**Flow controlled ventilation attenuates lung injury in a porcine model of ARDS – a preclinical randomized controlled study**

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**Abbreviation List**

ARDS acute respiratory distress syndrome

BAL broncho-alveolar lavage

CI cardiac index

CRS compliance of the respiratory system

CT computed tomography

ELWI extra-vascular lung water index

FCV flow controlled ventilation

FiO2 fraction of inspired oxygen

MAP mean arterial pressure

MPAP mean pulmonary arterial pressure

MV minute volume

PaCO2 arterial partial pressure of carbon dioxide

PaO2 arterial partial pressure of oxygen

PEEP positive end-expiratory pressure

Ptrach tracheal pressure

RR respiratory rate

SP-A surfactant protein A

TP total protein

VCV volume controlled ventilation

VT tidal volume

**SDC 1: Detailed description of methods**

The study was approved by the Regierungspräsidium Freiburg (file reference G-16/77) and conducted in accordance with European law (EU-Directive 2010/63). The animals were kept in a housing facility for 10 to 14 days prior to the experiment (free access to water and food, natural circadian light). The experiments started at the same time in the morning and were finished in the afternoon. The randomization sequence was generated by a computer algorithm and was kept in closed envelopes until disclosure.

**Anesthesia and surgical instrumentation**

19 German landrace hybrid pigs (body weight 40 - 50 kg) were premedicated with an intramuscular injection of ketamine (20 mg·kg-1) and midazolam (0.5 mg·kg-1) after fasting for a period of 12 hours with water ad libitum. Propofol (2-4 mg·kg-1) and vecuronium (0.2 mg·kg-1) were injected to allow for tracheal intubation with a standard endotracheal tube (ETT) with an inner diameter of 8.0 mm. Midazolam (0.5‑1.5 mg·kg‑1·h-1), ketamine (10-30 mg·kg-1·h-1), and fentanyl (3-6 µg·kg-1·h-1) maintained anaesthesia and vecuronium (0.2-0.4 mg·kg-1·h-1) provided neuromuscular blockade. A balanced electrolyte solution (Sterofundin ISO, B. Braun AG, Melsungen, Germany) was infused (10 ml·kg-1·h-1). A catheter placed in the femoral artery (5F, Pulsion Medical Systems, Feldkirchen, Germany) was used for systemic blood pressure monitoring and transcardiopulmonary thermodilution (PiCCO2, Pulsion Medical Systems, Feldkirchen, Germany). A pulmonary artery catheter (7F, Edwards Lifesciences, Irvine, California, USA) was placed via the right external jugular vein. The left external jugular vein was cannulated with an 8.5F catheter for injection of oleic acid. A vesical catheter was inserted via mini laparotomy for collecting urine.

**Ventilation and induction of lung injury**

The lungs of all animals were ventilated (Evita 4, Dräger medical, Lübeck, Germany) with identical settings (VCV, FiO2 of 0.3, tidal volume (VT) 7 ml·kg-1, positive end-expiratory pressure (PEEP) 5 cmH2O, I:E ratio of 1:1.2, and respiratory rate (RR) adjusted to reach an end-tidal partial pressure of CO2 of 35-45 mmHg) until the surgical instrumentation was complete. Before induction of lung injury, FiO2 was increased to 0.8 and PEEP to 9 cmH2O. Lung injury was then induced with central venous injection of oleic acid (Oleic Acid PharmaGrade, Sigma-Aldrich Co. LLC, Munich, Germany) as previously described.(1) In brief, the oleic acid was thoroughly mixed with a 5% solution of glucose (G5%, B. Braun AG, Melsungen, Germany) in the ratio of 1:2 (Oleic Acid:Glucose) and then continuously administered with an infusion rate of 30 to 60 ml·h-1. If hemodynamic instability occurred (mean arterial pressure (MAP) < 60 mmHg), the infusion was paused to allow recompensation. The level of lung injury was determined from frequent arterial blood gas measurements (cobas b 123, Roche Diagnostics, Mannheim, Germany). The intended level of lung injury was achieved when the ratio of PaO2 to the fraction of inspired oxygen (FiO2) remained stable between 100 and 150 mmHg for 20 minutes. Beginning with the induction of lung injury, the respiratory rate was adjusted to maintain arterial blood pH above 7.2.

**Experimental protocol**

Figure 1 shows the timeline of the experiment. After establishing lung injury, baseline parameters were recorded for all animals (with identical ventilation settings for all animals). The random allocation to either VCV (control) or FCV was then disclosed and the ETT was disconnected from the ventilator for 30 seconds and pulmonary edema fluid removed via active tracheal suction (all animals). For the animals of the control group, the ETT was reconnected to the standard ventilator. For the animals of the FCV group, a tracheal ventilation tube (Tritube, Ventinova Medical B.V., Eindhoven, The Netherlands) was placed inside the ETT and was connected to a prototype ventilator capable of providing FCV (Evone, Ventinova Medical B.V., Eindhoven, The Netherlands). Ventilation was started in both groups with identical settings (FiO2: 0.8, PEEP: 9 cmH2O, VT: 7 ml·kg-1, I:E ratio: 1:1.2) and maintained for three hours. No tracheal suctioning or recruitment maneuvers were performed during the observation period. Then, a dynamic computed tomography (CT) scan was taken under the designated ventilation mode and samples of lung tissue and broncho-alveolar lavage (BAL) fluid were collected as described below. The level of lung injury was evaluated concerning different aspects: Capability of gas exchange (where PaO2 served as primary endpoint), alveolar wall thickness, total protein concentration and surfactant protein A (SP-A) concentration of BAL fluid, extravascular lung water index (ELWI), and cell infiltration into lung tissue. The aeration of lung tissue was determined, based on the CT scans as described below.

**Respiratory and hemodynamic parameters**

Beginning with the baseline measurement, respiratory and hemodynamic parameters were recorded every 30 minutes: PaO2 and PaCO2 were determined. Flow rate was recorded with a sample rate of 100 Hz for five minutes from the respective ventilator to calculate VT, RR, and minute volume (MV). In the FCV group, tracheal pressure (Ptrach) was measured directly via a dedicated pressure measurement lumen of the Tritube with its opening at the distal end of the tube. In the control group airway pressure was recorded and Ptrach was calculated as described previously.(2) Briefly, the measured flow rate and the non-linear resistance coefficients were used to calculate the pressure drop across the endotracheal tube. For VCV, plateau pressure was determined after every inspiration at the end of the inspiratory plateau. FCV included an end-inspiratory occlusion (1 sec.) after every 10th inspiration to determine the tracheal plateau pressure. The dynamic compliance of the respiratory system was calculated by multiple linear regression analysis. Recorded hemodynamic parameters included heart rate (HR), MAP, and mean pulmonary arterial pressure (MPAP). Cardiac index (CI) and ELWI were determined via threefold injection of 15 ml of cold saline (ca. 8°C) and subsequently calculated based on the predicted body surface area for pigs.(3)

**Computed tomography**

After three hours of ventilation, a thoracic CT scan was performed as a dynamic sequence (always taken at the same thoracic cross-sectional level, approx. 30 mm caudal to the carina) under the designated ventilation mode as described previously.(4) In brief, RR was decreased to 6 min-1 to reduce movement artefacts. During 45 seconds 60 images with a layer thickness of 9 mm were reconstructed, hence the images taken in total represent 45 seconds of real time ventilation.

These scans were further analyzed to calculate histograms of Hounsfield unit (HU) distribution in the range of -1000 to 0 HU for independent and dependent lung regions separately. First, independent and dependent lung regions were manually divided by drawing a line through the ventral walls of the main bronchi. Then, the image was manually cut along the border between pulmonary tissue and the chest wall. The remaining image data was then automatically analyzed and subdivided with steps of 50 HU from 0 to -1000 HU to create histograms. These data were then summarized for four distinctive lung compartments as described previously:(5) (I) Overinflated lung tissue with HU from -1000 to -900, (II) normally aerated lung tissue with HU from -900 to ‑500, (III) poorly aerated lung tissue with HU from -500 to -100, and (IV) non-aerated lung tissue with HU from -100 to 0.

**Broncho-alveolar lavage**

After the CT scan, the thorax was opened via sternotomy and the right lower lobe and the right middle lobe of the lung were clamped and excised. Subsequently, the animals were euthanized with a lethal dose of potassium chloride.

The lobar bronchus of the right middle lobe was cannulated and 20 ml of saline was injected. Subsequent aspiration yielded approximately 5 ml of bronchoalveolar lavage (BAL) fluid. The total protein concentration and the concentration of SP-A of BAL fluid were determined using commercially available kits (Pierce BCA protein assay kit, Thermo Fisher Scientific, Darmstadt, Germany and SFTPA assay kit, Antibodies-Online GmbH, Aachen, Germany) according to the manufacturer’s instructions.

**Histopathology**

Representative tissue samples (obtained by blinded study personnel) of the right lower lobe were fixated in 2% paraformaldehyde in phosphate buffered saline for 1 hour and incubated in 30% sucrose over night at 4°C. After slow freezing in embedding medium (Tissue-Tek O.C.T., Sakura Finetek, Alphen van den Rijn, The Netherlands) cryosections of 6 µm were submitted to haematoxylin/eosin staining. The alveolar wall thickness and cell infiltration rate were determined in 5 images from each tissue sample utilizing AxioVision software (ver. 4.8, Carl Zeiss Micro Imaging, Jena, Germany). Measurements were performed in 5 high power fields, randomly assigned to each image, by an examiner blinded to the randomized allocation of the animals.

**Statistical analysis**

An a priori sample size calculation based on the assumption of an effect size of 1.5 standard deviations in the primary end point, an intended power of 80%, a type 1 error of 5%, and an equal sample ratio resulted in n=7 for each group. Data and statistical analyses were done off line with MATLAB (R2017b, MathWorks Inc., Natick, MA, USA). A linear mixed effects model(6) was applied to the measurements, with the group allocation as a fixed effect and a random intercept by-subject. For repeated measurements a random intercept by-time was added to the model. Statistical significance (p<0.05) between the two groups was determined via a likelihood ratio test comparing the full model with a model reduced by the fixed effect. The goodness of fit of the applied linear mixed effects model was assessed by visual inspection of the plotted residuals and by calculating adjusted R2 values (see Table E1). Additionally, the standard error given for the estimate allows for assessment of the uncertainty of the modelled mean. For consistency and comparability, all data are reported as mean ± standard error of the mean (SEM).

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**SDC 2:** R2 values for the linear mixed effects model to assess goodness of fit.

|  |  |
| --- | --- |
| **Variable** | **R2 adjusted** |
| paO2 [mmHg] | 0.77714 |
| paCO2 [mmHg] | 0.87396 |
| pH | 0.92874 |
| PIPtrach [mbar] | 0.94293 |
| Ptrach plat [mbar] | 0.96085 |
| Ptrach mean [mbar] | 0.66634 |
| PEEPtrach [mbar] | 0.3693 |
| ΔPtrach [mbar] | 0.94827 |
| MV [l·min-1] | 0.95549 |
| VT [ml·kg-1] | 0.85452 |
| CRSdyn [ml·mbar-1] | 0.94244 |
| RR [min-1] | 0.86008 |
| Expiratory time [norm.] | 0.55816 |
| Heart rate [min-1] | 0.88879 |
| MAP [mmHg] | 0.65573 |
| MPAP [mmHg] | 0.8751 |
| Cardiac index [l·min‑1·m‑2] | 0.72239 |
| ELWI [ml·kg-1] | 0.78501 |
| Alveolar wall thickness [µm] | 0.82484 |
| Infiltrated cells [n·field-1] | 0.9321 |
| C(TP)BAL [norm.] | 0.40397 |
| C(SP-A)BAL [norm.] | 0.81418 |

PaO2: arterial partial pressure of oxygen; paCO2: arterial partial pressure of carbon dioxide; PIPtrach: peak inspiratory tracheal pressure; Ptrach: tracheal pressure; PEEPtrach: tracheal positive endexpiratory pressure; ΔPtrach: difference between plateau Ptrach and PEEPtrach; MV: minute volume; VT: tidal volume; CRSdyn: dynamic respiratory system compliance; RR: respiratory rate; MAP: mean arterial pressure; MPAP: mean pulmonary arterial pressure; ELWI: extravascular lung water index; C(TP)BAL: concentration of total protein in broncho-alveolar lavage fluid; C(SP-A)BAL: concentration of surfactant protein A in broncho-alveolar lavage fluid.

**SDC 3:** Body weight, medication, and fluid infusion for FCV and control group. Data presented as mean ± SEM

|  |  |  |  |
| --- | --- | --- | --- |
| **Variable** | **FCV** | **Control** | **p-value** |
| Body weight [kg] | 45 ± 2 | 48 ± 2 | 0.14 |
| Oleic acid [ml·kg-1] | 0.27 ± 0.09 | 0.35 ± 0.09 | 0.40 |
| Midazolam [mg·kg-1·h-1] | 1.09 ± 0.06 | 1.22 ± 0.04 | 0.06 |
| Ketamine [mg·kg-1·h-1] | 22.7 ± 1.2 | 23.6 ± 0.9 | 0.49 |
| Fentanyl [µg·kg-1·h-1] | 5.3 ± 0.4 | 5.5 ± 0.3 | 0.57 |
| Vecuronium [mg·kg-1·h-1] | 0.22 ± 0.01 | 0.21 ± 0.01 | 0.22 |
| Fluid infusion [ml·kg-1·h-1] | 11.7 ± 1.1 | 12.4 ± 1.0 | 0.51 |

FCV: flow-controlled ventilation.

**SDC 4:** Data for the group allocated, but excluded animals

|  |  |  |  |
| --- | --- | --- | --- |
| **Variable** | **No. 1** | **No. 2** | **No. 3** |
| Last data collection | T30 | T60 | T150 |
| PaO2 [torr] (kPa) | 67 (8.9) | 99 (13.2) | 163 (21.8) |
| PaCO2 [torr] (kPa) | 62 (8.3) | 51 (6.8) | 49 (6.5) |
| pH | 7.25 | 7.35 | 7.29 |
| PIPtrach [cmH2O] | 34 | 33 | 30 |
| Plateau Ptrach [cmH2O] | 33 | 31 | 29 |
| Ptrach mean [cmH2O] | 20 | 20 | 19 |
| PEEPtrach [cmH2O] | 9 | 9 | 9 |
| ΔPtrach [cmH2O] | 24 | 22 | 20 |
| MV [l·min-1] | 7.4 | 6.8 | 6.9 |
| VT [ml·kg-1] | 7.4 | 6.5 | 7.1 |
| CRSdyn [ml·cmH2O-1] | 13 | 14 | 19 |
| RR [min-1] | 22 | 22 | 18 |
| Expiratory time [norm.] | 1.3 | 1.3 | 1.2 |
| Heart rate [min-1] | 98 | 89 | 96 |
| MAP [mmHg] | 81 | 90 | 87 |
| MPAP [mmHg] | 51 | 44 | 39 |
| Cardiac index [l·min‑1·m‑2] | 3.9 | 4.2 | 4.6 |
| ELWI [ml·kg-1] | 17 | 13 | 12 |

FCV: flow-controlled ventilation; PaO2: arterial partial pressure of oxygen; PaCO2: arterial partial pressure of carbon dioxide; PIPtrach: peak inspiratory tracheal pressure; Ptrach: tracheal pressure; PEEPtrach: tracheal positive endexpiratory pressure; ΔPtrach: difference between plateau Ptrach and PEEPtrach; MV: minute volume; VT: tidal volume; CRSdyn: dynamic respiratory system compliance; RR: respiratory rate; MAP: mean arterial pressure; MPAP: mean pulmonary arterial pressure; ELWI: extravascular lung water index.

**SDC 5:** Baseline paO2 and cause for exclusion of the excluded animals

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **No.** | **BL paO2**  **[torr]** | **Last data collection** | **Group allocation** | **Cause for exclusion** |
| No. 1 | 112 | T30 | FCV | Instable ventilation, FiO2 instable, defective initiation of inspiration; due to software malfunction. |
| No. 2 | 87 | T60 | FCV | Malignant arrhythmia (ventricular tachycardia), probably due to hypokalemia (K+ 3.1 mmol) |
| No. 3 | 99 | T150 | FCV | Sudden termination of ventilation during preparation for transport to CT scanner, caused by a software malfunction. |
| No.4 | 67 | BL | - | BL criteria not matched, no intervention or control treatment |
| No. 5 | 63 | BL | - | BL criteria not matched, no intervention or control treatment |

BL: Baseline; FCV: flow-controlled ventilation.