Breath-Synchronized Nebulized Surfactant in a Porcine Model of Acute Respiratory Distress Syndrome

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Supplemental Methods

Nebulized Surfactant Drug and Breath-Synchronized Aerosol Delivery System

In this study, we used a bovine surfactant substrate, SF-RI 1 (Alveofact, Lyomark Pharma, Oberhaching, Germany) is commonly used in neonates. SF-RI-1 is composed of approximately 90% phospholipids and 1% surfactant protein B and C. A single vial of powdered SF-RI 1 (108 mg) was reconstituted with 2.4 mL of the supplied HCO_3 buffered saline diluent and placed into the nebulizer reservoir.

Surfactant was delivered with AeroFact™ nebulizer delivery system (Aerogen Pharma, San Mateo, CA). The prototype AeroFact™ nebulizer uses a Photo-Defined Aperture Plate (PDAP) composed of a two-layer vibrating mesh architecture with approximately 20,000 precision-formed apertures. This allows for increased liquid output while generating small aerosol droplet sizes. This nebulizer synchronizes drug delivery with a ventilator breath to prevent drug loss to the system during exhalation by generating aerosol for the first 80% of inspiration. Nebulizer actuation and generation of aerosol on inhalation is based on flow changes detected with a flow sensor placed within the inspiratory limb of the ventilator (Figure 1A). The Aerogen PDAP Controller and Synchronizer can be adjusted to generate aerosol early or late in the inspiratory phase. This technology does not require an external flow source to operate and therefore has no impact on inspiratory volume and pressure delivery or ability of the patient to trigger ventilator breaths. Following completion of nebulization with each dose, 2 drops of normal saline were placed on the mesh and nebulized to prevent residue from forming on the PDAP.

Nebulizer ability to Nebulize High Dose Surfactant

In a fume hood, we ran 3 separate nebulizers in continuous output mode to evaluate durability and longevity based on the nebulizer's ability to function without failure when nebulizing 20 doses (~48 mL or 2,160 mg) of surfactant.

Particle Sizing

A high-performance, multistage, cooled, next generation impactor (NGI, TSI Incorporated, Shoreview, MN) was used to quantify the distribution of aerosol droplet size produced by the prototype AeroFact™ nebulizer and classify into respirable size fractions. The vacuum flow was adjusted with a frit resistor (S/N 511197-9, Cole Palmer, Vernon Hills, IL) at 15 L/min and confirmed with a TSI 5200 Flow and Pressure Analyzer (TSI Industries, Shoreview, MN). The NGI uses eight different particle trays with 45 mm glass fiber filter substrates to collect aerosol droplets. A standard leak test was performed per manufacturer's specifications before each test. 108 mg of SF-RI 1 suspension was aerosolized by the AeroFact™ nebulizer in continuous output mode and aerosol was collected on pre-weighed and pre-conditioned vacuum dried (30 minutes) glass fiber filters (P/N AP2504700, 45 mm diameter, 1.2 µm thickness, Millipore Sigma, St. Louis, MO). Three nebulizers were tested in triplicate (n = 9). Following nebulization, drug mass was quantified gravimetrically at each of the individual filter stages. We then calculated median mass aerodynamic diameter (MMAD), Geometric Standard Deviation (GSD) using a log-probit equation, fine particle fraction (FPF, %) based on proportional mass of respirable particles (MMAD <5.4 µm) and total drug mass delivered to the impactor stages.

Delivered Lung Dose

Nebulizers were placed at the proximal airway between the Y piece and endotracheal tube (Figure 1A and E1). The Aerogen PDAP Controller was set in the breath-synchronized mode and the Synchronizer was adjusted so that aerosol was delivered only within the initial 80% of the inspiratory phase. SF-RI 1 suspension was aerosolized in triplicate with three different nebulizers (n = 9) in the breath-synchronized mode and collected on a filter.

Ventilator settings used were: peak flow of 40 L/min, inspiratory time (It) of 1 second, inspiratory to expiratory time ratio (I:E) of 1:1, positive end expiratory pressure (PEEP) of 15 cmH_2O, respiratory rate (RR) of 30 breaths/min and tidal volume (V_T) of 500 mL. Following heated humidification of inspired gases for 20 minutes with a humidifier (MR 850, Fisher Paykel Healthcare, Auckland, New Zealand) and proprietary heated-wire patient circuits, a hygrometer was placed at the patient Y piece to verify optimal humidification and temperature controlled conditioning of inspiratory gases (39°C; relative humidity: 100%). The ventilator was double filtered with high-efficiency particulate air filters as a safety precaution to prevent any exhaled aerosol from entering the expiratory valve. A 7.5 mm endotracheal tube was attached to a low dead space filter housing with a pre-weighed and pre-conditioned, vacuum dried (30 minutes), low resistance filter (PARI, Sternberg, Germany) that was used to collect inspired aerosol. After nebulization, the filter was removed and absolute drug mass (µg) captured in the filter was quantified using gravimetric analysis prior to and following heating/drying with a vacuum pump at 50°C for 12 hours. The recovered filter mass was then frozen (-

20°C) and surface-active phospholipid content was quantified based on phosphatidylcholine content.

Gravimetric Analysis Assay

We measured the delivered gravimetric mass to quantify aerosolized surfactant for studies in vitro. Weight difference represents the entirety of the solid drug mass delivered to impactor stage filters and lung model filters, which constitutes the active ingredients of surfactant. Roughly 2.4-2.5 mL (or g) of saline and $NaHCO_3$ diluent is added to a mere 118.8 mg (nominally, 108 mg) of SF-RI 1. Inhaled drug mass was weighed with a laboratory balance from pre-weighed and pre-conditioned, vacuum dried (30 minutes) 45 mm glass fiber filters (Impactor) and the lung model filter with an AX205 Delta Range Lab balance (Mettler Toledo, Columbus, OH). Following nebulization to NGI or lung model collected aerosol dose and the filter was then vacuum dried for 2 hours with the next generation impactor (NGI) filters and >10 hours for lung model filters or until the mass did not change. We assessed gravimetric analysis and mass changes to filters with the analytical scale. The drying process evaporated the fluid from the NaCl/$NaHCO_3$ diluent.

Phospholipid Analysis

Phosphatidylcholine is the major lipid found in SF-RI 1, accounting for 80% (~78 mg) of total phospholipids. We quantified phosphatidylcholine levels in the lung model filters and porcine lung tissue. Phosphatidylcholine was measured using commercially

available enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions (ab83377, Abcam, Cambridge, MA). Filters from studies evaluating delivered lung dose *in-vitro* were eluted with 0.1M NaCl and 0.1M $NaHCO_3$ to recover drug bound to the lung model filter. Lung tissue was homogenized with protease inhibitors in phosphate-buffered saline and centrifuged to remove particulates. The resulting solutions were used in the assay and colorimetric values measured at OD450 nm with wavelength correction set to 540 nm using a multi-mode microplate reader (SpectraMax M3, Molecular Devices, San Jose, CA).

Animal Preparation

All experimental animal procedures were conducted according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by Seattle Children's Institutional Animal Care and Use Committee. Experiments were carried out on mixed breed Yorkshire piglets (S&S Farms, Ramona, CA) with weight of 34.3 ± 0.6 kg (n = 8). Pigs were initially sedated with an intramuscular injection of ketamine (30 mg/kg), xylazine (3 mg/kg) and atropine (0.02 mg/kg) and placed in supine position on a surgical table. Continuous electrocardiogram (ECG), oxygen saturation (SpO_2), and rectal temperature were recorded on a PowerLab 16/30 recorder (AD Instruments, Colorado Springs, CO). Temperature was maintained at the normal porcine body temperature range of 38-39°C with a heating pad and a heat lamp. After the administration of local anesthesia (0.5% bupivacaine) subcutaneously around the trachea, animals were tracheotomized with a cuffed 7.5 mm endotracheal tube and connected to a ventilator (Draeger V500, Draeger medical, Lubeck, Germany) in volume assist/control mode with

FiO_2 = 1.0, respiratory rate = 20 breaths/min, inspiratory time = 1.0 second, PEEP = 5 cmH_2O and tidal volume (V_T) adjusted to target 8 mL/kg. The ventilator rate was set at 20/min prior to lavage and adjusted to maintain pH between 7.35 - 7.45, remaining at that setting for the remainder of the experiment. A 20-22-gauge angiocatheter was inserted into the right femoral artery and the left external jugular vein for continuous recording of arterial blood pressure, fluid/drug infusion and blood samples.

Anesthesia was maintained by continuous venous infusion of propofol (loading, 1-2 mg/kg; first 15 minutes, 15 mg/kg/hr to achieve plasma equilibrium concentration; 15- 60 minutes, 12 mg/kg/hr and then maintain at 4-10 mg/kg/hr) combined with bolus of fentanyl (1-2 µg/kg intramuscular injection every 1-2 hours) and vecuronium bromide (0.02-0.04 mg/kg intravenous injection every 30-60 minutes). Analgesia and sedation were titrated to maintain anesthetic plane based on toe pinch and increases in heart rate or blood pressure. Additional doses of vecuronium bromide were administered to prevent spontaneous breathing efforts. A urinary catheter was also inserted via a small lower abdominal incision of the bladder.

Arterial blood gases (ABG: pH, $PaCO_2$, PaO_2, and SaO_2) were measured at regular intervals by a Radiometer ABL 800 (Radiometer America, Brea, CA). An 8 Fr pulmonary artery catheter (Edwards Lifesciences Corp. Irvine, CA) was inserted via the right external jugular vein and connected to the Vigilance II monitor (Edwards Lifesciences Corp.) for the measurement of central venous pressure and mean pulmonary artery pressure (mPAP).

A 12 Fr esophageal balloon catheter (Cardinal Healthcare, Dublin, OH) was positioned in the lower esophagus and proper placement confirmed using the occlusion

technique as previously described (1). Changes in esophageal pressures were used to estimate changes in pleural pressures, and end-inspiratory/expiratory transpulmonary pressures were used in the calculation of static lung compliance measurements. A Hans Rudolph calibrated, differential pressure pneumotachometer was also placed in series for flow, V_T, and airway pressure measurements. The elastance, resistance and static compliance respiratory system (including chest wall and lung) were monitored intermittently using the mechanical ventilator.

Disease severity (PaO_2/FiO_2 and oxygenation index (OI: (FiO_2*mean airway pressure*100)/PaO_2), and ventilation efficiency Index (VEI: 3800/((PIP-PEEP)*RR*$PaCO_2$) (2), ABG, hemodynamics, and lung mechanics were obtained at time zero and every 30 minutes following nebulization (Figure 1B). Peridosing events or physiologic 'clinical deterioration' (e.g. bradycardia, hypoxic events) associated with nebulization were established *a priori* and continuously monitored and recorded following nebulization according to the guideline (3).

ARDS Model by the Repeated Lavage

Induction of lung lavage was performed to induce surfactant-deficiency. A detailed description of this model is published elsewhere (4). Prior to lavage, PEEP was reduced to 2 cmH_2O and tidal volume to 8 mL/kg to induce injury. FiO_2 was 1.0 to induce oxidative stress and pulmonary injury. The airway lavage model consisted of whole lung lavage with 50 mL/kg prewarmed (38°C), normal saline given to an animal via funnel in the supine position and from a height of 30 cm above the lungs and the BALF containing endogenous surfactant is removed by lowering the funnel and recovering

BALF. Lung lavage was repeated until PaO_2/FiO_2 <100 was able to be maintained over 1hour. ABG analysis was performed pre-lavage, post lavage (1hour) and each time point prior to sacrificing the animal. Following lavage, animals remained on V_T 6 mL/kg, FiO_2 1.0 and PEEP 5 cmH_2O. If PEEP 5 cmH_2O was not tolerated (SpO_2<90%), then PEEP was adjusted to maintain SpO_2 between 90-94% prior to initial post-lavage ABG and remained on that PEEP setting for the remainder of study.

Electric Impedance Tomography (EIT)

Global and regional expiratory EIT measurements were obtained with the PulmoVista 500 (Draeger, Lubeck, Germany) at a sampling frequency of 20 HZ. The pre-use testing, application and utilization of the EIT device was done according to the manufacturer's recommendations. To ensure adequate function and high-quality measurement, sixteen ECG electrodes and a reference lead were applied to each subject using a small amount of electrode gel, approximately 30 minutes prior to recording measurements. Pediatric radiolucent ECG monitoring electrodes (Red Dot 3M; #2282) were placed in a straight line, circumferentially around the chest and back at the level of the 5^{th} intercostal space, and the reference electrode was placed on the abdomen above the navel and attached to the PulmoVista. Changes in global and regional expiratory EIT data between baseline and at each timepoint were analyzed offline to assess changes in end-expiratory lung volume (ΔEELV) related to alveolar re-recruitment (recovery), or destabilization in lung volumes (deterioration) using EIT Data Analysis Tool (Draeger, Lubeck, Germany). Investigators reviewed the raw data for each condition (two minutes) and calculated mean ± SEM values for global changes in

end-expiratory impedance waveform by selecting a stable period that included a minimum of 20 spontaneous breaths.

The end-expiratory lung impedance (EELI) is representative of end-expiratory lung volume (EELV) based on previously established linear relationships between impedance and functional residual capacity (FRC) change within the lungs (5-10). The EELI was calculated as the trough value in the global impedance waveform. The relative cross-sectional change in global (all regions) EELI or 'ΔEELI' was calculated as the difference between the baseline (post-lavage) and at each 30 minutes time period following nebulization. The EELI value was then scaled to respective lung volume (mL) changes measured at the proximal airway and expressed as the end-expiratory lung volume (mL).

Regional distribution of ventilation was evaluated by assessing the EELI value as a proportion (%) of the global EIT between four cross-sectional horizontal regions of interest: dorsal, mid-dorsal, ventral, and mid-ventral. Since ARDS has been shown to affect dorsal or 'dependent' lung regions, alveolar recruitment was quantified by further regional assessment of lung impedance in dependent lung regions. Prior studies in humans and animals have shown that the increase in EELV after treatment favors the dorsal (dependent) lung regions, suggesting a gravity-dependent distribution of exogenous surfactant (11-13).

Porcine Lung Samples

After median sternotomy, a vascular perfusion system was created by placing two cannulae into right heart ventricle (16 Fr, infusion site) and bi-cava (20-24 Fr, drainage

site). Whole body was perfused with cold normal saline from the right heart ventricle to wash out the blood from the vascular system at the end of the protocol. Animals were euthanized with euthasol (100 mg/kg). The lungs were removed *en bloc* while ventilated at peak inspiratory pressure of 20 cmH_2O. After taking gross morphological photos, the left lung was catheterized with a 14 Fr catheter and lavaged with 20 mL normal saline. Recovered BALF was removed using gentle suction. The airway instillation technique was used for the fixation of lung in an established manner (14). The left lower lobe was infused with 10% neutral formaldehyde fixative at a constant pressure of 25 cmH_2O, immersed in a container of formaldehyde for 72 hours, then placed in 70% ethanol and embedded in paraffin for histological analysis. The right lower lobe was frozen in liquid nitrogen and stored at -80°C until further processing. After excision of the right middle lobe, samples were rapidly weighed to obtain the “wet” lung weight, and then samples were oven dried (65°C) for 48 hours to determine the stable “dry” lung weight. The wet-to-dry (W/D) weight ratio was used to measure extravascular lung water resulting from injury/inflammation (15).

Porcine Sample Preparation

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes at several time points (pre, post-lavage and endpoint of experiment), and centrifuged at 3,000 rpm and 4°C for 10 minutes. The aliquot supernatant (plasma) was stored at -80°C for further analysis. Frozen lung tissue was homogenized in phosphate-buffered saline containing a protease inhibitor cocktail and centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant (equal total protein concentration of 4 mg/ml) was used for the

assay. BALF samples were placed on ice immediately after collection, and centrifuged at 800 g for 5 minutes at 4°C. The supernatants with protease inhibitor cocktail were stored at -80°C for later inflammatory cytokine measurements.

Histopathologic Evaluation

Left lower lobe samples were embedded in paraffin, and the tissue was sectioned at 5 μm thickness. Hematoxylin and eosin (H&E) staining of slides from three separate lung sites per animal were examined under light microscopy and extent of lung injury was reviewed by a pulmonary pathologist (GD) blinded to treatment group.

A semi quantitative grading scale was used to score lung injury for this experiment, modified from that proposed by the American Thoracic Society and a study of acute lung injury in animals (16, 17) (Table 1 and E1, Figure 3H). Also recorded were relevant histologic phenotypes that have recently been described in severe COVID-19 ARDS, including vascular thrombi, multinucleated giant cells and reactive alveolar pneumocytes (18). Review occurred on H&E slides at x20 magnification, with the whole slide evaluated. Features assessed were: A) Neutrophils in the alveolar space, B) Neutrophils in the interstitial space, C) Hyaline membranes, D) Proteinaceous debris filling the airspaces, E) Alveolar septal thickening, F) Capillary congestion and alveolar hemorrhage and G) Lobular remodeling (including fibrosis and smooth muscle hyperplasia). Grading was as follows: Zero = absent, 1 = mild or localized injury, 2 = moderate injury encompassing several fields, 3 = severe injury encompassing most fields. To generate a lung injury score, the sum of each of the seven independent variables shown in Tables 1 and E1 was tabulated. Images were captured with a digital

camera mounted on a Nikon Eclipse 80i microscope and using NIS-Elements Advanced Research Software v4.13 (Nikon Instruments Inc.).

Cytokine Measurement

Inflammatory markers in plasma, BALF, and lung tissue were analyzed in duplicate using the Eve Technologies Discovery Assay Pig Cytokine Array (Eve Technologies, Calgary, AB, Canada) to measure levels of 13 different cytokines (GM-CSF, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, and TNF-α).

Statistical Analysis

Statistical analysis was performed using PRISM 8 (Graph Pad Software Inc., San Diego, CA). Two-way analysis of variance (ANOVA) was used for the evaluation of the effects of group and time and their interaction and both Sidak and Dunnett multiple comparisons tests were performed on these data. Other data were compared between 2 groups and analyzed with unpaired t-tests. The criterion for significance was $P < 0.05$ for all comparisons.

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