Am J Transl Res 2016;8(6):2826-2837
www.ajtr.org /ISSN:1943-8141/AJTR0026498

Original Article

Extracorporeal membrane oxygenation improves survival in a novel 24-hour pig model of severe acute respiratory distress syndrome

Joaquín Araos[1], Leyla Alegría[1], Patricio García[2], Felipe Damiani[1], Pablo Tapia[3], Dagoberto Soto[1], Tatiana Salomon[4], Felipe Rodriguez[5], Macarena Amthauer[5], Benjamín Erranz[6], Gabriel Castro[5], Pamela Carreño[7], Tania Medina[7], Jaime Retamal[1], Pablo Cruces[7,8], Guillermo Bugedo[1], Alejandro Bruhn[1]

[1]Departamento de Medicina Intensiva, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile; [2]Carrera de Kinesiología, UDA Ciencias de la Salud, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile; [3]Unidad de Pacientes Críticos, Hospital La Florida, Santiago, Chile; [4]Unidad de Cuidados Intensivos Pediátricos, Clinica Alemana, Santiago, Chile; [5]Centro de Pacientes Críticos, Hospital Clínico de Red de Salud UC CHRISTUS, Santiago, Chile; [6]Centro de Medicina Regenerativa, Facultad de Medicina, Clínica Alemana Universidad del Desarrollo, Santiago, Chile; [7]Unidad de Cuidados Intensivos Pediátricos, Hospital El Carmen, Dr. Luis Valentín Ferrada, Santiago, Chile; [8]Centro de Investigación de Medicina Veterinaria, Escuela de Medicina Veterinaria, Facultad de Ecología y Recursos Naturales, Universidad Andres Bello, Santiago, Chile

Received February 23, 2016; Accepted April 27, 2016; Epub June 15, 2016; Published June 30, 2016

Abstract: Extracorporeal membrane oxygenation (ECMO) is increasingly being used to treat severe acute respiratory distress syndrome (ARDS). However, there is limited clinical evidence about how to optimize the technique. Experimental research can provide an alternative to fill the actual knowledge gap. The purpose of the present study was to develop and validate an animal model of acute lung injury (ALI) which resembled severe ARDS, and which could be successfully supported with ECMO. Eighteen pigs were randomly allocated into three groups: sham, ALI, and ALI + ECMO. ALI was induced by a double-hit consisting in repeated saline lavage followed by a 2-hour period of injurious ventilation. All animals were followed up to 24 hours while being ventilated with conventional ventilation (tidal volume 10 ml/kg). The lung injury model resulted in severe hypoxemia, increased airway pressures, pulmonary hypertension, and altered alveolar membrane barrier function, as indicated by an increased protein concentration in bronchoalveolar fluid, and increased wet/dry lung weight ratio. Histologic examination revealed severe diffuse alveolar damage, characteristic of ARDS. Veno-venous ECMO was started at the end of lung injury induction with a flow > 60 ml/kg/min resulting in rapid reversal of hypoxemia and pulmonary hypertension. Mortality was 0, 66.6 and 16.6% in the SHAM, ALI and ALI + ECMO groups, respectively ($p < 0.05$). This is a novel clinically relevant animal model that can be used to optimize the approach to ECMO and foster translational research in extracorporeal lung support.

Keywords: ECMO, ARDS, mechanical ventilation

Introduction

Despite the increasing use of extracorporeal membrane oxygenation (ECMO) in acute respiratory distress syndrome (ARDS), there is quite low evidence about its efficacy or how to optimize its application [1, 2]. Although the number of patients receiving ECMO is increasing steadily worldwide [3], it is still difficult to perform controlled studies in these patients. At this stage, the few published large clinical trials have been focused on trying to demonstrate the general efficacy of ECMO to improve survival [4]. However, other relevant aspects of care such as mechanical ventilation, during ECMO, or the introduction of technological innovations in extracorporeal circulation, have not been well studied and current practices are widely variable and based mainly on uncontrolled studies or on general recommendations [5-7]. Animal models and translational research can provide an alternative to explore the optimal care and novel interventions during ECMO.

A few animal studies with ECMO have been published during the last years exploring physiologic consequences of ECMO on coagulation, pharmacokinetics or organ function [8-11]. Some of these studies have used acute lung injury models. However, the reported models do not appear well suited or relevant to study optimal approaches to care during ECMO. Major limitations of current models are doubtful clinical relevance concerning severe ARDS, insufficient duration of the models to detect more subtle modifications, or insufficient severity compared to ARDS patients treated with ECMO [10, 12].

The goal of the present study was to develop and characterize a large animal model of acute lung injury which resembled clinical severe ARDS, refractory to conventional mechanical ventilation, and which could be supported successfully with high flow veno-venous ECMO (vvECMO) for at least 24 hours.

Materials and methods

This study was conducted with the approval of the Pontificia Universidad Católica de Chile Animal Ethics Committee, approval number 12-029. All experiments were performed in accordance to the *Guide for the Care and Use of Laboratory Animals*, 8th Edition, from the National Academy of Sciences of the United States of America.

Animal preparation

Eighteen pigs (*Sus scrofa domestica*) (30±5 kg) were used in the study. Animals were housed at the research facility the day before, fasted for 12 hours before experiments, with water access *ad libitum*. Animals were pre medicated with ketamine (20 $mg \cdot kg^{-1}$) + xylazine (2 $mg \cdot kg^{-1}$) + Atropine (0.05 $mg \cdot kg^{-1}$) intramuscularly. Once sedated, a catheter was placed in the marginal ear vein, and anesthesia induced with a combination of fentanyl (30 $\mu g \cdot kg^{-1}$), midazolam (0.25 $mg \cdot kg^{-1}$) and atracurium (0.5 $mg \cdot kg^{-1}$) intravenously. Pigs were then intubated with an endotracheal tube (6.0-7.0 ID), and connected to a mechanical ventilator in volume controlled ventilation (VCV) mode (Dräger Evita XL®, Lübeck, Germany). Initial ventilatory settings included positive end-expiratory pressure (PEEP) of 5 cmH_2O, tidal volume (Vt) 10 $ml \cdot kg^{-1}$, and an I:E ratio 1:2. Respiratory rate (RR) was initially set at 16-18$\cdot min^{-1}$, and adjusted thereafter to keep $PaCO_2$ between 30-50 mmHg. Inspired oxygen fraction (FiO_2) was kept at 1.0 throughout all the experiment. Anesthesia was maintained with a continuous intravenous infusion of a solution consisting of midazolam 2 $mg \cdot ml^{-1}$, fentanyl 20 $\mu g \cdot ml^{-1}$, and ketamine 20 $mg \cdot ml^{-1}$, set at 0.5 $ml \cdot kg^{-1} \cdot h^{-1}$ during invasive procedures and induction of lung injury, and at 0.25 $ml \cdot kg^{-1} \cdot h^{-1}$ thereafter until the end of the experiment. Depth of anesthesia was assessed regularly by checking for movements or hemodynamic response to a painful stimulus. Muscle paralysis was maintained with a continuous infusion of atracurium (0.5 $mg \cdot kg^{-1} \cdot h^{-1}$) throughout the experiment. At the time of instrumentation, 30 $mg \cdot kg^{-1}$ of cephazolin was administered intravenously and repeated every 8-hours thereafter. Animals received normal saline at 10 $ml \cdot kg^{-1} \cdot h^{-1}$ during preparation and while inducing lung injury, and 2 $ml \cdot kg^{-1} \cdot h^{-1}$ during the 24-hour study period. The body temperature of the animals was kept at 38±1°C.

Under sterile conditions, the left carotid artery and left external jugular vein were surgically exposed for insertion of arterial and pulmonary artery catheters, respectively. A pulmonary artery catheter was placed under direct pressure curve guidance. A percutaneous cystostomy was placed to measure urine output. After completing instrumentation, baseline data was collected. Electrocardiogram, arterial blood pressure, pulmonary artery pressure, cardiac output, heart rate, pulse oximetry, and core temperature were monitored periodically.

Induction of lung injury

A 2-hit model of ARDS was applied, starting with lung lavages to deplete the lungs of alveolar surfactant, followed by injurious mechanical ventilation. With animals under deep anesthesia and fully monitored, we performed repeated lung lavages with warm saline (30 $ml \cdot kg^{-1}$, 39°C), alternating 2 in supine and 2 in prone position. The setting of mechanical ventilation between lavages was the same as that described above. Subsequent lavages were performed if necessary until PaO_2 felt below 250 mmHg for at least 15 minutes while in supine position. Subsequently, a two-hour period of injurious ventilation was started in pressure control ventilation, with PEEP 0 cmH_2O

and inspiratory pressure of 40 cmH_2O, at a respiratory rate of 10·min^{-1}, I:E of 1:1, and FiO_2 1.0. The first hour was in prone position and the second in supine position. After completing this two-hour period, ventilator settings were returned back to those used at baseline for 10 minutes and a full assessment of all variables was registered (time 0-T0), before starting the 24-hour study period.

Extracorporeal membrane oxygenation (ECMO) setup

The ECMO equipment included a magnetic Medtronic Bio-Medicus® 540 centrifuged pump (Eden Praire, MN, USA), a coagulation monitor (Hemochron® Response, ITC, USA), and a heat exchanger HU-35 (Maquet, USA). The circuit comprised a HILITE® 2400LT polymethylpentene hollow fiber membrane oxygenator, 0.65 m^2 (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. Pressure transducers were placed before and after the membrane, and a negative pressure transducer was connected to the drainage line.

In animals allocated to ECMO, cannulation was performed during the second hour of injurious ventilation, while pigs were in supine position. Under sterile conditions, the right external jugular vein was surgically exposed and a 23-F bicaval dual lumen (BCDL) catheter (AVALON ELITE®, Maquet, USA) was inserted and directed towards the inferior vena cava, and secured at 18 cm from the tip. In pilot experiments we observed that the infusion port consistently remained facing the right atrium at this depth. Anticoagulation was induced with an intravenous heparin bolus (100 IU·kg^{-1}), followed by a continuous infusion targeting an activated clotting time (ACT) of 180-220 s. The BCDL catheter was connected to the circuit after T0 measurements and extracorporeal circulation started progressively. The pump was adjusted to target a blood flow > 65 ml/kg/min, but keeping pressure in the drainage line above -70 mmHg. Heat exchanger was set at 38°C. The initial sweep gas flow (FiO_2 1.0) was set at 1:1 with blood flow, and then titrated to keep an arterial $PaCO_2$ between 40±10 mmHg. In pilot experiments we observed that hypotension was frequent after connection to ECMO, and that moderate doses of noradrenaline were required despite adequate fluid loading. Therefore, our protocol established that a noradrenaline infusion was started promptly after connection to ECMO in case mean arterial pressure felt below 65 mmHg. If hypotension persisted despite noradrenaline (0.1 mcg·kg^{-1}·min^{-1}), or if pulse pressure variation was > 15%, animals received a fluid challenge with normal saline 2 ml·kg^{-1}. Further fluid challenges were decided according to fluid and vasopressor responsiveness.

Study protocol

After instrumentation and baseline measurements, 18 animals were randomly allocated to one of the three following study groups:

Sham: animals were ventilated with the same parameters as baseline for 3 hours (to parallel the time spent in inducing lung injury in the other groups), but without inducing lung injury. Thereafter, the 24-hour study period was started with conventional mechanical ventilation: VCV, Vt 10 ml·kg^{-1}, PEEP 5 cmH_2O, RR adjusted to $PaCO_2$ 30-50 mmHg, I:E ratio 1:2 (n = 6).

Acute lung injury (ALI): lung injury was induced as described above, followed by the 24-hour study period with conventional mechanical ventilation: VCV, Vt 10 ml·kg^{-1}, PEEP 5 cmH_2O, RR adjusted to $PaCO_2$ 30-50 mmHg, I:E ratio 1:2 (n = 6).

Acute lung injury + ECMO (ALI + ECMO): lung injury was induced as described above, but performing cannulation during the second hour of injurious ventilation. Thereafter, ECMO was started at T0, along with conventional mechanical ventilation for the 24-hour study period: VCV, Vt 10 ml·kg^{-1}, PEEP 5 cmH_2O, RR as set at baseline, I:E ratio 1:2 (n = 6).

Monitoring, sampling and measurements

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 fluids, as well as infusion drugs for hemodynamic support were recorded and registered hourly for the 24-hour study period.

Table 1. Hemodynamic variables

Variable	SHAM	ALI	ALI + ECMO
HR ($\cdot min^{-1}$)			
Baseline	96 [78, 131]$^{\#}$	91 [68, 106]	69 [50, 92]
T0	76 [69, 92]	117 [92, 152]	81 [60, 121]
T3	60 [52, 88]	145 [120, 204]*	137 [110, 159]$^{*,\#}$
T12	74 [57, 145]	123 [107, 139]	114 [90, 130]
T24	77 [64, 113]	98 [91, 104]	108 [77, 121]
MAP (mmHg)			
Baseline	90 [80.5, 104]	99 [85, 141]	102 [75, 105]
T0	103 [91, 109]	90 [87, 106]	81 [66, 116]
T3	94 [85, 103]	98 [86, 124]	70 [59, 82]$^{\#}$
T12	82 [70, 100]	93 [89, 96]	71 [62, 81]$^{\#}$
T24	71 [64, 81]$^{\#}$	64 [58, 69]	67 [61, 93]$^{\#}$
CO ($L\cdot min^{-1}$)			
Baseline	2.5 [2.2, 2.9]	2.9 [2.6, 3.2]	3.1 [2.9, 3.3]
T0	2.5 [2.2, 2.9]	3.5 [3.3, 4.3]	2.7 [2.7, 3.8]
T3	2.7 [2.3, 3.1]	4.3 [3.7, 4.8]	NM
T12	3.2 [2.1, 3.8]	3.8 [3.7, 3.8]	NM
T24	2.4 [2.0, 2.8]	3.6 [3.6, 3.6]	NM
mPAP (mmHg)			
Baseline	17 [17, 24]	15 [14, 18]$^{\#}$	21 [18, 23]$^{\#}$
T0	21 [19, 24]	39 [33, 40]*	38 [37, 40]*
T3	22 [18, 23]	34 [31, 36]*	29 [23, 31]$^{\#}$
T12	24 [17, 26]	24 [20, 28]	19 [18, 20]$^{\#}$
T24	23 [16, 24]	21 [20, 21]	17 [14, 18]
NA ($ug\cdot kg^{-1}\cdot min^{-1}$)			
Baseline	0.00 [0.00, 0.00]	0.00 [0.00, 0.00]	0.00 [0.00, 0.00]
T0	0.00 [0.00, 0.00]	0.00 [0.00, 0.00]	0.01 [0.00, 0.04]
T3	0.00 [0.00, 0.00]	0.00 [0.00, 0.00]	0.12 [0.10, 0.15]$^{\#,*,£}$
T12	0.00 [0.00, 0.00]	0.00 [0.00, 0.00]	0.14 [0.10, 0.14]$^{\#,*,£}$
T24	0.00 [0.00, 0.00]	0.00 [0.00, 0.00]	0.23 [0.16, 0.33]$^{\#,*,£}$

All results show median [percentile 25, 75] except for ALI group at T12, T24 (median[range]). $^{\#}p < 0.05$ compared to T0; $^{*}p < 0.05$ compared to SHAM; $^{£}p < 0.05$ compared to ALI. *HR*, Heart rate; *MAP*, mean arterial pressure; *CO*, Cardiac output; *mPAP*, mean pulmonary arterial pressure; *NA*, Noradrenaline; *NM*, not measured.

Blood was drawn for blood gas and biochemical analysis at baseline, T0, T3, T12 and T24. Non-bronchoscopic bronchoalveolar lavages (BAL) with 0.5 $ml\cdot kg^{-1}$ of warm saline were performed at T0, T3 and T24. Plasma and cell-free BAL fluid (BALF) samples were obtained by centrifugation and immediately frozen and kept at -80°C for further analysis. Changes in BALF total protein content were determined as a surrogate of alveolar-capillary membrane permeability. Total protein content in BALF was measured using the Bradford method [13]. Plasma and BALF urea concentrations were determined in order to account for the dilution of BALF samples. Epithelial lining fluid (ELF) protein concentrations were calculated using the following equation: [protein in ELF] = ([protein in BALF] x [plasma urea])/[BALF urea] [14].

At the end of the 24-hour study period, animals were euthanized by an overdose of thiopental and T-61 euthanasia solution (Intervet International B.V, Netherlands). Immediately after euthanasia, a thoracotomy was performed in order to access the lungs. Lungs were kept inflated at an end inspiratory pressure of 20 cmH_20 by clamping the endotracheal tube. The left bronchus was tightly sutured in order to separate the left from the right lung. Samples were obtained from the dependent and non-dependent regions of the left superior, middle and inferior lobes, for total lung water estimation [15]. Briefly, all sections were weighted immediately upon extraction and then dried for 24 hours at 120°C. After weighting the dry portions of the lungs, a wet-dry-weight ratio was obtained and reported as an average of all sections. The right lung was filled with formaldehyde at 20 cmH_20.

Lung tissue histology

Tissue samples from the dependent region of the right middle lung lobe were kept in cold formaldehyde for 24 hours and then embedded in paraffin for microscopic assessment of acute lung injury markers according to a recommended score ranging from 0 (healthy) to 1 (maximal injury), which includes alveolar wall thickening and the presence of inflammatory cells, pro-

Table 2. Respiratory variables

Variable	SHAM	ALI	ALI + ECMO
PaO_2 (mmHg)			
Baseline	331 [314, 414]	383 [360, 424]#	333 [276, 437]#
T0	365 [331, 437]	35.5 [32.8, 40.3]*	47.0 [35.0, 125]*
T3	352 [310, 392]	46.5 [38.3, 53.3]*	98.0 [75.8, 198]#
T12	390 [348, 452]	73.5 [44.0, 103]*	182 [94.0, 321]#
T24	314 [200, 426]	107 [80.0, 133]	255 [111, 314]#
$PaCO_2$ (mmHg)			
Baseline	35.9 [33.6, 40.9]	44.6 [42.6, 57.1]	36.8 [34.4, 45.7]
T0	35.9 [32.8, 40.7]	49.9 [46.0, 59.8]*	33.7 [31.1, 45.3]£
T3	42.6 [38.3, 43.8]#	64.1 [57.2, 70.1]	33.0 [29.1, 36.1]£
T12	43.2 [38.9, 49.6]	52.2 [38.9, 65.5]	33.7 [29.2, 34.5]*,£
T24	34.6 [31.4, 46.7]	48.0 [42.4, 53.1]	34.7 [28.5, 39.4]
SatO2 (mmHg)			
Baseline	100 [100, 100]	100 [100, 100]#	100 [100, 100]#
T0	100 [100, 100]	62 [55, 70]*	89 [62, 99]*
T3	100 [100, 100]	74 [62, 83]*	97 [94, 100]£,#
T12	100 [100, 100]	91 [84, 97]*	99 [98, 100]
T24	100 [100, 100]	98 [96, 99]	99 [97, 100]
MV ($L{\cdot}min^{-1}$)			
Baseline	5.2 [4.6, 7.8]	4.8 [4.5, 5.3]#	6.1 [5.2, 6.5]
T0	5.1 [4.8, 7.3]	5.5 [5.3, 6.4]	6.7 [5.8, 8.8]
T3	5.1 [4.9, 7.2]	5.9 [5.2, 8.3]	5.8 [4.6, 7.0]#
T12	6.3 [5.6, 7.7]	7.4 [6.7, 8.1]	5.9 [4.9, 6.7]
T24	6.8 [5.1, 7.9]	7.4 [6.7, 8.1]	6.6 [6.2, 7.4]
Pplat (cmH_2O)			
Baseline	13 [13, 16]	12 [12, 15]#	14 [13, 16]#
T0	15 [14, 17]	25 [22, 27]*	28 [22, 33]*
T3	15 [13, 18]	26 [25, 27]*	30 [27, 33]*
T12	16 [14, 18]	22 [20, 24]	31 [25, 31]*
T24	16 [13, 18]	27 [23, 30]*	28 [23, 30]*
Ppeak (cmH_2O)			
Baseline	20 [18, 24]	17 [16, 18]#	17 [16, 19]#
T0	20 [15, 22]	30 [29, 31]*	38 [31, 43]*
T3	22 [17, 23]#	31 [31, 33]*	34 [30, 41]*
T12	23 [20, 24]#	33 [30, 35]*	32 [29, 35]*
T24	23 [20, 25]#	35 [32, 39]*	34 [27, 35]*
Pmean (cmH_2O)			
Baseline	9 [8, 10]	8 [7, 8]*,#	8 [8, 8]*,#
T0	9 [8, 9]	11 [11, 13]*	15 [13, 15]*
T3	8 [8, 9]	12 [12, 14]*	11 [10, 14]*
T12	9 [8, 10]	11 [10, 11]	12 [11, 20]*
T24	9 [8, 11]	12 [11, 12]	12 [11, 16]

All results show median [percentile 25, 75] except for ALI group at T12, T24 (median [range]). #$p < 0.05$ compared to T0; *$p < 0.05$ compared to SHAM; £$p < 0.05$ compared to ALI. MV, minute ventilation; Pplat, plateau pressure; Ppeak, peak pressure; Pmean, mean airway pressure.

teinaceous debris, and hyaline membranes in the alveolar space [15].

Statistical analysis

All data are presented as median [percentile 25, 75]. Comparison between groups was performed by Kruskal Wallis test, followed by Bonferroni *post hoc* test. Since several animals of group ALI died before completing the study period, we analyzed changes along time by comparing all time points versus T0 using Wilcoxon signed-rank test, instead of performing a multiple comparison analysis. Survival analysis was performed by Log-rank test (Mantel-Cox). A *P*-value < 0.05 was considered to be statistically significant. All analyses were performed using Statistical Package for Social Science (SPSS) Statistics 20.0.

Results

Characteristics of the acute lung injury model

Induction of acute lung injury required 8 [7, 9] lavages to reach a PaO_2/FiO_2 < 250. After the 2-hour period of injurious ventilation, animals developed severe hypoxemia, increased airway pressures and moderate pulmonary hypertension (**Tables 1** and **2**). Due to persistent hypoxemia 4 of 6 animals died in the ALI group before completing the study period (**Figure 1**). The 2 animals that survived evolved with tachycardia and increased car-

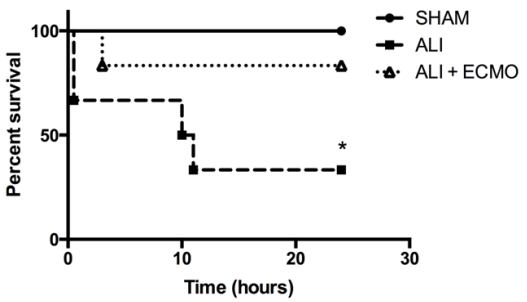


Figure 1. Survival curves. Survival curves obtained from SHAM, ALI and ALI + ECMO groups, with six animals per group. Statistical analysis was performed by Log-rank test. *p < 0.05 compared to SHAM.

diac output during the first hours but their oxygenation slowly improved regaining a PaO_2 > 60 mmHg by the end of the study period. In contrast, airway pressures remained high until the end of the experiment. Protein concentration in the ELF was markedly increased in both ALI and ALI + ECMO groups, and remained high throughout the study period (**Figure 3A**).

Only 2 animals from the ALI group and 5 from the ALI + ECMO group completed the study period and had lung tissue samples retrieved for analysis. Pulmonary wet/dry weight ratios were increased, indicating significant lung edema in all the animals subjected to ALI (**Figure 3B**). Histology revealed a severe pattern of diffuse alveolar damage, the pathologic hallmark of ARDS, with presence of hyaline membranes, alveolar wall thickening and extensive infiltration with inflammatory cells, as shown in **Figure 2**.

Concerning biochemical data, induction of ALI was associated to a non-significant trend towards increased lactate at T0, and increased serum glutamic oxaloacetic transaminase at T12 and T24.

Performance of extracorporeal membrane oxygenation

Cannulation could be performed successfully in all animals. Connection to ECMO was followed by rapid reversal of hypoxemia and pulmonary hypertension (**Tables 1** and **2**). However, hemodynamic instability was common during the first hour, with variable degrees of hypotension, which exhibited no response to fluids and therefore required moderate doses of noradrenaline. Thereafter, most animals regained hemodynamic stability, but remained dependent on noradrenaline until the end of the study period. One animal evolved with progressive hypotension non-responsive to vasopressor and fluids, and died before T2 (**Figure 1**). The other five animals completed the study period. Total fluid loading was 2472 ml [2188, 2933], 3532 ml [3470, 3549], and 3015 ml [2683, 3234], in the SHAM, ALI and ALI + ECMO groups, respectively ($p < 0.05$ for SHAM vs ALI). Urine output was 1500 ml [945,2225], 2495 ml [2100, 2890], 1405 ml [888, 1566], in the SHAM, ALI and ALI + ECMO groups, respectively (no significant differences). When analysing biochemistry data in the ECMO group the only relevant observation was a trend towards increased creatinine concentrations, with no other side effects (**Table 3**). Oxygenation continued to improve throughout the 24-hour study period. At T24 PaO_2 was 255 mmHg [111, 314] while mixed venous PO_2 was 77 mmHg [56, 100]. However, airway pressures remained high up to the end of the experiment indicating that lung compliance did not improve.

Extracorporeal blood flow reached 2.1 $L{\cdot}min^{-1}$ [2.0, 2.2] at T1, which corresponded to 68.1 % [66.3, 69.4] of the last cardiac output measured at T0, just before starting ECMO. The pump speed was 2970 rpm [2305, 3000], with pressure in the drain line at -60 mmHg [-71, -43]. Pre and post membrane pressures were 160 mmHg [97, 171] and 136 mmHg [82, 148], while pre and post membrane partial pressures of oxygen were 37 mmHg [36.8, 37.8] and 143.5 mmHg [121, 183.8], respectively. As ventilator settings were not modified in the ALI + ECMO group after starting ECMO, sweep gas flow had to be decreased to 0.7 $L{\cdot}min^{-1}$ [0.5, 1.3] to avoid hypocapnia. However, in 2 separate pilot experiments not included in the present analysis, near-apneic ventilation (minute ventilation < 1 $L{\cdot}min^{-1}$) was applied during the 24-hour study period. By increasing sweep gas flow, PaO_2 and $PaCO_2$ could be kept at rather normal levels (Supplementary Figure 1).

Discussion

We have developed a reproducible porcine 24-hour model of severe acute respiratory failure supported with ECMO, which resembles

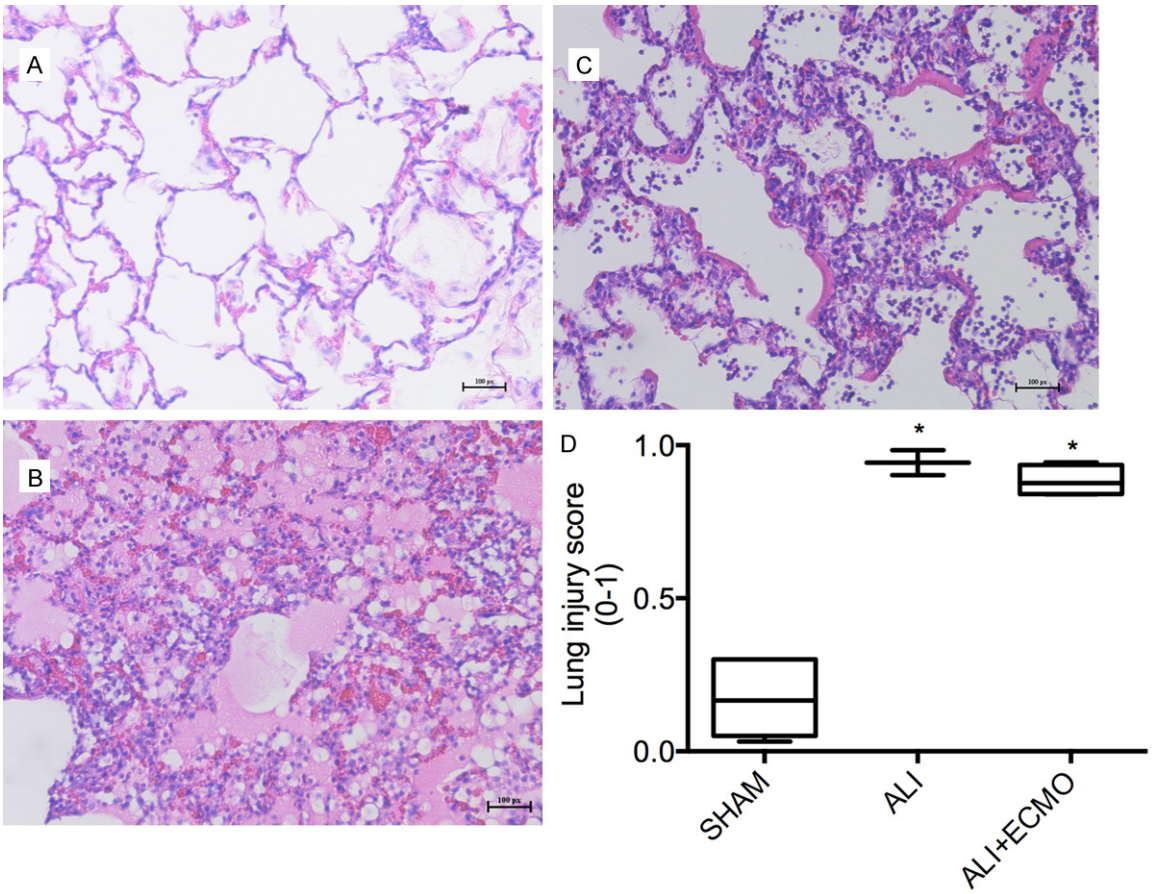


Figure 2. Histologic evaluation of lung sections. Representative histologic examinations of lung samples obtained from the dependent region of the right middle lobe of animals from the SHAM (A), ALI (B) and ALI + ECMO (C) groups. A normal lung architecture is observed in (A), whereas diffuse alveolar damage, as represented by the presence of inflammatory cells, both in the interstitial and alveolar space, capillary congestion, alveolar flooding, and the presence of hyaline membranes, are observed in (B and C). Lung injury was quantified by a validated score which includes alveolar wall thickening, the presence of inflammatory cells, proteinaceous debris, and hyaline membranes in the alveolar space, with a range from 0 to 1 [15] (D). Data corresponds to median [percentile 25, 75]. *$p < 0.05$ compared to SHAM.

several features of clinical severe ARDS. Acute lung injury was induced by repeated saline lavage, followed by a 2-hour period of injurious mechanical ventilation, with both hits applied alternating supine and prone position to promote a more diffuse injury. The model is characterized by severe hypoxemia refractory to mechanical ventilation with PEEP 5 cmH_2O and FiO_2 1.0, exhibiting a high lethality when not supported with ECMO. A high flow vvECMO could be provided with minimal recirculation, rapidly correcting hypoxemia and improving survival.

Our goal was to develop a model of ARDS characterized histologically by diffuse alveolar damage, and functionally by severe hypoxemia and decreased compliance, to reproduce the context in which ECMO is applied clinically, but without significant hemodynamic instability, so that vvECMO could be well tolerated. A few models of ALI supported with ECMO have been reported, including surfactant depletion [12, 16], endotoxin [17], oleic acid infusion [18], and smoke inhalation [10], among others. Concerning optimal species, sheep and pigs are the preferred options. Although sheep offer advantages in terms of an easier cannulation, pigs have some physiologic advantages for translation to humans concerning coagulation [19]. It is clear that all animal models have significant limitations and the optimal choice will depend on the particular goals and the research context.

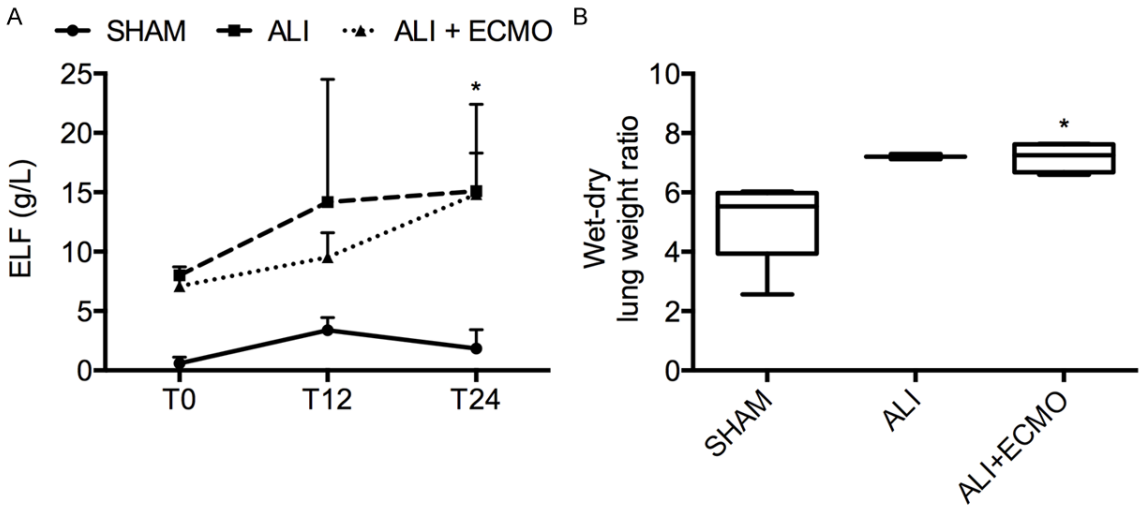


Figure 3. Surrogates of alveolar membrane barrier integrity (A). Epithelial lining fluid protein concentration calculated from non-bronchoscopic bronchoalveolar lavage samples, with correction for dilution by Urea (B). Wet/dry weight ratio obtained from the left lungs of the three study groups (A, B). Data corresponds to median [percentile 25, 75]. *p < 0.05 compared to SHAM. ELF (Epithelial lining fluid).

After some preliminary experiments and analysing the published data we decided that surfactant depletion was the best option for our goal. However, lung inflammation is usually low in surfactant depletion models. Therefore we decided to add a second hit: injurious ventilation. With this double hit model significant lung inflammation has been described [20]. In pilot experiments we observed that this model led to marked diffuse alveolar damage and severe hypoxemia, however, injury had an excessive gravitational distribution, and hypoxemia was easily reversible even with low PEEP levels. For this reason we decided to perform the double hit injury alternating supine and prone position, so that dorsal and ventral regions were equally affected, and promoting a more widespread development of atelectasis. This approach resulted in severe hypoxemia and a high mortality as observed in the ALI group. In contrast, hypoxemia was rapidly corrected in the ALI + ECMO group, and 5/6 animals could complete the 24-hour study period. Furthermore, at the end of the 24-hour study period animals were in good condition and the model could have easily been extended. Interestingly, although gas exchange in the native lungs had partially recovered, as reflected by the large difference between PaO_2 and mixed venous PO_2 at T24, compliance remained low. We speculate that improvement in oxygenation may have not been due to reopening of atelectatic lung, but instead to a better ventilation-perfusion relationship.

Since one of our goals was to develop a model of ECMO, with the ability to provide full lung support if necessary, one of the major challenges was cannulation. Cervical veins in the pig are rather small compared to other species, while femoral veins are difficult to cannulate because of their deep location. The largest vein is the jugular external vein, which measures 5 to 8 mm diameter for 30 kg pigs. We aimed to have a high blood flow extracorporeal setting with low recirculation, so that full support could be provided. Therefore we decided to use a BCDL catheter. After trying different sizes we found that the largest cannula we could place was a 23 French. In the first experiments we used transthoracic ultrasound to make sure that the cannula was directed towards the inferior vena cava and not into the right ventricle. However, thereafter we were able to introduce it correctly without ultrasound, just by advancing blindly the wire guide at least 40 cm without observing arrhythmias. The cannula was advanced with the introducer over the wire guide and secured at 18 cm from the tip. In *post mortem* analysis we observed that by using this approach the cannulas were correctly placed towards the inferior vena cava and the return port remained at the right atrium level in

Table 3. Biochemistry data

Variable	SHAM	ALI	ALI + ECMO
Creatinine ($mg \cdot dL^{-1}$)			
Baseline	1.18 [1.17, 1.97]	0.81 [0.73, 0.88]*	1.03 [0.90, 1.04]
T0	1.05 [0.79, 1.09]	0.91 [0.76, 1.06]	1.03 [0.78, 1.13]
T12	1.17 [1.13, 1.53]#	1.16 [0.93, 1.39]	1.33 [1.17, 2.37]
T24	1.33 [0.84, 2.48]#	1.15 [0.99, 1.31]	1.66 [1.24, 2.38]
BUN ($mg \cdot dL^{-1}$)			
Baseline	8 [7, 25]	11 [9, 12]	8 [8, 9]
T0	9 [8, 12]	9 [7, 11]	8 [7, 9]
T12	23 [16, 25]#	13 [9, 17]	18 [15, 20]
T24	23 [15, 36]#	12 [9, 15]	21 [17, 29]
SGOT ($U \cdot L^{-1}$)			
Baseline	54 [45, 132]	25 [21, 29]	21 [15, 34]
T0	66 [54, 69]	122 [91, 152]	30 [16, 68]
T12	88 [58, 141]#	154 [141, 166]	73 [56, 142]
T24	99 [61, 139]#	266 [162, 370]	117 [85, 200]
Bilirubin ($mg \cdot dL^{-1}$)			
Baseline	0.15 [0.15, 0.15]	0.16 [0.15, 0.16]	0.15 [0.15, 0.15]
T0	0.15 [0.14, 0.16]	0.17[0.15, 0.18]	0.15 [0.15, 0.15]
T12	0.15 [0.13, 0.15]	0.15 [0.15, 0.15]	0.15 [0.15, 0.17]
T24	0.15 [0.13, 0.15]	0.14 [0.12, 0.15]	0.15 [0.15, 0.15]
Urea ($g \cdot L^{-1}$)			
Baseline	0.17 [0.15, 0.53]	0.21 [0.19, 0.23]	0.17 [0.17, 0.19]
T0	0.19 [0.17, 0.24]	0.19 [0.15, 0.23]	0.17 [0.15, 0.19]
T12	0.49 [0.34, 0.54]#	0.28 [0.19, 0.36]	0.37 [0.31, 0.42]
T24	0.49 [0.28, 0.57]#	0.75 [0.19, 1.31]	0.45 [0.37, 0.61]
pH			
Baseline	7.53 [7.43, 7.60]	7.48 [7.39, 7.49]#	7.52 [7.42, 7.58]
T0	7.51 [7.45, 7.58]	7.31 [7.27, 7.37]*	7.42 [7.34, 7.49]
T12	7.37 [7.33, 7.43]#	7.43 [7.37, 7.49]	7.49 [7.43, 7.50]
T24	7.43 [7.36, 7.48]	7.49 [7.48, 7.50]	7.45 [7.40, 7.45]
$HCO3^-$ ($mEq \cdot L^{-1}$)			
Baseline	30.3 [27.8, 33.1]	33.2 [32.3, 35.9]	30.6 [29.6, 34.2]#
T0	29.5 [28.5, 30.9]	26.8 [23.3, 29.9]	24.8 [20.5, 26.6]*
T12	25.5 [23.8, 89.9]	34.0 [29.9, 38.0]	24.0 [20.9, 26.1]
T24	23.7 [20.4, 29.1]#	36.6 [33.2, 40.0]	23.0 [20.2, 25.3]
BE ($mEq \cdot L^{-1}$)			
Baseline	8.0 [3.5, 11.5]	10 [8.0, 12]	8.0 [6.0, 12]#
T0	6.5 [4.5, 8.0]	2.0 [0.0, 8.3]	2.0 [0.0, 6.0]
T12	2.0 [1.5, 3.5]#	10 [7.0, 13]	2.0 [-2.0, 3.0]
T24	3.5 [2.8, 6.5]	13.5 [10, 17]	2.0 [-3.0, 2.0]£
Lactate ($mmol \cdot L^{-1}$)			
Baseline	1.6 [1.2, 2.0]	1.4 [1.0, 1.5]	1.5 [1.1, 1.8]#
T0	1.6 [1.2, 2.3]	2.1 [1.8, 2.8]	3.2 [2.9, 4.5]*
T12	2.3 [1.7, 4.3]	0.9 [0.8, 1.1]	1.5 [1.4, 2.4]#
T24	1.5 [0.9, 1.9]	1.3 [1.3, 1.4]	1.2 [1.0, 1.9]#

All results show median [percentile 25, 75] except for ALI group at T12, T24 (median [range]). #$p < 0.05$ compared to T0; *$p < 0.05$ compared to SHAM; £$p < 0.05$ compared to ALI. BUN, blood urea nitrogen; SGOT, Serum glutamic oxaloacetic transaminase; BE, base excess.

all cases. Although we could not perform reliable calculations of recirculation, because of the impossibility of obtaining representative pure venous blood samples from the vena cava, the very low pre-membrane PO_2 indicates that recirculation was probably very low [21].

In the present study we did not modify ventilator settings after connecting the animals to ECMO, as usually done in the clinical scenario, because our goal at this stage was to assess the isolated effect of extracorporeal circulation and gas exchange in our ALI model, without the additional influence of protective ventilation. However, in two separate pilot experiments we were able to induce near-apneic ventilation keeping normal arterial blood gases, as shown in Supplementary Figure 1, indicating that the ECMO setting applied is able to provide full lung support. In fact, the following studies will compare different strategies of lung protection during ECMO in this model. In addition, we decided to keep FiO_2 at 1.0 during all the experiment and in all the groups, even after ECMO connection, to avoid the potential influence of applying different inspired oxygen concentrations on gas exchange and lung injury. Although this may deviate from clinical practice and certainly is not an ideal setting to protect the

lungs, we thought for the purpose of research validity it was important to minimize the potential covariates.

The major strengths of our model are first, its severity, which is important concerning potential translations to patients with severe respiratory failure treated with ECMO. Animals subjected to ALI without ECMO developed severe hypoxemia and most of them died before completing the study period. A second attribute is hemodynamic stability, which otherwise could preclude using vvECMO. Although we had to use moderate doses of Noradrenaline after starting ECMO, most animals regained stability. A third strength of our model is the use of a single BCDL catheter. Cannulation could be performed without advanced imaging, which frequently is not available in most research labs. In addition, we could avoid using the femoral veins, in which due to their deep location, it is cumbersome to place large cannulas in large animals [10, 22]. Fourth, histologic findings in the injured lungs indicate diffuse alveolar damage, as expected in ARDS, which is one of the main indications for vvECMO in adults. Finally, with the ECMO configuration applied we obtained minimal recirculation and a relatively high extracorporeal blood flow (> 60 $ml \cdot kg^{-1} \cdot min^{-1}$), which allows full extracorporeal lung support. This is important to explore more extreme forms of lung protection as near-apneic ventilation.

However, the model also has limitations. It is rather expensive, highly demanding and labor-intensive, therefore requiring a large research team. Experimental preparation takes 5 to 6 hours. Changing repeatedly between supine and prone position during induction of lung injury is laborious. Insertion of a 23 F cannula in the external jugular vein is challenging and requires dissecting the vein very distally towards the sternal notch, where it has a larger diameter. Once vvECMO is started measurements of cardiac output by thermodilution are no longer reliable due to indicator loss into the extracorporeal circulation. In addition, connection to ECMO while the animals are hypoxemic and hypercapnic is followed by a short period of hemodynamic instability, which requires vasopressor support. Almost all animals regained stability with moderate vasopressor support but one of them developed progressive hypotension and finally died early during the model. However, this is a price worth to pay in order to have a clinically relevant scenario.

Conclusions

The porcine model of ALI induced by repeated saline lavage, followed by injurious ventilation, exhibits severe hypoxemia that is refractory to conventional mechanical ventilation, and has a high lethality, accurately resembling severe ARDS in humans. The present model can be successfully rescued by vvECMO, and supported for 24 hours, providing an adequate model for translational research in extracorporeal lung support.

Acknowledgements

AB, PC, JA, PG and GB acknowledge support from Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) through grant 1130428. JA acknowledges support from Programa Nacional de Becas de Posgrado, from Comisión Nacional de Investigación Científica y Tecnológica (CONICYT).

Disclosure of conflict of interest

None.

Authors' contribution

JA, PC and AB participated in all aspects of the study and wrote the manuscript. LA organized the experiments, and performed animal experiments. FD performed statistical analysis and assisted in the animal experiments. PG, PT, TS, BE, GC, TM and PC assisted and performed animal experiments. DS contributed to biochemical analysis. FR and MA managed the ECMO circuit. JR contributed to study design and animal experiments. GB contributed to study design and placed catheters and cannulas. All authors read and approved the final manuscript.

Address correspondence to: Dr. Alejandro Bruhn, Departamento de Medicina Intensiva, Facultad de Medicina, Pontificia Universidad Católica de Chile, Marcoleta 367, 3° Piso, Centro de Pacientes Críticos, Santiago, Chile. Tel: 56-223543972; E-mail: abruhn@med.puc.cl

References

[1] Australia and New Zealand Extracorporeal Membrane Oxygenation (ANZ ECMO) Influenza

Investigators, Davies A, Jones D, Bailey M, Beca J, Bellomo R, Blackwell N, Forrest P, Gattas D, Granger E, Herkes R, Jackson A, McGuinness S, Nair P, Pellegrino V, Pettilä V, Plunkett B, Pye R, Torzillo P, Webb S, Wilson M, Ziegenfuss M. Extracorporeal Membrane Oxygenation for 2009 Influenza A(H1N1) Acute Respiratory Distress Syndrome. JAMA 2009; 302: 1888-1895.

[2] Brodie D and Bacchetta M. Extracorporeal membrane oxygenation for ARDS in adults. N Engl J Med 2011; 365: 1905-1914.

[3] Sauer CM, Yuh DD and Bonde P. Extracorporeal membrane oxygenation use has increased by 433% in adults in the United States from 2006 to 2011. ASAIO J 2015; 61: 31-36.

[4] Peek GJ, Mugford M, Tiruvoipati R, Wilson A, Allen E, Thalanany MM, Hibbert CL, Truesdale A, Clemens F, Cooper N, Firmin RK and Elbourne D. Efficacy and economic assessment of conventional ventilatory support versus extracorporeal membrane oxygenation for severe adult respiratory failure (CESAR): a multicentre randomised controlled trial. Lancet 2009; 374: 1351-1363.

[5] Marhong JD, Munshi L, Detsky M, Telesnicki T and Fan E. Mechanical ventilation during extracorporeal life support (ECLS): a systematic review. Intensive Care Med 2015; 41: 994-1003.

[6] Marhong JD, Telesnicki T, Munshi L, Del Sorbo L, Detsky M and Fan E. Mechanical ventilation during extracorporeal membrane oxygenation. An international survey. Ann Am Thorac Soc 2014; 11: 956-961.

[7] Schmidt M, Stewart C, Bailey M, Nieszkowska A, Kelly J, Murphy L, Pilcher D, Cooper DJ, Scheinkestel C, Pellegrino V, Forrest P, Combes A and Hodgson C. Mechanical ventilation management during extracorporeal membrane oxygenation for acute respiratory distress syndrome: a retrospective international multicenter study. Crit Care Med 2015; 43: 654-664.

[8] Hayes RA, Foley S, Shekar K, Diab S, Dunster KR, McDonald C and Fraser JF. Ovine platelet function is unaffected by extracorporeal membrane oxygenation within the first 24 h. Blood Coagul Fibrinolysis 2015; 26: 816-822.

[9] Ni L, Chen Q, Zhu K, Shi J, Shen J, Gong J, Gao T, Yu W, Li J and Li N. The influence of extracorporeal membrane oxygenation therapy on intestinal mucosal barrier in a porcine model for post-traumatic acute respiratory distress syndrome. J Cardiothorac Surg 2015; 10: 20.

[10] Shekar K, Fung YL, Diab S, Mullany DV, McDonald CI, Dunster KR, Fisquet S, Platts DG, Stewart D, Wallis SC, Smith MT, Roberts JA and Fraser JF. Development of simulated and ovine models of extracorporeal life support to improve understanding of circuit-host interactions. Crit Care Resusc 2012; 14: 105-111.

[11] Shekar K, Roberts JA, Barnett AG, Diab S, Wallis SC, Fung YL and Fraser JF. Can physicochemical properties of antimicrobials be used to predict their pharmacokinetics during extracorporeal membrane oxygenation? Illustrative data from ovine models. Crit Care 2015; 19: 437.

[12] Iglesias M, Jungebluth P, Petit C, Matute MP, Rovira I, Martinez E, Catalan M, Ramirez J and Macchiarini P. Extracorporeal lung membrane provides better lung protection than conventional treatment for severe postpneumonectomy noncardiogenic acute respiratory distress syndrome. J Thorac Cardiovasc Surg 2008; 135: 1362-1371.

[13] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-254.

[14] Rennard SI, Basset G, Lecossier D, O'Donnell KM, Pinkston P, Martin PG and Crystal RG. Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. J Appl Physiol (1985) 1986; 60: 532-538.

[15] Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS and Kuebler WM. An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. Am J Respir Cell Mol Biol 2011; 44: 725-738.

[16] Sanchez-Lorente D, Go T, Jungebluth P, Rovira I, Mata M, Ayats MC and Macchiarini P. Single double-lumen venous-venous pump-driven extracorporeal lung membrane support. J Thorac Cardiovasc Surg 2010; 140: 558-563, 563 e551-552.

[17] Song J, Palmer K and Sun B. Effects of inhaled nitric oxide and surfactant with extracorporeal life support in recovery phase of septic acute lung injury in piglets. Pulm Pharmacol Ther 2010; 23: 78-87.

[18] Langer T, Vecchi V, Belenkiy SM, Cannon JW, Chung KK, Cancio LC, Gattinoni L and Batchinsky AI. Extracorporeal gas exchange and spontaneous breathing for the treatment of acute respiratory distress syndrome: an alternative to mechanical ventilation?*. Crit Care Med 2014; 42: e211-220.

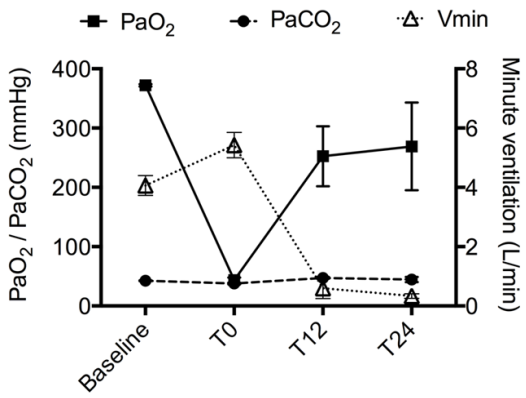
[19] Peek GJ, Scott R, Killer HM, Jarvis MA, Kolvekar S, Forbes D and Firmin RK. A porcine model of prolonged closed chest venovenous extracorporeal membrane oxygenation. ASAIO J 1999; 45: 488-495.

[20] Ballard-Croft C, Wang D, Sumpter LR, Zhou X and Zwischenberger JB. Large-animal models

of acute respiratory distress syndrome. Ann Thorac Surg 2012; 93: 1331-1339.

[21] Abrams D, Bacchetta M and Brodie D. Recirculation in venovenous extracorporeal membrane oxygenation. ASAIO J 2015; 61: 115-121.

[22] Hayes D Jr, Yates AR, Duffy VL, Tobias JD, Mansour HM, Olshove VF Jr and Preston TJ. Rapid placement of bicaval dual-lumen catheter in a swine model of venovenous ECMO. J Invest Surg 2014; 27: 27-31.



Supplementary Figure 1. Pilot experiments using near-apneic ventilation. PaO_2, $PaCO_2$ (left axis) and minute ventilation (right axis) along the 24-hour study period, corresponding to two pilot animal experiments performed using the same model, but applying a near-apneic ventilation strategy (mean and SEM).