Supplementary Digital Content

Low spontaneous breathing effort during Extracorporeal Membrane Oxygenation in a porcine model of severe Acute Respiratory Distress Syndrome

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1. Supplementary Methods

The study was approved by the Animal Ethics Committee of Pontificia Universidad Católica de Chile (150811040). The experimental protocol complies the basics principles of the Chilean Law 20.380 about animal protection; the Terrestrial Animal Health Sanitary Code (OIE, 24a Edition, 2015); the Directive 2010/63/EU in Europe on the protection of animals used for scientific purposes; and the Guide for the Care and Use of Laboratory Animals (NRC, 8a Edition, 2011). At the same time, this study complies with the Three Rs (3Rs) principles (Replacement, Reduction, and Refinement) for the ethical use of animals in testing.

Preparation and maintenance Phase

Twelve female pigs (*sus scrofa domestica*, 30±5 kg) were used in the study. Animals were housed at the research facility the day before, fasted for solid food 12 hours before experiments, with water access ad libitum. Early at the morning, animals were pre-medicated with intramuscular ketamine (20 mg/kg) and xylazine (2 mg/kg) and then anesthetized with a combination of intravenous fentanyl (30 ug/kg), midazolam (0.25 mg/kg) and atracurium (0.5 mg/kg). Pigs were then intubated with an endotracheal tube (6.5 ID), and connected to a mechanical ventilator (Dräger Evita XL®, Lübeck, Germany) in volume-controlled ventilation (VCV) mode: positive end-expiratory pressure (PEEP) of 5 cmH_2O, tidal volume (Vt) 10 ml/kg, I:E ratio 1:2, respiratory rate (RR) was initially set at 16-18 breaths/min to keep $PaCO_2$ between 30- 50 mmHg. Inspired oxygen fraction (FiO_2) was kept at 1.0 throughout the whole experiment. Anesthesia was maintained with a continuous intravenous infusion of a solution consisting of midazolam 2 mg/ml, xylazine 1 mg/ml and ketamine 20 mg/ml, set at 0.5 ml/kg/h during invasive procedures and induction of lung injury and at 0.25-0.5 ml/kg/h thereafter until the end of the experiment. Depth of anesthesia was assessed regularly by checking for movements and hemodynamic response to a painful stimulus (*e.g.* pinch toe). Muscle paralysis was maintained with a continuous infusion of atracurium (0.5 mg/kg/h) throughout the experiment. At the time of instrumentation, a dose of 1 gr of cephazolin was administered intravenously and repeated every 8 hours thereafter.

Under sterile conditions, the left carotid artery was surgically exposed for insertion of an arterial catheter. A pulmonary artery catheter was placed through the right femoral vein with ultrasound guidance. After

completing instrumentation, a 32-electrode belt for electrical impedance tomography (EIT) was fitted at the mid-thoracic level to assess lung impedance distribution (EIT-Pioneer Set, Swisstom, Switzerland). An esophageal balloon catheter (Cooper Surgical, Inc., USA) was placed in the lower third of the esophagus, inflated, and checked by a dynamic occlusion test (1). Before each measurement, the optimal balloon filling volume was reassessed to obtain the largest tidal swing in esophageal pressure (Pes) (1.0 to 2.0 ml) (2). The Pes signal was monitored through a pneumotach (Data Acquisition System, Hans Rudolf, Inc., USA). Respiratory variables, including $ETCO_2$ and VCO_2, were assessed by a non-invasive cardiac output (NICO®) monitor (Novametrix Medical Systems Inc., Wallingford, CT, U.S.A. Animals received normal saline at 30 ml/kg during the first hour of preparation, followed by 20 ml/kg/h during induction of lung injury and until T_0, and then 10 ml/kg/h during the 24-hour study period.

Induction of lung injury

After the preparation phase, a 2-hit model of lung injury was induced under deep anesthesia. First, repeated lung lavages with warm saline (30 ml/kg, 39°C) were performed until PaO_2/FiO_2 felt below 100 for at least 15 minutes. MV settings between lavages remained the same as during the preparation phase described above. Subsequently, two hours of injurious ventilation was started in pressure-controlled ventilation, with PEEP 0 cmH_2O, inspiratory pressure of 40 cmH_2O, RR of 10/min, I:E 1:1 and FiO_2 100%. After completing the two hours, ventilator settings were switched back to those used at baseline, and after 10 minutes, before starting ECMO, a full assessment of all variables was registered (time 0).

Extracorporeal membrane oxygenation (ECMO) support

The ECMO equipment included a magnetic Medtronic Bio-Medicus® 540 centrifuged pump (Eden Prairie, MN, USA), a coagulation monitor (Hemochron® Response, ITC, USA), and a heat exchanger HU-35 (Maquet, USA). The circuit comprised a HILITE® 2400LT polymethyl pentene hollow fiber membrane oxygenator, 0.65 m2 (MEDOS, Stolberg, Germany), polyvinyl chloride ¼-inch lines coated with rheoparin, and a Rotaflow 32 head pump (Maquet, USA). The circuit was primed with saline.

Cannulation was performed during the second hour of injurious ventilation. Under sterile conditions, the right external jugular vein was surgically exposed and a 23-F double-lumen cannula (AVALON ELITE®, Maquet, USA) was inserted and directed towards the inferior vena cava. Anticoagulation was induced with heparin with an intravenous bolus (100 IU/kg), followed by a continuous infusion targeting an activated clotting time (ACT) of 180–220s (Hemochron® Response, ITC, USA). The cannula was connected to the circuit after time 0 measurements and extracorporeal circulation started progressively. The pump was adjusted to target a blood flow at 60-70 ml/kg/min. Heat exchanger was set at 38 °C. The initial sweep gas flow (FiO_2 1.0) was adjusted to $PaCO_2$ 30-50 mmHg.

During the first 3 hours, all animals were ventilated with near-apneic ventilation (pressure control ventilation, PEEP 10 cmH_2O, driving pressure 10 cmH_2O, RR 5/min, I:E ratio 1:2) (3) and keeping muscle paralysis. After T_3, animals were allocated by simple randomization into 2 groups:

I. Near-apneic ventilation, which continued with the same settings previously described (time 0 to time 3).
II. Spontaneous Breathing: in this group, neuromuscular blockade was stopped, and sweep gas flow decreased until regaining respiratory efforts, without modifying sedation. Thereafter, ventilation was switched to Pressure Support Ventilation (PSV) (PS 10 cmH_2O, PEEP 10 cmH_2O) and sweep gas flow adjusted to keep $PaCO_2$ 30-50 mmHg. The goals in this group were to keep the animals with continuous spontaneous breathing efforts and without agitation. Although no specific targets were defined for esophageal pressure swings or tidal volumes, in pilot experiments we observed that moderate to high breathing efforts were generally associated with agitation and that most pigs maintained regular low spontaneous breathing efforts without agitation at respiratory rates of 30-50 breaths/minute.

Physiological measurements and sample collection

Heart rate, pulse oximetry, core temperature, arterial blood pressure, pulmonary artery pressure, respiratory rate, and respiratory mechanics, ventilator and ECMO settings, anesthetic drugs, and maintenance fluid, as well as infusion drugs for hemodynamic support, were registered at baseline, after completing lung injury

(time 0), and at 3, 6, 12 and 24 hours of the study period (time 3, time 6, time 12 y time 24). Blood was drawn for arterial and mixed venous blood gas analysis (i-STAT®-1 immunoready) samples at baseline, time 0, time 3, time 6, time 12 and time 24

Airway pressure and flow signals, as well as $ETCO_2$, VCO_2, and Pes signals were transferred to a laptop, and integrated, recorded, and analyzed by a custom LabVIEW-based software (National Instruments™, USA).

EIT images were recorded at 50 Hz at each time point during the protocol. Lung images were divided into four symmetrical nonoverlapping regions of interest (ROIs): (1) ventral, (2) central–ventral, (3) central–dorsal, and (4) dorsal regions. ROIs have equal height and each corresponds to 25% of the anteroposterior diameter. Values represent percent changes in local impedance as compared with a peak-expiratory reference image taken at the beginning of each acquisition. Changes in impedance (ΔZ) during the study were defined as the difference between end-inspiratory and end-expiratory lung impedance. End-expiratory lung impedance variations along time relative to baseline values were also assessed and expressed as % end-expiratory lung impedance.

Tissue sampling and Histological analysis

At the end of the protocol, animals were euthanized under deep anesthesia by an overdose of thiopental and T- 61 solution IV (Intervet International GMBH, Germany). A thoracotomy and a laparotomy were performed, and the lungs were excised in a block. The right lung was separated and fixed in cold formalin for 24 hours, followed by extraction of representative lung tissue samples from six areas (upper, non-dependent middle, central middle, dependent middle, non-dependent lower, and dependent lower), which were embedded in paraffin for histologic preparation. The left lung was dissected as above extracting two samples from each of the six areas: one sample was weighted and dried for 3 days at 50ºC to calculate gravimetric lung edema (wet/dry ratio), and the other sample was frozen at -80ºC for later analysis.

To assess lung injury, tissue slices were cut from paraffin blocks, stained with hematoxylin and eosin, and observed with light microscopy. A validated score (4) was used to evaluate 3 parameters of lung injury: a)

intra-alveolar neutrophil exudate, b) alveolar disruption and c) intra-alveolar hemorrhage; each of these categories received a score ranging from 0 to 3, where 0 corresponds to no pathologic alteration, 1 corresponds to mild, 2 corresponds to moderate and 3 corresponds to severe pathologic alteration (Supplementary Figure 1, Supplemental Digital Content 1). Ten random areas were evaluated for each section at 100x magnification and its values averaged. Histological assessment was performed by a board-certified pathologist, who was blinded to time and group assignment.

A commercially available ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) was used to evaluate IL-1b and IL-8 levels in lung tissue homogenates from the middle region of the left lung (dependent and non-dependent).

Statistical analysis

As we had no reliable data about the potential impact on lung injury, of a strategy of low spontaneous breathing effort compared to near-apneic ventilation, during ARDS assisted with ECMO, sample size calculation was based on effect estimates obtained from a previous study in which we compared a near-apneic ventilation strategy with a less protective ventilation using the same animal model.[17] In that study which included 6 animals per group, the near apneic ventilation group had a mean histological lung injury score of 0.7±0.3, vs 1.3±0.2 observed in the group ventilated with Vt of 6 ml/kg. Accordingly, we calculated that six animals per group were required to find a 50% difference in histologic lung injury scores between groups, using *t-test* family, two-sided test, and equal number of animals per group, with 80% power and an alpha level of 5%.

The normality of the variables was tested by the Shapiro-Wilk test. Data measured along time were analyzed, when appropriate, using repeated measures two-way ANOVA, followed by Tukey's multiple comparisons test; or Friedman Test of variance for repeated measures, followed by pairwise comparisons using Dunn post hoc test with Bonferroni correction; both for differences between groups and along time (time 0 compared to Baseline and all other time points compared to time 0). Data derived from lung tissue analysis were compared with the Mann Whitney test. Outliers were detected checking standardized

residuals, but no action was taken. Statistical analysis was performed with GraphPad Prism 7 (GraphPad Software, USA) for all analyses, with a two-tailed p-value <0.05 and 95% confidence intervals.

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Supplementary Table 1. Relative regional impedance distribution

Variable	Time					
	Baseline	0	3	6	12	24
Ventral (%)						
Near-apneic ventilation	3.7 ± 0.3	4.8 ± 0.6	6.6 ± 1.3	5.3 ± 0.6	4.7 ± 1.3	4.7 ± 0.6
Spontaneous breathing	3.7 ± 0.4	5.5 ± 2.7	6.2 ± 2.7	7.7 ± 2.9	5.2 ± 2.0	6.5 ± 2.4
Central Ventral (%)						
Near-apneic ventilation	26.3 ± 0.9	34.8 ± 1.8	36.8 ± 2.2	35.4 ± 2.1	31.2 ± 4.7	31.5 ± 3.1
Spontaneous breathing	27.0 ± 1.9	40.3 ± 4.8§	31.1 ± 1.1	30.3 ± 3.1†	27.1 ± 4.4†	29.0 ± 3.5†
Central Dorsal (%)						
Near-apneic ventilation	47.8 ± 0.9	49.0 ± 1.2	42.3 ± 2.7	44.7 ± 0.9	49.4 ± 3.1	44.8 ± 3.0
Spontaneous breathing	47.9 ± 0.3	44.0 ± 5.4	46.0 ± 3.2	43.1 ± 3.3	45.7 ± 3.3	42.2 ± 3.8
Dorsal (%)						
Near-apneic ventilation	22.1 ± 1.5	11.4 ± 2.0§	14.3 ± 2.6	14.6 ± 2.8	14.8 ± 2.5	19.0 ± 3.5
Spontaneous breathing	21.4 ± 1.8	10.2 ± 2.2§	16.6 ± 1.0	18.9 ± 2.6	21.9 ± 5.2†	22.3 ± 3.6†

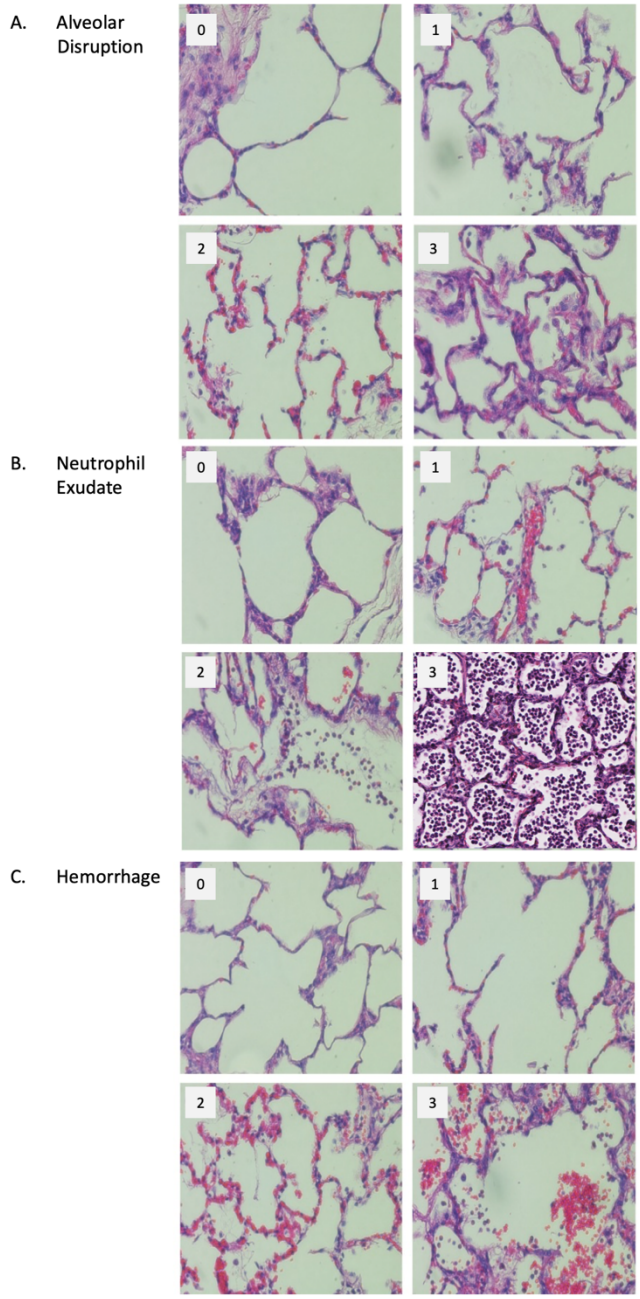
Values are expressed as mean ± standard error of the mean.
***** p<0.05 comparing groups. All time points were compared to time 0. § p < 0.05 for time 0 compared to baseline, † p < 0.05 compared to time 0.

Supplementary Table 2. Histological parameters of lung injury in different lung areas.

Variable	Lung zone					
	1 Upper	2 Non-dependent middle	3 Central middle	4 Dependent middle	5 Non-dependent lower	6 Dependent lower
Alveolar Disruption						
Near-apneic ventilation	0.9 (0.7-1.7)	1.0 (0.4-1.5)	1.1 (0.8-1.4)	1.2 (1.0-1.4)	1.1 (1.0-1.5)	1.1 (1.1-1.9)
Spontaneous breathing	0.9 (0.4-2.6)	1.4 (0.9-1.6)	1.2 (0.5-2.0)	1.0 (1.0-1.7)	1.0 (1.0-1.9)	1.0 (1.0-1.9)
Neutrophil Exudate						
Near-apneic ventilation	0.9 (0-1.0)	1.0 (0.1-1.0)	1.0 (0.1-1.4)	1.0 (0.1-2.2)	1.0 (0.2-1.0)	0.7 (0-1.0)
Spontaneous breathing	0.9 (0-1.0)	1.1 (0.9-1.1)	1.2 (0.7-1.7)	1.5 (1.0-2.0)	1.1 (0.2-1.7)	1.0 (0-1.9)
Hemorrhage						
Near-apneic ventilation	0.2 (0-1.0)	0.4 (0-1.0)	0.3 (0-1.0)	0.7 (0-1.0)	0.7 (0-1.0)	0.7 (0-1.0)
Spontaneous breathing	0.9 (0-1.3)	1.0 (0-1.3)	1.0 (0.6-1.1)	1.2 (1.0-1.6)	1.0 (0.8-1.7)	1.0 (0.-1.0)
Global Score						
Near-apneic ventilation	0.6 (0.2-1.2)	0.7 (0.4-1.2)	0.8 (0.4-1.3)	0.9 (0.5-1.4)	0.9 (0.7-1.0)	0.9 (0.5-1.1)
Spontaneous breathing	0.7 (0.5-1.6)	1.2 (0.7-1.4)	1.0 (0.8-1.6)	1.2 (1.0-1.6)	1.1 (0.7-1.6)	0.9 (0.3-1.3)

Values correspond to scores for alveolar disruption, neutrophil effusion and hemorrhage (from 0 = normal, to 3 = maximal alteration), in each areas of the right lung. Data are expressed as median and range.
* p < 0.05 comparing groups

Supplementary Figure 1.



Supplementary Figure 1. Representative images of histological lung tissue analysis by parameter. A 3-parameter score was used to evaluate lung injury, including alveolar disruption (Panel A), intra-alveolar neutrophil exudate (Panel B), and intra-alveolar hemorrhage (Panel C). 0 corresponds to no pathologic alteration, 1 corresponds to mild, 2 corresponds to moderate and 3 corresponds to severe pathologic alteration (Magnification 200X).