Sweden) in volume-controlled mode with VT=7 mL/kg, minute ventilation=150 mL/min, inspiratory-to-expiratory ratio=1:2, fraction of inspired oxygen=0.4, and PEEP=1 cmH2O for 5 min. Arterial blood (300 μL) was drawn into a heparinized syringe to determine arterial oxygen partial pressure (PaO2), arterial carbon dioxide partial pressure (PaCO2), bicarbonate levels, and arterial pH (pHa) (ABL80 FLEX, Radiometer, Denmark). This was defined as the baseline time point for data collection (Baseline). Rats were then randomly assigned to one of two groups (n=5/group) to receive the following mechanical ventilation parameters: 1) VT=7 mL/kg with PEEP=3 cmH2O without RMs (Low-VT/Moderate-PEEP/RM-); or 2) VT=7 mL/kg with PEEP=6 cmH2O also without RMs (Low-VT/High-PEEP/RM-). After group allocation, laparotomy was performed and animals were ventilated for 4 hours. The respiratory rate was adjusted to reach a minute ventilation of 150 mL/min. At the start of mechanical ventilation and 3 hours thereafter, a standardized bowel manipulation was performed as follows: under sterile conditions, lateral retractors were carefully placed, the bowel was gently taken out of the abdominal cavity and reintroduced within 20 seconds. The retractors were left in place and the abdominal cavity continuously humidified with warmed saline at 37°C. Respiratory system mechanics, heart rate (HR), MAP, and the amount of fluids infused were measured hourly. At the end of the experiment, animals were killed by injection of sodium thiopental (60 mg/kg) and the lungs extracted for post-mortem analysis.

*Data acquisition and processing*

Airflow and airway pressure were continuously recorded throughout the experiments with a computer running software written in LabVIEW® (National Instruments; Austin, Texas, USA). VT was calculated by digital integration of the flow signal.1 All signals were filtered, amplified (SC-24, SCIREQ, Montreal, QC, Canada), and sampled at 200 Hz with a 12-bit analogue-to-digital converter (National Instruments; Austin, TX, USA). Peak (Ppeak,RS) and mean (Pmean,RS) airway pressures, as well as respiratory system plateau pressure (Pplat,RS), were computed offline by a routine written in MATLAB (Version R2007a; The Mathworks Inc., Natick, MA, USA).2

*Measurement of driving pressure, energy, and mechanical power*

Respiratory system driving pressure (ΔPRS) was calculated as the difference between Pplat,RS and minimal pressure per cycle.3 Energy (E) applied by the ventilator to the respiratory system, was calculated by numerical integration of the inspiratory airway pressure-volume curve *versus* volume (the area between the inspiratory limb of the pressure volume curve and the volume axis). Accordingly mechanical power (PV) applied by the ventilator was calculated by multiplication of energy by respiratory rate RR as PV = E ∙ RR.4,5

*Histology*

At euthanasia, heparin (1000 IU) was injected into the tail vein, the trachea was clamped at end-expiration, and lungs were removed *en bloc*. The left lung was frozen in liquid nitrogen and submerged in Carnoy’s solution.6,7 Slices 4 μm thick were stained with haematoxylin and eosin. Lung morphometric analysis was performed using an integrating eyepiece with a coherent system consisting of a grid with 100 points and 50 lines of known length coupled to a conventional light microscope (Olympus BX51; Olympus Latin America, Brazil). The volume fractions of the lung occupied by collapsed alveoli, normal pulmonary areas, or hyperinflated structures were determined by the point-counting technique at a magnification of ×200 across 10 random, non-coincident microscopic fields.8

*Immunohistochemistry*

The right lower lung was immersed in immunohistochemistry solution. To evaluate the degree of epithelial cell damage, E-cadherin (the major transmembrane protein of the adherens junction) was analysed.9 Immunohistochemical procedures were performed on lung sections using a mouse polyclonal antibody against E-cadherin (cat. 610181, BD Transduction Laboratories™, 1:300). Visualization and image capture were performed using a light microscope (Eclipse E800, Nikon, Japan) coupled to a digital camera (Evolution, Media Cybernetics Inc., Bethesda, MD, USA) and Q-Capture 2.95.0 graphic interface software (version 2.0.5; Quantitative Imaging, Surrey, BC, Canada). Expression of E-cadherin was analysed using ImagePro Plus software (version 4.5.1, Media Cybernetics, Rockville, MD, USA).

*Molecular biology*

The right middle lung was flash-frozen by immersion in liquid nitrogen and stored at −80ºC for quantification of mRNA expression. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed to measure the expression in lung tissue of type III procollagen (PCIII), a biological marker associated with fibrogenesis; amphiregulin, a marker of pulmonary stretch; receptor for advanced glycation end products (RAGE), a marker of damage inflicted to type I epithelial cells; and vascular cell adhesion molecule 1 (VCAM-1), a marker of endothelial cell damage. Total RNA was extracted from frozen lung tissue slices with the RNeasy plus® mini kit (Qiagen, USA). RNA concentration was measured by spectrophotometry in a Nanodrop ND-1000 system (Thermo Fisher Scientific, USA). First-strand complementary DNA was synthesized from total RNA using the QuantiTect® Reverse Transcription Kit (Qiagen, USA). Relative mRNA levels were measured with a SYBR green detection system using ABI 7500 real-time PCR (Applied Biosystems, USA). Samples were measured in triplicate. Relative gene expression was calculated as a ratio of the average gene expression levels compared with the reference gene (acidic ribosomal phosphoprotein P0, *36B4*)10 and expressed as fold change relative to healthy non-operated and non-ventilated controls (NO-NV).

The following primers (Integrated DNA Technologies, San Diego, CA) were used: amphiregulin (sense 5′-TTTCGCTGGCGCTCTCA-3′ and antisense 3′- TTCCAACCCAGCTGCATAATG-5′); PCIII (sense 5′-ACCTGGACCACAAGGACAC-3 and antisense 5′-TGGACCCATTTCACCTTTC-3′); RAGE (sense 5′-TGAACTCACAGCCAATGTCC-3′ and antisense 5′-ACAACTGTCCCTTTGCCATC-3′); VCAM-1 (sense 5′- TGCACGGTCCCTAATGTGTA and antisense 5′- TGCCAATTTCCTCCCTTAAA), and acidic ribosomal phosphoprotein P0, 36B4 (sense 5′-AATCCTGAGCGATGTGCAG-3′ and antisense 5′-GCTGCCATTGTCAAACAC-3′).

*Enzyme-linked immunosorbent assay (ELISA)*

The right upper lung was flash-frozen in liquid nitrogen and stored at −80ºC for ELISA. Tumour necrosis factor (TNF)-α levels were quantified by ELISA in the lung homogenate. All procedures were done according to the manufacturer’s protocol (Peprotech, London, UK) and normalized to total protein as assessed by Bradford’s reagent (Sigma-Aldrich, St Louis, MO, USA).

*Statistical analysis*

Variables were tested for normality using the Kolmogorov–Smirnov test. Parametric data were expressed as means ± standard deviation (SD), and nonparametric data as median (interquartile range). The main results were presented as treatment effects (difference in means between groups) and their 95% confidence intervals (CIs). To compare respiratory system mechanics and blood gas analysis over time, two-way repeated-measures ANOVA followed by the Bonferroni post-hoc test was used. For interactions between group and time, we adopted a less conservative p-value (p<0.10) as significant. To compare the NO-NV group with each ventilatory strategy, Student *t*-tests followed by the Bonferroni–Holm procedure were used (p-value adjusted for 4 comparisons, p<0.0125). The percentage of alveolar collapse, E-cadherin, and the expression of biological markers among mechanical ventilation groups were compared using the Kruskal–Wallis test followed by Dunn’s post hoc test. Correlations were assessed using Spearman’s test, as data were distributed non-parametrically. All tests were performed in GraphPad Prism version 6.07 (GraphPad Software, La Jolla, CA, USA). The significance level was set at 5%.

The sample size calculation of each group was based on our experimental experience, which allows detection of significant differences with the smallest possible number of animals, and on the respiratory effects observed in a previous study in rodents using comparable ventilator settings. 1-5,7 A sample size of five to seven animals per group would provide the appropriate power (1-β = 0.8) to identify significant (α = 0.05) differences in Pplat,RS between ventilatory strategies based on low VT associated with 1) low PEEP without RMs and 2) high PEEP with RMs, taking into account an effect size d = 1.76, a two-sided test, and a sample size ratio = 1 (G\*Power 3.1.9.2, University of Düsseldorf, Germany).

**References**

1. Riva DR, Oliveira MB, Rzezinski AF et al. Recruitment maneuver in pulmonary and extrapulmonary experimental acute lung injury. *Crit Care Med* 2008; **36**:1900-1908.

2. Silva PL, Moraes L, Santos RS et al. Recruitment maneuvers modulate epithelial and endothelial cell response according to acute lung injury etiology. *Crit Care Med* 2013; **41**:e256-265.

3. Samary CS, Santos RS, Santos CL et al. Biological Impact of Transpulmonary Driving Pressure in Experimental Acute Respiratory Distress Syndrome. *Anesthesiology* 2015; **123**:423-433.

4. Gattinoni L, Tonetti T, Cressoni M et al.Ventilator-related causes of lung injury: the mechanical power. *Intensive Care Med* 2016;**42**:1567-75.

5. Cressoni M, Gotti M, Chiurazzi C et al. Mechanical Power and Development of Ventilator-induced Lung Injury. *Anesthesiology* 2016 May;124(5):1100-8

6. Passaro CP, Silva PL, Rzezinski AF et al. Pulmonary lesion induced by low and high positive end-expiratory pressure levels during protective ventilation in experimental acute lung injury. *Crit Care Med* 2009; **37**:1011-1017.

7. Santos CL, Moraes L, Santos RS et al. The biological effects of higher and lower positive end-expiratory pressure in pulmonary and extrapulmonary acute lung injury with intra-abdominal hypertension. *Crit Care* 2014; **18**:R121.

8. Cruz-Orive LM, Weibel ER. Recent stereological methods for cell biology: a brief survey. *Am J Physiol* 1990; **258**:L148-156.

9. Samary CS, Moraes L, Santos CL, et al. Lung Functional and Biologic Responses to Variable Ventilation in Experimental Pulmonary and Extrapulmonary Acute Respiratory Distress Syndrome. *Crit Care Med* 2016.

10. Akamine R, Yamamoto T, Watanabe M et al. Usefulness of the 5' region of the cDNA encoding acidic ribosomal phosphoprotein P0 conserved among rats, mice, and humans as a standard probe for gene expression analysis in different tissues and animal species. *J Biochem Biophys Methods* 2007; **70**:481-486.