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Impact of high tidal volume ventilation on surfactant metabolism and lung injury in infant rats

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Abstract: The poorly understood tolerance towards high tidal volume (V_T) ventilation observed in critically ill children and age-equivalent animal models may be explained by surfactant homeostasis. The aim of our prospective animal study was to test whether high V_T with adequate positive end-expiratory pressure (PEEP) is associated with surfactant de novo synthesis and secretion leading to improved lung function and whether extreme mechanical ventilation affects intra-cellular lamellar body formation and exocytosis. Fourteen days old rats were allocated to five groups: non-ventilated controls, PEEP 5 cmH₂O with V_T of 8, 16, and 24 mL/kg, respectively, and PEEP 1 cmH₂O with V_T 24 mL/kg. Following 6 hours of ventilation, lung function, surfactant proteins and phospholipids, and lamellar bodies were assessed by forced oscillation technique, quantitative real-time polymerase chain reaction, mass spectrometry, immunohistochemistry, and transmission electron microscopy, respectively. High V_T (24 mL/kg) with PEEP of 5 cmH₂O improved respiratory system mechanics and was not associated with lung injury, elevated surfactant protein expression, or surfactant phospholipid content. Extreme ventilation with V_T 24 mL/kg and PEEP 1 cmH₂O produced a mild inflammatory response and correlated with higher surfactant phospholipid concentrations in bronchoalveolar lavage fluid without affecting lamellar body count and morphology. Elevated phospholipid concentrations in the potentially most injurious strategy (V_T 24 mL/kg, PEEP 1 cmH₂O) need further evaluation and might reflect accumulation of biophysically inactive small aggregates. In conclusion, our data confirm the resilience of infant rats towards high V_T -induced lung injury and challenge the relevance of surfactant synthesis, storage, and secretion as protective factors.

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Title Page

Title: Impact of high tidal volume ventilation on surfactant metabolism and lung injury in infant rats

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Keywords: Ventilator-induced lung injury, surfactant metabolism, animal model, respiratory system diagnostic techniques, electron microscopy

27 **ABSTRACT**

28 The poorly understood tolerance towards high tidal volume (V_T) ventilation observed
29 in critically ill children and age-equivalent animal models may be explained by
30 surfactant homeostasis. The aim of our prospective animal study was to test whether
31 high V_T with adequate positive end-expiratory pressure (PEEP) is associated with
32 surfactant *de novo* synthesis and secretion leading to improved lung function and
33 whether extreme mechanical ventilation affects intracellular lamellar body formation
34 and exocytosis. Fourteen days old rats were allocated to five groups: non-ventilated
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36 cmH₂O with V_T 24 mL/kg. Following 6 hours of ventilation, lung function, surfactant
37 proteins and phospholipids, and lamellar bodies were assessed by forced oscillation
38 technique, quantitative real-time polymerase chain reaction, mass spectrometry,
39 immunohistochemistry, and transmission electron microscopy, respectively. High V_T
40 (24 mL/kg) with PEEP of 5 cmH₂O improved respiratory system mechanics and was
41 not associated with lung injury, elevated surfactant protein expression, or surfactant
42 phospholipid content. Extreme ventilation with V_T 24 mL/kg and PEEP 1 cmH₂O
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44 phospholipid concentrations in bronchoalveolar lavage fluid without affecting lamellar
45 body count and morphology. Elevated phospholipid concentrations in the potentially
46 most injurious strategy (V_T 24 mL/kg, PEEP 1 cmH₂O) need further evaluation and
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48 our data confirm the resilience of infant rats towards high V_T -induced lung injury and
49 challenge the relevance of surfactant synthesis, storage, and secretion as protective
50 factors.

51

52 INTRODUCTION

53 The key concept of protective mechanical ventilation strategies implies the
54 application of so-called low tidal volumes (V_T) and cautious oxygen delivery to ensure
55 adequate gas exchange without causing additional ventilator or operator-induced
56 lung injury (3). However, application of higher V_T in pediatric acute respiratory
57 distress syndrome (ARDS) patients seems not to be associated with increased
58 mortality (14, 25) and a recent study in burned pediatric patients found more
59 ventilator-free days and less atelectasis after exposure to ventilation with a V_T of up
60 to 15 mL/kg (42). Hence, clinical practice questions and partially contradicts general
61 recommendations on ideal V_T in critically ill children.

62 Few experimental studies have systematically investigated the impact of different V_T
63 on ventilator-induced lung injury (VILI) in infantile and pediatric models. Results from
64 these age-specific animal studies also challenge the concept of protective low V_T
65 ventilation. In fact, high V_T ventilation in both one-hit (10, 27) and two-hit models (8,
66 41) was associated with improved lung function without aggravated inflammation
67 when compared to control animals. *Ex vivo* and *in vitro* studies suggest that
68 mechanical stretch of alveolar epithelial type II (AE2) cells promotes both surfactant
69 *de novo* synthesis and calcium-induced secretion of surfactant proteins (SPs) and
70 phospholipids (PLs) (1, 36). Thus, alterations of alveolar surfactant homeostasis
71 might play a key role with regards to high V_T susceptibility in children. The impact of
72 high V_T ventilation on top of zero positive end-expiratory pressure (PEEP) has been
73 investigated by Milos et al. (31) in the pulmonary surfactant system of adult rats.
74 They demonstrated a decrease in intracellular surfactant-secretory granules, termed
75 lamellar bodies (LBs), in AE2 cells and impaired biophysical surfactant properties
76 related to VILI.

Given the poorly understood age-specific tolerance towards high V_T ventilation, the aim of our study was to compare the impact of different mechanical ventilation strategies on surfactant metabolism in an infantile (non-neonatal) *in vivo* rat model. To minimize the well-known limitations of rodent models, we tried to optimize the translational value of this study by applying multimodal methodologies, including ventilation strategies with clinically significant PEEP levels, and ensuring that all ventilated animals were alive at the end of the 6-hour protocol. Our first hypothesis was that improved lung function after high V_T ventilation on top of adequate PEEP (5 cmH₂O) is associated with stretch-induced surfactant *de novo* synthesis and release. The second hypothesis was that 6 hours of ventilation with both extreme V_T and low PEEP results in less intracellular LB formation and exocytosis leading to impaired surfactant function.

MATERIALS AND METHODS

Animals. The research protocol was approved by the Cantonal Veterinary Office of Zurich and was performed within the Ethical Principles and Guidelines for Experiments on Animals of the Swiss Academy of Medical Sciences and the Swiss Academy of Sciences. Infant outbred specific pathogen-free Wistar rats of either sex were purchased from Charles River Laboratories International, Inc. (Sulzfeld, Germany) at the age of 7 days. Animals were housed in single cages with their dams and littermates under 12 h light-dark regime with food and water *ad libitum*. After an acclimatization period of 6-8 days, pups were weighed (mean 39.7 (SD 3.9) g) at the age of 13-15 days (equivalent to lung development in humans at the age of 12-24 months) and introduced into the experiment (6).

Preparation of animals, mechanical ventilation, and monitoring. Preparation and monitoring of rat pups as well as standardization procedures such as anesthesia, lung volume history, and measurements of respiratory system mechanics at baseline were performed as previously published (28). Briefly, rat pups were anesthetized with a solution containing ketamine (75 $\mu\text{g/g}$ body weight) and xylazine (12 $\mu\text{g/g}$ body weight). Then, a tracheostomy was performed and pups were connected to a computer-controlled ventilator (flexiVent®, Scireq, Montreal, Canada) in supine position on a heating mat (Physitemp Instruments, Incorporation, TCAT-2LV Temperature Controller, Clifton, New Jersey, USA). Non-ventilated control (NVC) pups underwent the same preparatory work except for connection to the ventilator. Lung volume history was standardized by applying two recruitment maneuvers up to 40 cmH_2O with 9-sec ramp and 3-sec plateau within two minutes. Next, the following baseline settings were adjusted: V_T ~ 8 mL/kg, PEEP 5 cmH_2O , respiratory rate (RR) 100 min^{-1} , and inspired oxygen fraction (FiO_2) 0.4. PEEP 5 cmH_2O was chosen to be adequate as lower PEEP levels facilitate alveolar derecruitment (9). Monitoring of heart rate (HR) and peripheral oxygen saturation (SpO_2) as well as body temperature control at 38°C were ensured with an animal pulse oximeter (MouseOx™, STARR Life Sciences Corporation™, Oakmont, PA, USA) and a thermocouple feedback sensor accompanied by a heating mat (Physitemp Instruments), respectively. A constant phase model (21) was applied to determine respiratory system impedance by the low-frequency forced oscillation technique provided by the flexiVent® system. This allowed calculation of airway resistance (R_{aw}), tissue damping (G), and tissue elastance (H). At each time point, 4 respiratory system input impedance spectra were collected within 120 sec and the resulting values were averaged. Anesthesia was maintained via top-ups every 2 h, ~ 15 min before each respiratory system mechanics measurement. Subcutaneous fluid replacement with 0.5 mL of saline solution was

conducted directly after each measurement of respiratory system mechanics. At the end of ventilation, mixed blood gas parameters, pH, and partial pressure of carbon dioxide ($p\text{CO}_2$) were determined via a portable blood analyzer (i-STAT[®], Axonlab, Abbott Laboratories, IL, USA) using few drops of whole blood. Euthanasia of the pups was induced by complete exsanguination under full anesthesia. After separation of the last pup from the litter, dams were euthanized via inhalation of carbon dioxide.

Experimental design. Animals were allocated to the following study groups: 1) NVC = non-ventilated controls ($n = 19$); 2) $V_T8\text{PEEP}5 = V_T 8 \text{ mL/kg}$, PEEP $5 \text{ cmH}_2\text{O}$, respiratory rate (RR) 100 min^{-1} ($n = 24$); 3) $V_T16\text{PEEP}5 = V_T 16 \text{ mL/kg}$, PEEP $5 \text{ cmH}_2\text{O}$, RR 40 min^{-1} ($n = 24$); 4) $V_T24\text{PEEP}5 = V_T 24 \text{ mL/kg}$, PEEP $5 \text{ cmH}_2\text{O}$, RR 20 min^{-1} ($n = 24$); and 5) $V_T24\text{PEEP}1 = V_T 24 \text{ mL/kg}$, PEEP $1 \text{ cmH}_2\text{O}$, RR 25 min^{-1} ($n = 22$). Following baseline measurements of respiratory system mechanics (study groups 2-5), pups were ventilated for 6 h with lung function measurement intervals of 2 h.

Sampling and processing of bronchoalveolar lavage fluid. After the 6-h-ventilation protocol, rat pups were disconnected from the ventilator and their lungs were lavaged three times with ice-cold $1.5 \text{ mL } 0.9\%$ saline through the endotracheal tube. Three bronchoalveolar lavage fluid (BALF) aliquots were pooled resulting in $\sim 4 \text{ mL}$. BALF was immediately centrifuged at $300 \times g$ for 5 min to remove cell debris. Supernatant was aliquoted, shock frozen in liquid nitrogen, and stored at -80°C for future analyses.

Mass spectrometric analyses of surfactant phospholipids. Surfactant PLs from BALF were determined by electrospray ionization tandem mass spectrometry as described elsewhere (3). PLs were extracted from BALF according to the Bligh and Dyer method (5). As internal standard 125 nmol di-eicosanoyl-phosphatidylcholine (Avanti

Polar Lipids, Alabaster, AL, USA) was added to BALF aliquots. BALF samples were dissolved in chloroform-methanol-water (20:78:2, vol/vol/vol) at a PL concentration of 3 $\mu\text{mol/L}$. Then, they were introduced into the triple quadrupole mass spectrometer (Thermo TSQ Quantum Discovery MAX, Thermo Fisher Scientific, Dreieich, Germany) via loop injection from the Finnigan Surveyor Autosampler Plus and MS Pump Plus equipped with a heated electrospray ionization interface (Thermo). The PL species were determined by selective reaction monitoring. The precursor scan of protonated phosphocholine head group was used to determine phosphatidylcholine (PC), lyso-PC, and sphingomyelin (SM) species. PLs were calculated as percentage of the total contained in the BALF sample in reference to internal standard. After determination of the PL classes using the sum of all individual molecular species related to the internal standard, the specific concentration in total BALF was calculated.

Lung injury biomarkers in BALF. The concentration of IL-1 β , IL-6, macrophage inflammatory protein (MIP)-2, and albumin in BALF was determined using commercial ELISA kits (RECYTMAG-65K-03 and RKT2MAG-37K-01, Merck KGaA, Darmstadt, Germany). All samples were analyzed in duplicates in a blinded fashion along with Luminex xMAP technology by EMD Millipore MILLIPLEX system according to the manufacturer's instructions with the following two modifications. We set up 8 standards using an additional lower point. Moreover, we added undiluted BALF samples in a volume of 50 μL and incubated with 25 μL antibody-coupled beads in sample wells over night at 4°C in a first incubation step.

Sampling, fixation, and preparation of lungs. Following laparotomy and thoracotomy, lungs were randomly assigned to SP mRNA evaluation by quantitative real-time PCR (qPCR) or to histological and immunohistochemical analyses with or without transmission electron microscopy (TEM). Lungs of 7-9 pups per group were removed

180 *en bloc* from the thoracic cavity to determine SP mRNA levels. The left and right
181 lungs were separated, shock frozen in liquid nitrogen, and stored at -80°C.

182 Lungs from 11-13 animals per study group were processed for histological,
183 immunohistochemical, and TEM studies. Right lungs were scheduled for histological
184 and immunohistochemical analyses and left lungs of 3-5 animals per group were
185 additionally appointed for TEM. Therefore, 6-9 lungs solely intended for light
186 microscopy were fixed *in situ* via instillation of 4% buffered paraformaldehyde through
187 the endotracheal tube at a constant pressure of 10 cmH₂O for 30-60 min. Then, right
188 lungs were isolated and submerged in the same fixative over night at 4°C. Lungs of
189 the remaining 3-5 animals were prepared both for light and electron microscopy.
190 From each animal, the left lung was instilled *in situ* via endotracheal tube with 2.5%
191 glutaraldehyde buffered in 0.2 M cacodylic acid buffer, pH 7.3, at a pressure of 10
192 cmH₂O for 30 min at 4°C, while the right main bronchus was ligated. Subsequently, a
193 ligature was placed at the left main bronchus, and the right main bronchus was untied
194 to fix the right lung with 4% buffered paraformaldehyde as described above. Finally,
195 lungs were removed from the thoracic cavity, left and right lungs were separated, and
196 submerged in the respective fixative over night at 4°C.

197 *Quantitative real-time PCR.* Total mRNA was isolated from lung tissues
198 homogenates using RiliaPrepTM RNA Cell Miniprep System (Promega Corporation,
199 Promega AG, Dübendorf, Switzerland) according to manufacturer's instructions.
200 Reverse-transcriptase PCR for cDNA synthesis was performed via Biometra's T-
201 Personal Thermal Cycler (Biocompare, South San Francisco, CA, USA) along with
202 the GoScriptTM Reverse Transcription System (Promega) using 1 µg of total RNA as
203 per manufacturer's manual. Twenty µL cDNA were diluted in 100 µL nuclease-free
204 water and stored at -20°C until further use. qPCR was performed on a CFX
205 ConnectTM Real-Time PCR Detection System (Bio-Rad Laboratories, Incorporation,

Bio-Rad Laboratories AG, Cressier, Switzerland). We used a PCR sample volume of 14 μ L, containing 2.6 μ L cDNA, 7 μ L 2x GoTaq[®] qPCR Master Mix (Promega), 3 μ L H₂O, and 0.7 μ L of each primer (10 μ M) pursuant to the following protocol. The initial activation step at 50°C for 2 min and at 95°C for 10 min was followed by 39 cycles of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 1 min, and dissociation at 95°C for 10 sec. Gene-specific primers for SP-A, -B, -C, and -D were synthesized according to published cDNA sequences (35) and originated from a commercial source (Microsynth AG, Balgach, Switzerland). The house keeping gene ribosomal protein L13a (RPL13a) was used as an internal control (40). Its sequence was already predefined by a commercial supplier (Microsynth) as MuRPL13aEx4f for the forward and RatRPL13aEx6r for the reverse primer. The sequences for all primers used for qPCR are listed in Table 1. The cycle threshold values were normalized to the RPL13a expression.

Processing of lungs for histological and immunohistochemical analyses. From the lungs exclusively processed for histology and immunohistochemistry, the right cranial, medial, and caudal lung lobes were dissected by a veterinary pathologist blinded to the study, and were subsequently routinely paraffin wax embedded. Additionally, accessory lobes of animals scheduled to both light and electron microscopic analyses were included into the same assessment and were similarly processed. Consecutive sections (3-5 μ m) were prepared and subjected to hematoxylin-eosin (HE) or immunohistochemical stain for the detection of AE2 cells. For immunohistochemistry, the horseradish peroxidase method was applied using an automated staining device (Dako Discovery XT, Ventana Medical Systems, Inc., Tucson, AZ, USA) with 3,3'-diaminobenzidine (Dako) as a chromogen and Meyer's hematoxylin counterstain. Slides were pretreated with acidic buffer (CC2, No. 950-123, Ventana Medical Systems) and then incubated with the SP-C antibody (rabbit

anti-human SP-C, 1:50, sc-13979, Santa Cruz Biotechnology Inc., Heidelberg, Germany) for 1 h at 37°C, followed by incubation with the OmniMap™ anti-Rb HRP kit (760-4311, Ventana Medical Systems) according to the manufacturer's protocol.

Histological and immunohistochemical image analyses. The HE and SP-C stained sections from the right lung lobes were scanned at a magnification of ×40 using a digital slide scanner (NanoZoomer-XR C12000; Hamamatsu Photonics K. K., Hamamatsu City, Japan). Then, histomorphometrical assessments of acute lung injury (ALI) were performed in a blinded fashion based on recommendations published by American Thoracic Society for experimental lung injury (32). Briefly, the following features were analyzed: alveolar inflammatory cells, edema, and hemorrhage, hyaline membranes, atelectasis, and overdistension. Three to four lobes from the right lung were assessed per animal and the averages used for statistical analyses. Taking into account heterogeneity of the lung, especially of infant rats at the stage of bulk alveolarization, proximal and distal areas were pursuant standardization representatively evaluated. Moreover, areas with large bronchioles and vessels were excluded from the analyses. For quantification of alveolar inflammatory cells, 5 non-overlapping regions of interest (ROIs) with a size of 0.56 mm² were randomly set in distal areas of a section at the ×40 magnification. In each ROI, the number of alveolar macrophages (AMs) and neutrophil granulocytes (NGs) was counted. The average number of inflammatory cells normalized to surface area of ROI was expressed as cells per square millimeter. Alveolar edema, hemorrhage, and hyaline membranes were identified by intra-alveolar pink homogenous fluid, erythrocytes, and eosinophilic glassy membranes lining alveolar walls, respectively. An injury score ranging from 0-3 (0 = none; 1 = mild, <25%; 2 = moderate, 25-75%; 3 = severe, >75%) was estimated using the same random ROIs from each HE section. Atelectasis and overdistension were analyzed via two separate ways. First,

atelectasis and overdistension scores were determined according to Kozian et al. (26) via a combination of extent and severity of a feature with the following modifications. An image software (ImageJ 1.51f, Wayne Rasband, National Institutes of Health, USA) was used to quantify the extent of the affected area of whole lung sections, which was categorized from 1-5 as follows: 1 (0-25%); 2 (>25–50%); 3 (>50–75%); 4 (>75% to almost complete); and 5 (complete). In addition, severity was graded from 0-3 (0 = normal; 1 = mild; 2 = moderate; 3 = severe). The extent and the severity of the feature were multiplied by each other resulting in a score ranged from 0 to 15. Second, morphometric analyses of alveolar number and relative surface area were quantified with the computer program VIS (Visiopharm Integrator System, Version 5.0.4. 1382; Visiopharm®, Hoersholm, Denmark). Therefore, 5 non-overlapping ROIs with a size of 0.905 mm² were randomly set both in proximal and distal areas each at the magnification of ×40. Alveolar lumina, walls, and counts were determined by a threshold classification in each ROI. Then, the alveolar number and surface area were normalized to lung parenchyma surface area, respectively).

In SP-C-stained sections, the number of AE2 cells was counted in cranial, medial, and caudal lung lobes in 10 non-overlapping randomly selected ROIs with a size of 0.026 mm² of proximal and distal areas each using the Visiopharm® software tool. A threshold classification allowed distinguishing between SP-C-positive cells and negative parenchyma. The number of AE2 cells was expressed as cells per square millimeter in relation to SP-C negative tissue surface area.

Transmission electron microscopy. For TEM, the left lungs were routinely prepared and ultrathin sections were viewed via a FEI Philips CM10 (TSS Microscopy, Hillsboro, OR, USA) operating with a Gatan Orius Sc1000 digital camera (Gatan, Inc., Version 3.30.2016.0, Gatan Microscopy Suite®, Pleasanton, CA, USA). Three blocks of approximately 1 mm³ were trimmed from cranial, medial, and caudal

284 locations of the left lung and routinely embedded in epoxy resin. Of each specimen,
285 toluidine blue stained semithin sections (0.9 μm) were prepared. Representative
286 areas were trimmed and ultrathin sections (90 nm) prepared, contrasted with lead
287 citrate (Merck) and uranyl acetate (Merck). Representative electron micrographs
288 were taken at a resolution of $\times 2,600$ of at least 9 AE2 cells per lung (3 AE2 cells per
289 location). Image analysis was carried out in a blinded fashion using digital viewing
290 software (ImageJ 1.51f) to determine surface areas of AE2 cells and of each
291 individual LB per AE2 cell in square micrometers as well as the number of LBs per
292 each individual AE2 cell. Then, the mean surface area of AE2 cells per group and the
293 total surface area of LBs per AE2 cell in square micrometers were calculated.
294 Furthermore, each TEM image was semi-quantitatively evaluated for differences in
295 the distribution of extracellular surfactant structures, mitotic AE2 (AE2_{mit}), and
296 intermediate alveolar epithelial (AE_{int}) cells. Only representative electron micrographs
297 containing at least one of AE cell types with observable nuclei (alveolar epithelial type
298 I (AE1), AE2, AE2_{mit}, and AE_{int} cells) fulfilled criteria of a ROI (0.0017536 mm²). The
299 numbers of each extracellular surfactant structure, AE2_{mit}, and AE_{int} were counted
300 based on their morphological appearance (7, 15, 16). Results were expressed as the
301 number of surfactant subtypes or cells per square millimeter. Following surfactant
302 subtypes were determined: tubular myelin (TM, lattice-like) and LB-like (LBL, densely
303 packed, single lamellae not differentiable) representing the biophysically active
304 surfactant forms called large aggregates (LAs), unilamellar (UL, one lamellae) and
305 lamellar vesicles (LV, with 2-3 lamellae) representing small aggregates (SAs) as
306 inactive surfactant subfraction, and multilamellar (ML, with >3 lamellae) and
307 multivesicular (MV, with >2 vesicles which are neither SA nor LA) surfactant forms
308 without clear classification, defined by Busley et al. (7) and Jörger et al. (23). ML and
309 MV were included into the LA subfraction for statistical analysis, since these subtypes

possess surface activity. AE2 cells lining alveolar surface, containing LBs, in phases before, during, and after cell division were recognized as AE2_{mit} cells, whereby sister cells were stated as one counting event (15). AE_{int} cells were identified by characteristics of both AE1 and AE2 cells with apical microvilli, LBs, and cytoplasmic extensions (16).

Statistical analysis. Statistics were conducted using the analysis software tool SigmaStat 3.5 (Systat Software Inc., Richmond, CA, USA). Normally distributed data were evaluated by repeated measures one-way ANOVA (over time) or by one-way ANOVA (at single time points) with Holm-Sidak *post hoc* tests and presented as means (SEM), if not otherwise stated. Non-normally distributed variables were compared via Kruskal-Wallis one-way ANOVA on ranks and reported as medians with interquartile range. Values were considered to be statistically different at $p < 0.05$.

RESULTS

Respiratory system mechanics. At baseline, there were no significant differences in airway resistance (R_{aw}), tissue damping (G), tissue elastance (H), and hysteresivity (η) between all groups ($p \geq 0.32$ in all cases; Figure 1). From the time point 0 onwards, R_{aw} did not change in the group V_T24PEEP1, only ($p = 0.99$). In the other three groups with a PEEP of 5 cmH₂O, we observed an inverse correlation between V_T and R_{aw} over time. Following allocation, G and H significantly increased over time in V_T8PEEP5 and V_T24PEEP1 groups. In contrast, we found a significant and continuous fall of G and stable H values in V_T16PEEP5 and V_T24PEEP5 groups. Finally, η significantly decreased in all groups over time. At the end of ventilation, we

observed significant inter-group differences where higher V_T on top of a PEEP 5 cmH₂O were associated with higher η values.

Vital parameters and blood gas analyses. SpO₂, HR, pH, and pCO₂ were in a physiological range and showed no significant differences between ventilated groups at the end of the ventilation protocol (Table 2).

Surfactant protein expression. SP expression did not significantly differ between all groups for SP-A, SP-B, and SP-D (Figures 2A, 2B, and 2D). For SP-C, the only statistically significant difference was found between NVC and V_T24PEEP5 (Figure 2C; representative immunohistochemical images of rat lungs are displayed in Figure 3).

BALF surfactant phospholipids. Specific concentrations of surfactant PL species (PC, DPPC, lyso-PC, and SM) in BALF were significantly higher in the group V_T24PEEP1 in comparison with all other groups (Figure 4A-D). Additionally, PC, DPPC, and SM were significantly lower in the groups V_T16PEEP5 and V_T24PEEP5 when compared to NVC.

Assessment of lung injury. Alveolar edema and hyaline membranes were not present in our lung sections ([Supplemental Figure S1](#)). Thus, only hemorrhage, atelectasis, and overdistension were reported for the histological lung injury score in Table 3 (hemorrhage: $p = 0.19$; atelectasis: $p = 0.15$; overdistension: $p = 0.49$). Additionally, atelectasis and overdistension were quantified by alveolar number and relative surface area per parenchyma surface area. Both parameters did not differ between ventilated groups ($p \geq 0.25$ in both cases; [Supplemental Figure S2](#)). After 6 h of ventilation, both PC/lyso-PC and PC/SM ratios as indicators of lung injury did not differ between all ventilated study groups ($p \geq 0.34$; Table 3). Furthermore, there was no significant inter-group difference in inflammatory cells counted in HE stained lung sections (Figures 5A and 5B). Regarding BALF biomarkers, we did not find any

significant differences for IL-1 β and albumin concentrations between all groups (Figures 5C and 5F). IL-6 levels were significantly lower in NVC, but did not differ between ventilated groups ($p = 0.09$; Figure 5D). In contrast, MIP-2 concentrations were significantly higher in the V_T24PEEP1 group when compared to NVC and V_T24PEEP5 (Figure 5E).

Alveolar epithelial cells and surfactant ultrastructures. We did not find any significant differences in the count and size of AE2 cells between all experimental groups (Figures 6A and 6B). Also, there was no evidence for increased number of AE2_{mit} (NVC: 59.5 (20.4) mm⁻²; V_T8PEEP5: 51.4 (11.3) mm⁻²; V_T16PEEP5: 49.4 (20.9) mm⁻²; V_T24PEEP5: 44.8 (21.2) mm⁻²; V_T24PEEP1: 77.0 (1.7) mm⁻²; $p = 0.82$) and AE_{int} cells (NVC: 122.3 (34.5) mm⁻²; V_T8PEEP5: 217.1 (42.2) mm⁻²; V_T16PEEP5: 173.9 (34.4) mm⁻²; V_T24PEEP5: 88.2 (24.8) mm⁻²; V_T24PEEP1: 193.7 (51.3) mm⁻²; $p = 0.11$) after mechanical ventilation ([Supplemental Figure S3](#)). Furthermore, we did not find any significant inter-group differences in the quantity and surface area of intracellular LBs (Figures 6C and 6D). Additionally, we did not detect any significant differences between study groups for extracellular surfactant forms, i.e. TM, LBL, ML, MV, UL, and LV surfactant subtypes (Figures 7, 8A and 8B).

DISCUSSION

We designed this *in vivo* study to investigate for the first time surfactant metabolism in infant rats exposed to extremely high V_T ventilation strategies over a period of 6 hours. We hypothesized that stretch-induced surfactant *de novo* synthesis and secretion correlate with improved lung function after high V_T ventilation on top of adequate PEEP. In addition, based on adult rat studies, we hypothesized that repetitive derecruitment during high V_T ventilation with very low PEEP results in altered number and morphology of LBs. Both hypotheses were not confirmed. Also,

in contrast to adult rodent studies, features of VILI after extreme ventilation strategies were mild.

Changes in respiratory system mechanics were objectified with the forced oscillation technique. This method provides data on mechanical properties of central and peripheral airways and lung tissue. R_{aw} describes the resistance in the conducting airways and its variations can be explained by changes in airway caliber (13). H is associated with alterations in lung volume and indicative for changes in lung tissue stiffness, whereas G reflects lung tissue resistance and is related to regional airspace inhomogeneity (23, 29). As expected, R_{aw} , G , and H improved with increasing V_T from 8 to 24 mL/kg on top of PEEP of 5 cmH₂O. Double and triple V_T most probably led to significant increased airway diameters, airway homogeneity, and end-expiratory lung volume. In contrast, applying an extreme V_T to a PEEP of 1 cmH₂O resulted in an immediate and rapid rise of R_{aw} most likely due to reduced airway calibers caused by low end-expiratory pressure and volume. Thereafter, ventilation with excessive V_T prevented a further rise of R_{aw} . While high V_T ventilation with adequate PEEP of 5 cmH₂O produced both stable H values and less heterogeneity expressed by a fall of G , PEEP reduction to 1 cmH₂O caused a disproportionate increase of H when compared to G . This PEEP-induced alteration of respiratory system mechanics explains the lower η values and reflects a substantial lung volume derecruitment. Lower η has also been observed in surfactant knockout (SP-C^{-/-}) and heterozygous SP-B-deficient and null SP-C adult mice (SP-B^{+/-}/SP-C^{-/-}) when compared to controls (17, 22). Although raw data of G and H were not provided in these studies, it is conceivable, that the behavior of η indicates reduced viscoelasticity and can be explained by a disproportionate rise of H due to loss of lung volume in SP-C^{-/-} and SP-B^{+/-}/SP-C^{-/-}-mice. Takahashi et al. used an adult mouse lavage model of acute lung injury (ALI) and demonstrated that higher H

values led to lower η when compared with non-lavaged controls (43). Noteworthy, they suggested that the rise of H with time depends on tissue and surface film viscoelasticity and is a sensitive index of derecruitment. Taken together, alterations in respiratory system mechanics in our study most likely reflect changes in lung volume.

Studies performed in adult rat models reported a strong inflammatory response and severe alveolar damage after a short period of up to 90 min of mechanical ventilation with high V_T and zero PEEP (10, 27, 34). In contrast to adult studies, we only found a significantly higher BALF MIP-2 concentration after 6 h of ventilation with the $V_T24PEEP1$ strategy. Moreover, none of our extreme mechanical ventilation strategies caused significant physical damage to peripheral lung units at the light microscopic level. The American Thoracic Society guidelines for determination of experimental ALI (30) state that at least three out of four main features should be identified to confirm lung injury. The presence of two aspects of ALI in our experimental setting, i.e. impaired lung function and elevated MIP-2, does not point towards significant VILI.

Nevertheless, the rise of MIP-2 might be indicative for an inflammatory process following the extreme ventilation strategy with $V_T24PEEP1$. As shown in a comparable *in vivo* ventilation adult rat model (11) and in previous human AM and AE2 cell culture studies (37, 47), MIP-2 (or its human homologue IL-8) release can be induced via prolonged mechanical stretch. Moreover, repetitive stretch of AE2 cells promotes their proliferation and transdifferentiation to AE1 cells over an intermediate AE cell form (38). In addition, Chess et al. demonstrated in an *in vivo* murine model that 6 h of mechanical ventilation with a moderate V_T of 10 mL/kg and PEEP of 3 cmH₂O were sufficient to induce enhanced proliferation of AE2 cells (46). Thus, we quantified the total number of AE2 cells on SP-C stained sections. Next, we semi-quantitatively determined intermediate AE cells and mitotic AE2 cells on

electron micrographs. The absence of significant differences between study groups suggests that proliferation and transdifferentiation most probably did not occur in our study, again challenging the view of high V_T -induced VILI.

Mechanical stretch of AE cells is also known to trigger inter- and intra-cellular calcium-signaling stimulating surfactant production and release in *in vitro* and *ex vivo* conditions (1, 33, 36). Martinez et al. demonstrated a V_T - and time-dependent increase of alveolar surfactant content and alterations of its biophysical properties in *in vivo* infant rats (28). Therefore, we determined SP mRNA and PL concentrations in BALF by qPCR and mass spectrometry, respectively. In contrast to our hypothesis, improved lung function as a result of high V_T was not associated with enhanced SP expression or elevated PL content in the high V_T groups with adequate PEEP (V_T 16PEEP5 and V_T 24PEEP5). Even though we found a statistically significant low PL concentration when comparing NVC versus both V_T 16PEEP5 and V_T 24PEEP5 groups, we consider this difference physiologically negligible. The decrease of PEEP from 5 to 1 led to a statistically significant larger surfactant PL pool. Intuitively, one might expect that high PL content is associated with enhanced surfactant activity leading to improved lung function, which was clearly not the case in our study. In line with our results, *in vivo* and *ex vivo* adult rat studies examining the impact of high V_T without PEEP on surfactant and lung function also demonstrated an impaired lung compliance accompanied by increased total surfactant pools in BALF. Further analyses of BALF revealed an elevated SA/LA ratio and poor surfactant surface tension lowering properties (31, 44, 45). LAs represent the biophysically active form and consist of TM, LBL, ML, and MV surfactant structures. SAs emerge as metabolic products from LAs and are composed of vesicular PLs, UL and LV with poor surface activity (7). Thus, we can only speculate whether the SA/LA ratio at the alveolar air-liquid interface was also altered in our study groups.

Mass spectrometric BALF analyses allowed to further differentiate between individual surfactant PL species such as PC, lyso-PC, and SM. SM breakdown products such as ceramide and sphingosine are potent inhibitors of protein kinase C, a pivotal enzyme in surfactant secretion (20). Likewise, disproportionate accumulation of lyso-PC, arising from PC via deacylation through phospholipase A2 (36), suppresses the ability of SPs to promote the insertion of PLs into the interfacial surfactant film favoring a destabilization of its functional 3D structure and accelerating SA formation (4). Moreover, alterations in surfactant PL profile are associated with alveolar instability and impaired lung function. In fact, ARDS patients and ALI animal models showed decreased PC with concomitantly increased SM and lyso-PC levels in BALF (12, 18, 19, 24, 39). In our study, both SM and lyso-PC content was significantly higher after V_{T24} PEEP1 ventilation when compared to all other groups. This finding might indicate and reflect VILI following an extreme ventilation strategy. Then again, and in contrast to the above-mentioned studies, the rise of SM and lyso-PC was accompanied by a substantial and proportional increase of PC leading to similar ratios between study groups. Hence, both the role and ratio of these PL subtypes in the development of VILI remains unclear and does not challenge the assumption of derecruitment-induced deterioration in respiratory system mechanics in our study.

To investigate intracellular LB pathways and extracellular SA/LA ratios, we performed TEM analyses of lung sections. TEM provides a thorough tool to visualize the profile of intra- and extracellular morphological surfactant subtypes and alveolar cells. The number, morphology, and composition of intracellular LBs and extracellular surfactant structures as well as AE2 cell lumina did not show any significant differences between study groups. These findings confirm our results obtained by SP mRNA and BALF PL analyses, i.e. no increase in surfactant synthesis and secretion

when doubling and tripling the V_T under adequate PEEP. Interestingly, as opposed to Milos et al. (31), no alterations in intracellular LB formation or exocytosis were detected after ventilation with extreme V_T 24PEEP1 at the ultrastructural level. Moreover, the ratio of extracellular SA and LA fractions was not affected by any ventilation strategy. Thus, our TEM analyses suggest that none of our extreme ventilation strategies had a direct impact on surfactant synthesis, storage, and secretion.

We have to mention the following limitations. First, 6 h of mechanical ventilation might still be too short to induce clinically significant alterations. An extension of our ventilation protocol is desirable, but hampered by logistical issues. However, to the best of our knowledge, so far only one *in vivo* study examined the impact of mechanical ventilation on surfactant metabolism over 3 h in infant rats (32). Second, although we assessed extracellular composition of SAs and LAs via TEM, we did not perform differentiated analyses of SA and LA surfactant fractions in BALF. Thus, it remains unclear whether the increased BALF PL concentrations are due to a high percentage of non-functional surfactant (SA). Third, we missed the opportunity to quantify extracellular surfactant subtypes via TEM in non-lavaged animals. Also, fixation of lungs with a conventional procedure using a glutaraldehyde column might have altered the extracellular surfactant pool. Hence, it is conceivable that both BALF withdrawal and the fixation technique modified the extracellular surfactant composition. Fourth, morphological structures were analyzed on two-dimensional images. Future age-specific studies should consider the more sophisticated design-based stereology to increase the accuracy for a quantitative and objective evaluation. Fifth, we did not assess surfactant surface activity, which might have been interesting to correlate with lung function measurements. Sixth, to maximize the utility of the model and to be able to compare the study results to existing rat studies, we chose

clinically rather unusual ventilator settings with V_T 16/21 ml/kg and PEEP 1 cmH₂O in the extreme ventilation groups. As these settings are generally avoided in clinical settings, the translational value of these study groups is limited. Seventh, the application of FiO₂ 0.4 to all ventilated animals might introduce bias into the comparison of non-ventilated controls vs. ventilated animals. However, this was accepted, as FiO₂ 0.4 needed to be applied to all ventilated animals to prevent desaturation and to maintain the animals in an adequate health until the end of protocol (2). Eighth, future studies might be strengthened by analyzing age-specific differences in de novo precursor fatty acid synthesis and AEC2 glycogen stores, expression of intracellular lipid transporters, and surfactant protein levels in BALF to specifically test exocytosis. Lastly, we did not objectify stretch-induced changes in transdifferentiation rate revealing ALI via AE1 cell-staining. However, this shortcoming was probably compensated by the absence of significant changes in the number of intermediate AE cells, inflammatory cells, and physical damage at both light and electron microscopic level.

In conclusion, we have shown for the first time that improved respiratory system mechanics after extreme high V_T ventilation with adequate PEEP was not related to increased surfactant synthesis, storage, and secretion after 6 h of ventilation in infant rats. In addition, none of our excessive ventilation strategies induced or fulfilled experimental ALI criteria. Moreover, in contrast to a comparable adult study, mechanical ventilation with V_T 24PEEP1 did not result in VILI-related intracellular alterations of lamellar body number and morphology. The potentially most injurious protocol with V_T 24PEEP1 led to a rather weak inflammation expressed by the biomarker MIP-2, only. The elevated BALF phospholipid concentration found in this group needs further evaluation and might reflect an accumulation of the biophysically inactive small aggregates. Taken together, this study did not reveal that

543 the resilience of infant rats towards development of VILI in response to high V_T
544 ventilation can be attributed to surfactant homeostasis. Future age-specific studies
545 should focus both on surfactant activity and small and large aggregate fractions in
546 order to deepen our knowledge about surfactant homeostasis and VILI.

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578 **Disclosure**

579 The authors declare that there are no conflicts of interests.

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TABLES

Table 1. Oligonucleotides for SPs and RPL13a used for qPCR

Gene	Forward primer	Reverse primer
SP-A	5'-CGGATCCAGTCCTCAGCTTGCAAGGATC-3'	5'-GGAATTCCGTTCTCCTCAGGAGTCCTCG-3'
SP-B	5'-CGGATCCGAGCAGTTTGTGGAACAGCAC-3'	5'-GGAATTCTGGTCCTTTGGTACAGGTTGC-3'
SP-C	5'-CGGATCCCATACTGAGATGGTCCTTGAG-3'	5'-GGAATTCTCTGGAGCCATCTTCATGATG-3'
SP-D	5'-CGGATCCCGGAAGAGCCTTTTGAGGATG-3'	5'-GGAATTCACAGTTCTCTGCCCCTCCATTG-3'
RPL13a	5'-GCGGATGAATACCAACCC-3'	5'-GTAGGCTTCAGCCGCACAAC-3'

SP = surfactant protein; RPL13a = ribosomal protein L13a; qPCR = quantitative real-time polymerase chain reaction.

Table 2. Vital parameters and blood gas analyses

Groups	SpO ₂ [%]	HR [bpm]	pH	pCO ₂ [kPa]
V _T 8PEEP5	99.1 (0.3)	287 (19)	7.35 (0.05)	5.3 (0.8)
V _T 16PEEP5	99.2 (0.4)	279 (20)	7.37 (0.09)	4.4 (1.4)
V _T 24PEEP5	99.2 (0.3)	277 (25)	7.35 (0.08)	4.7 (1.4)
V _T 24PEEP1	99.2 (0.3)	286 (16)	7.37 (0.07)	4.6 (0.9)
P-value	0.872	0.198	0.611	0.070

Values are group means (SD). N = 22-24 per ventilation group. P-values for each parameter are outlined in the bottom row. V_T = tidal volume; PEEP = positive end-expiratory pressure; SpO₂ = peripheral oxygen saturation; HR = heart rate; pCO₂ = partial pressure of carbon dioxide; bpm = breaths per minute.

Table 3. Histological lung injury scores and BALF surfactant PL ratios

Group	Hemorrhage	Atelectasis	Overdistension	Total PC/lyso-PC	Total PC/SM
NVC	1.0 (1.0 - 2.3)	7.0 (4.8 - 8.3)	0.0 (0.0 - 1.3)	54.9 (43.8 - 211.1)	15.1 (14.5 - 21.7)
V _T 8PEEP5	3.0 (1.0 - 3.0)	6.0 (4.8 - 7.0)	1.0 (0.0 - 1.0)	68.3 (54.1 - 170.0)	14.5 (13.4 - 20.1)
V _T 16PEEP5	3.0 (1.5 - 3.0)	6.0 (2.5 - 6.8)	0.0 (0.0 - 0.0)	62.8 (49.7 - 147.5)	14.3 (13.2 - 18.8)
V _T 24PEEP5	3.0 (1.3 - 3.0)	6.0 (3.0 - 6.0)	0.0 (0.0 - 0.8)	62.0 (52.2 - 65.6)	14.0 (13.8 - 14.3)*
V _T 24PEEP1	3.0 (2.0 - 3.0)	6.0 (6.0 - 7.8)	0.0 (0.0 - 1.0)	58.1 (50.7 - 141.7)	14.2 (13.7 - 20.9)

Values are group medians (interquartile range). Lung injury scores including hemorrhage, atelectasis, and overdistension were determined in hematoxylin-eosin stained lung sections (n = 11-13 per study group). Concentrations of surfactant phospholipid (PL) species were measured in bronchoalveolar lavage fluid (BALF) by mass spectrometry (n = 13-18 per treatment group). The sum of phosphatidylcholine (PC) and dipalmitoyl-PC (DPPC) concentrations forming total PC were then related to lyso-PC and sphingomyelin (SM). * displays a significant difference between V_T24PEEP5 and NVC, analyzed by Kruskal-Wallis one-way ANOVA on ranks, where p < 0.05. NVC = non-ventilated control; V_T = tidal volume; PEEP = positive end-expiratory pressure.

FIGURE LEGENDS

Figure 1. Respiratory system mechanics represented by (A) airway resistance (R_{aw}), (B) tissue damping (G), (C) tissue elastance (H), and (D) hysteresivity (η) as a function of time on the ventilator. The following groups were assessed by the forced oscillation technique: V_T 8 mL/kg and PEEP 5 cmH₂O (V_T 8PEEP5), V_T 16 mL/kg and PEEP 5 cmH₂O (V_T 16PEEP5), V_T 24 mL/kg and PEEP 5 cmH₂O (V_T 24PEEP5), and V_T 24 mL/kg and PEEP 1 cmH₂O (V_T 24PEEP1). Data are expressed as group means $\pm$ SEM. N = 22-24 per treatment group. * indicates significant differences compared to the time point of allocation (0 h), # displays significant differences between study groups at the end of the protocol, § shows significant differences between ventilated groups at the end of the protocol, data sets were analyzed by one-way RM ANOVA and one-way ANOVA, respectively, where $p < 0.05$. V_T = tidal volume; PEEP = positive end-expiratory pressure.

Figure 2. Relative expression of (A) surfactant protein (SP)-A, (B) -B, (C) -C, and (D) -D to the house keeping gene ribosomal protein L13a (RPL13a) determined by arbitrary units with a quantitative real-time PCR (qPCR). Data are displayed as vertical point plots and box plots with median, 10th, 25th, 75th, and 90th percentiles. N = 5-9 per treatment group. * indicates a significant difference between V_T 24PEEP5 and NVC for SP-C, $p < 0.05$, determined by one-way ANOVA. NVC = non-ventilated control; V_T = tidal volume; PEEP = positive end-expiratory pressure.

Figure 3. Representative immunohistochemical images of rat lung sections stained for surfactant protein (SP)-C, a marker of alveolar epithelial type II (AE2) cells, from (A) non-ventilated controls (NVC) and ventilated animals with (B) V_T 8PEEP5, (C)

V_T16PEEP5, (D) V_T24PEEP5, and (E) V_T24PEEP1. AE2 cells are located at the corners of alveolar walls and can be identified by brown color (*arrows*). V_T = tidal volume; PEEP = positive end-expiratory pressure. Original magnification ×40, *bar* = 50 μm.

Figure 4. Specific concentrations of phospholipid (PL) species in bronchoalveolar lavage fluid (BALF) assessed by mass spectrometry. (A) phosphatidylcholine (PC), (B) dipalmitoyl-PC (DPPC), (C) lyso-PC, and (D) sphingomyelin (SM). Data are displayed as vertical point plots and box plots with median, 10th, 25th, 75th, and 90th percentiles. N = 13-18 per treatment group. * shows a significant difference between V_T24PEEP1 and all other groups, # displays a significant difference between NVC and the V_T16PEEP5 and V_T24PEEP5 groups for PC, DPPC, and SM, analyzed by one-way ANOVA, where p < 0.05. NVC = non-ventilated control; V_T = tidal volume; PEEP = positive end-expiratory pressure; bw = body weight.

Figure 5. (A) Alveolar macrophages (AMs) and (B) neutrophil granulocytes (NGs) evaluated by histological analysis from hematoxylin-eosin (HE) stained lung sections and (C-F) BALF biomarker concentrations. Data are displayed as vertical point plots and box plots with median, 10th, 25th, 75th, and 90th percentiles. N = 11-13 for inflammatory cells, n = 18-20 for IL-1β, IL-6, and MIP-2, n = 13-17 for albumin per treatment group. * indicates a significant difference between NVC and all ventilated groups, # shows a significant difference between V_T24PEEP1 and both NVC and V_T24PEEP5 groups, determined by one-way ANOVA, where p < 0.05. NVC = non-ventilated control; V_T = tidal volume; PEEP = positive end-expiratory pressure; IL = interleukin; MIP = macrophage inflammatory protein.

Figure 6. Numbers and surface areas of alveolar epithelial type II (AE2) cells and lamellar bodies (LBs). (A) Number of AE2 cells per parenchyma surface area was evaluated in study groups by immunohistochemical analysis using an anti-SP-C antibody. (B) Mean surface area of AE2 cells, (C) number of LBs per AE2 cell, and (D) total surface area of LBs per AE2 cell were examined via transmission electron micrographs. Data are displayed as vertical point plots and box plots with median, 10th, 25th, 75th, and 90th percentiles. N = 10-13 for immunohistochemical analyses, n = 3-5 per strategy for TEM. P-values were estimated by one-way ANOVA. NVC = non-ventilated control; V_T = tidal volume; PEEP = positive end-expiratory pressure.

Figure 7. Representative transmission electron micrographs of intra- and extracellular surfactant forms from the following groups: (A) V_T16PEEP5, (B) V_T8PEEP5, and (C) V_T24PEEP1. Lamellar bodies (LBs) within alveolar epithelial type II (AE2) cells as well as extracellular tubular myelin (TM), LB-like (LBL), multilamellar (ML), multivesicular (MV), and unilamellar (UL) surfactant forms were found in all experimental groups without a significant inter-group difference. V_T = tidal volume; PEEP = positive end-expiratory pressure; AE_{int} = intermediate alveolar epithelial. Original magnification ×2,600.

Figure 8. Biophysically active and inactive surfactant forms assessed by semi-quantitative analysis in transmission electron micrographs. (A) and (B) Number of small (SA) and large (LA) surfactant aggregates, respectively. No significant differences in the quantity of large and small surfactant aggregates were seen. Surface-active tubular myelin (TM), LB-like (LBL), multilamellar (ML), and multivesicular (MV) surfactant forms were summarized as LAs. Biophysically inactive surfactant including unilamellar (UL) and lamellar vesicles (LV) were outlined as SAs.

834 Data are displayed as vertical point plots and box plots with median, 10th, 25th, 75th,
835 and 90th percentiles. N = 3-4 per treatment group. P-values were determined by one-
836 way ANOVA. V_T = tidal volume; PEEP = positive end-expiratory pressure.

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838 **Supplemental Information**

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