**Supplemental Digital Content 1**

**Detailed Methods**

**Study approval**

This study was approved by the Animal Care and Use Committee (CEUA: 086/17) of the Health Sciences Center, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the U.S. National Academy of Sciences *Guide for the Care and Use of Laboratory Animals*. The present study followed the ARRIVE guidelines for reporting of animal research.[1](#_ENREF_1)

**Animal preparation and experimental protocol**

Sixty-four male Wistar rats (8-10 weeks, weight 376 ± 52 g) were anesthetized by inhalation of 2% sevoflurane (Sevorane®; Cristália, Itapira, SP, Brazil), and received intratracheal (i.t.) instillation of *Escherichia coli* lipopolysaccharide (O55:B5, LPS Ultrapure, Invivogen), 200 µg suspended in saline solution to a total volume of 200 µL[2](#_ENREF_2), to induce acute respiratory distress syndrome (ARDS). After 24 h, during the morning, animals were premedicated intraperitoneally (i.p.) with 10 mg/kg diazepam (Compaz, Cristália, Itapira, SP, Brazil), followed by 100 mg/kg ketamine (Ketamin-S+, Cristália, Itapira, SP, Brazil) and 2 mg/kg midazolam (Dormicum, União Química, São Paulo, SP, Brazil). After local anesthesia with 2% lidocaine (0.4 mL), a midline neck incision and tracheostomy were performed. Eight rats were used for molecular biology analysis and were not mechanically ventilated (non-ventilated, NV).

An intravenous (i.v.) catheter (Jelco 24G, Becton, Dickinson and Company, New Jersey, NJ, USA) was inserted into the tail vein, and anesthesia induced and maintained with midazolam (2 mg/kg/h) and ketamine (50 mg/kg/h). Additionally, 10 mL/kg/h Ringer’s lactate (B. Braun, Crissier, Switzerland) was administered i.v. Gelafundin® 4% (B. Braun, São Gonçalo, RJ, Brazil) was administered intravenously (in 0.5-mL increments) to maintain mean arterial pressure (MAP) ≥ 70 mmHg. A second catheter (18G, Arrow International, USA) was then placed in the right internal carotid artery for blood sampling and gas analysis (Radiometer ABL80 FLEX, Copenhagen NV, Denmark), as well as monitoring of MAP (Networked Multiparameter Veterinary Monitor LifeWindow 6000 V; Digicare Animal Health, Boynton Beach, FL, USA). A 30-cm-long water-filled catheter (PE-205, Becton, Dickinson and Company) with side holes at the tip, connected to a differential pressure transducer (UT-PL-400, SCIREQ, Montreal, QC, Canada), was used to measure the esophageal pressure (Pes). Proper positioning was assessed with the “occlusion test”.[3](#_ENREF_3) Heart rate (HR), MAP, and rectal temperature were continuously monitored (Networked Multiparameter Veterinary Monitor LifeWindow 6000V, Digicare Animal Health, Florida, USA). Body temperature was maintained at 37.5±1 °C using a heating bed.

Animals were paralyzed with pancuronium bromide (2 mg/kg, i.v.), and their lungs mechanically ventilated (V500; Dräger Medical, Lübeck, Germany) in volume-controlled mode (VCV) with constant inspiratory airflow, tidal volume (VT)=6 mL/kg, positive end-expiratory pressure (PEEP)=3 cmH2O, RR adjusted to PaCO2=35–45 mmHg (around 70 bpm), FiO2=0.4, and an inspiratory-expiratory ratio of 1:2 **(figure 1**). Arterial blood gases (300 µl) were determined using a Radiometer ABL80 FLEX analyzer (Copenhagen NV, Denmark) and respiratory system mechanics were assessed (INITIAL). Animals were then randomly assigned to receive (n=8/group): mechanical ventilation with a protective strategy (VT=6mL/kg) for 2 hours (Control); VT=6mL/kg during hour 1 followed by an abrupt increase of VT=22mL/kg until 2 hours (No adaptation time); VT=6mL/kg during the first 30 minutes followed by a gradual VT increase up to 22mL/kg for 30 minutes, then constant VT=22mL/kg until 2 hours (Shorter adaptation time); and a more gradual VT increase, from 6mL/kg to 22mL/kg during 1 hour followed by VT=22mL/kg until 2 hours (Longer adaptation time). The VT size of 22 mL/kg was chosen because it has been shown to promote VILI yet allow survival for 2 h.[4](#_ENREF_4) Respiratory system mechanics were measured every 30 min and at the end of the experiment (FINAL). At FINAL, arterial blood gases were also analyzed, heparin (1000 IU) was injected into the tail vein. The trachea was then clamped at the same airway pressure used at end-expiration (PEEP = 3 cmH2O) and all animals were killed by an overdose of sodium thiopental (60 mg/kg i.v.) followed by transection of the abdominal aorta and vena cava. Lungs were then extracted for histology and molecular biology analyses.

In order to dissociate the effects of the gradual increase of VT from the association between gradual VT increase and VTH maintenance, we also evaluated 1 h mechanical ventilation effects. For this purpose, 24 animals were ventilated in groups A, C, and D (n=8/group) and euthanized at 1 hour. Heterogeneity index, DAD score, and gene expression of biomarkers were then measured.

**Echocardiography**

Shaved animals were placed in the dorsal recumbent position. Transthoracic echocardiography was performed by an expert (N.N.R.) blinded to group allocation, using an UGEO HM70A system (Samsung, São Paulo, Brazil) equipped with a linear phased-array probe (8–13 MHz). Measurements were obtained in accordance with American Society of Echocardiography guidelines [5](#_ENREF_5). Images were obtained from the subcostal and parasternal views. The left ventricular ejection fraction (EF%) and cardiac output (L/min) were calculated in one-dimensional mode analysis of the left ventricle guided by the parasternal short-axis view. Pulsed-wave Doppler was used to measure the ratio between pulmonary acceleration time (PAT) and pulmonary ejection time (PET), which is an indirect index of pulmonary arterial hypertension. Heart rate was assessed from the subcostal view.

**Data Acquisition and Processing**

A pneumotachograph (internal diameter = 1.5 mm, length = 4.2 cm, distance between side ports = 2.1 cm) was connected to the tracheal cannula for airflow (V´) measurements.[6](#_ENREF_6) The pressure gradient across the pneumotachograph was determined using a SCIREQ differential pressure transducer (UT-PDP-02, SCIREQ, Montreal, QC, Canada). Airflow, airway pressure (Paw), and Pes were continuously recorded throughout the experiments with a computer running custom-made software written in LabVIEW (National Instruments, Austin, TX).[7](#_ENREF_7),[8](#_ENREF_8) Briefly, VT was calculated by digital integration of the airflow signal obtained from a custom-made pneumotachograph[6](#_ENREF_6) that was connected to the Y-piece of the ventilator tubing, while RR was calculated from the Pes swings as the frequency per minute of each type of breathing cycle.

Respiratory system and lung mechanics were assessed every 30 min by occluding the airways at end-inspiration for 5 seconds until a respiratory system plateau pressure (Pplat,RS) and transpulmonary plateau pressure (Pplat,L), respectively, were reached.[7](#_ENREF_7) Respiratory system driving pressure (P,RS) was calculated as the difference between Pplat,RS (post end-inspiratory pause) and PEEP. Transpulmonary pressure (P,L) was calculated as the difference between the Paw and Pes, whereas transpulmonary driving pressure (ΔP,L) was the difference between the transpulmonary pressure at end-inspiration (post-inspiratory pause) and end-expiration.

**Measurement of Mechanical Power, Energy, and Driving Pressure**

The mechanical power applied by the ventilator to the respiratory system was calculated as the product of energy and RR. The mechanical energy, in turn, was calculated using the following equation: Energy,L = ΔP,2L/Est,L, where Est,L is the static lung elastance; Energy,L = ΔP,2L/(ΔP,L/VT) = ΔP,LxVT, which is the area of a rectangle.[9](#_ENREF_9),[10](#_ENREF_10) Therefore, one must compute the area of the rectangle and divide the result by 2. Values were converted to mJ and multiplied by RR to obtain the mechanical power in mJ/min. This equation estimates the elastic work (the major component of total work) without taking into account resistive properties or PEEP.

Cumulative power was calculated for 1 and 2 hours. All signals were amplified in a four-channel signal conditioner (SC-24, SCIREQ, Montreal, QC, Canada), and sampled at 200 Hz with a 12-bit analogue-to-digital converter (National Instruments; Austin, Texas, USA). Mechanical data were computed offline by a routine written in MATLAB (Version R2007a; The Mathworks Inc., Natick, Massachusetts, USA).

**Histology**

The lungs and heart were removed *en bloc.* The left lung was frozen in liquid nitrogen and immersed in formaldehyde solution (4%), embedded in paraffin, cut longitudinally in the central zone by means of a microtome into three slices, each 4 μm thick, and stained with hematoxylin–eosin for histological analysis.[7](#_ENREF_7),[11](#_ENREF_11) Photomicrographs at magnifications of ×100, ×200, and ×400 were obtained from eight non-overlapping fields of view per section using a light microscope (Olympus BX51, Olympus Latin America Inc., Brazil). Diffuse alveolar damage (DAD) was quantified using a weighted scoring system by two investigators (V.M. and V.L.C.) blinded to group assignment and independently, as described elsewhere.[12](#_ENREF_12) Briefly, scores of 0 to 4 were used to represent atelectasis, ductal overdistension, interstitial edema, inflammation, and detachment of airway epithelium, with 0 standing for no effect and 4 for maximum severity. Additionally, the extent of each scored characteristic per field of view was determined on a scale of 0 to 4, with 0 standing for no visible evidence and 4 for complete involvement. Scores were calculated as the product of severity and extent of each feature, on a range of 0 to 16. The cumulative DAD score was calculated as the sum of each score characteristic and ranged from 0 to 80.

Airspace enlargement was assessed by measuring the mean linear intercept (Lm) between alveolar walls at a magnification of ×400.[13](#_ENREF_13) To characterize the heterogeneity of airspace enlargement, the mean linear intercept (D2 of Lm) was computed from 20 airspace measurements [14](#_ENREF_14), according to Equation 1:

 (1)

where μ is the mean, σ the variance of airspace diameters, and γ the skewness of the diameter distribution. After D2 calculation, the heterogeneity index (β) was derived from D2 and Lm  by their ratio.[15](#_ENREF_15)

**Biological Markers**

Quantitative real-time reverse transcription polymerase chain reaction (PCR) was performed by an investigator (F.C.) blinded to group assignment, to measure biomarkers associated with inflammation (interleukin [IL]-6), mechanical pulmonary stretch (amphiregulin) [16](#_ENREF_16), epithelial cell damage (club cell secretory protein 16 [CC16]), endothelial cell damage (vascular cell adhesion molecule-1 [VCAM-1]), ECM connection to epithelial cells (syndecan-1)[17](#_ENREF_17), metalloproteinase (MMP)-9[18](#_ENREF_18), and fibrosis (decorin)[19](#_ENREF_19) in lung tissue (for primers, see **Supplemental Digital Content 3 – table 1**). Central slices of the right lung were cut, collected in cryotubes, flash-frozen by immersion in liquid nitrogen, and stored at −80 °C. Total RNA was extracted from frozen tissues using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), following the manufacturer’s recommendations. RNA concentrations were measured by spectrophotometry in a Nanodrop ND-1000 system (Thermo Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized from total RNA using a Quantitec reverse transcription kit (Qiagen, Hilden, Germany). Relative mRNA levels were measured with a SYBR green detection system in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, California, USA). Samples were run in triplicate. For each sample, the expression of each gene was normalized to the acidic ribosomal phosphoprotein P0 (*36B4*) housekeeping gene[20](#_ENREF_20) and expressed as fold change relative to respective NV animals, using the 2–∆∆Ct method, where ΔCt = Ct (target gene) – Ct (reference gene).[21](#_ENREF_21)

**Statistical analysis**

The sample size was calculated to allow detection of the differences in IL-6 among ventilatory strategies, which was expected between VT = 6 and 22 mL/kg, based on previous work from our group.[7](#_ENREF_7) A sample size of 8 animals per group would provide the appropriate power (1 − β = 0.8) to identify statistically significant differences in IL-6 (adjusted α = 0.025 for two comparisons), taking into account an effect size d = 2.0, a two-sided *t-*test, and a sample size ratio = 1 (G\*Power 3.1.9.2, University of Düsseldorf, Düsseldorf, Germany).

The primary outcome was the difference in gene expression of IL-6 among ventilatory strategies, whereas the secondary outcomes were lung function, heterogeneity index, diffuse alveolar damage score, and expression of genes related to alveolar stretch, epithelial and endothelial cell injuries, and extracellular matrix damage.

Data were tested for normality using the Kolmogorov-Smirnov test with Lilliefors’ correction, while the Levene median test was used to evaluate the homogeneity of variances. If both conditions were satisfied, time-dependent differences among groups were determined with mixed linear models based on a random intercept for each animal followed by Bonferroni tests. Molecular biology variables and DAD score were assessed with the Kruskal-Wallis test followed by Dunn’s test. Spearman correlation was performed. Parametric data were expressed as mean ± SD, while nonparametric data were expressed as median (interquartile range). Mixed linear models and multiple linear regression analyses were carried out in IBM SPSS Statistics for Windows, Version 19.0 (IBM Corp., USA). All the other tests were carried out in GraphPad Prism 6.00 (GraphPad Software, La Jolla, CA, USA). No outliers were excluded from the analysis. Significance was established at *p*<0.05.

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