**Impact of tidal volume strategy at birth on initiating lung injury in preterm lambs**

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1. **Detailed Methodology**
2. **Results: Online Supplementary Figure 1**

**DETAILED METHODOLOGY**

All techniques and procedures were approved by the Animal Ethics Committee of the

Murdoch Children’s Research Institute, Melbourne, Australia (10 May 2021; A940) in accordance with National Health and Medical Research Council guidelines (Australia). The ARRIVE Statement for this study is available at Tingay, David (2022): University of Melbourne. [https://doi.org/10.26188/22590724.v1](https://protect-au.mimecast.com/s/ipRSCq717ycX0lJ7hZaxw1?domain=doi.org). Some aspects of the methodology have been reported in detail previously.(1-4)

***Experimental Instrumentation***

Surfactant-deficient 124-127d preterm Border-Leicester cross lambs (term ~145d) were studied. All were born via Caesarean section under general anaesthesia to ewes that received 11.7 mg of betamethasone 24 and 48 hours prior to delivery.(3) The head and chest of the lamb was exteriorised and instrumented. This included insertion of a carotid arterial and external jugular line, placement of a 3 mm flow probe around the contralateral carotid artery (Transonic, AD Instruments, Sydney, Australia), intubation (4.0 cuffed endotracheal tube) and applying a custom-built electrical impedance tomography (EIT) belt around the chest as described previously.(5, 6) Lambs were then fully exteriorised, dried and placed supine on heat pads on the ewes abdomen (under heat lamp) with placental support maintained. A 4 mm and 6 mm flow probe were placed around an umbilical artery and vein respectively as part of another study. Lung liquid was passively drained before commencing respiratory support. Temperature was monitored via a rectal thermistor and external heating adjusted accordingly. Anaesthesia and analgesia were maintained throughout the study period with ketamine and midazolam infusions.

***Measurements***

Heart rate, carotid artery and umbilical vessel flow, airway pressure, gas flow and tidal volume (VT) at the airway opening (Florian, Acutronic Medical Systems AG, Hirzel, Switzerland) were measured continuously from birth. Global and regional lung volume changes were acquired by EIT (Pioneer System, Sentec AG, Landquart, Switzerland) at 48 scans/s.(5-7) Arterial blood analysis was performed at 5 minutes and then every fifteen minutes from birth.

***Randomisation process***

Lambs were randomly assigned to a ventilation strategy before delivery in a two-step process. Initial randomisation occurred when obstetric ultrasound scans were performed at the farm (usually 1-2 months before delivery of ewes to the research facility) to facilitate even allocation of lambs across groups by gestational age (days), singleton and twin pregnancy, and delivery order if a twin pregnancy. As sex could not be predicted before delivery, reallocation was allowed prior to foetal instrumentation, if intra-group imbalances in sex were identified, and only after half of the allocated sample size for each group was studied. Further, lambs with severe growth restriction and/or severe foetal acidosis (defined as a cord pH <7.20) were excluded due to the independent impact each may have on lung injury and need for larger sample sizes (principal of reduction). Where possible lambs were replaced with the same group.

***Ventilation Strategies and general management after birth***

Lambs were randomly assigned before delivery to receive one of three PPV strategies at birth with 0.21 fractional inspired heated and humidified oxygen concentration:

**1) Static 7 ml/kg VT (VTstatic):** PPV delivered at 7 ml/kg based on estimated birth weight for the duration of the study. PPV was delivered with an inspiratory time of 0.5 s, bias flow 4-6 L/min, rate 60 inflations per minute in Volume Targeted Ventilation (VTV) mode at 7 ml/kg with a maximum positive inflation pressure (PIP) of 50 cmH2O (SLE5000; SLE Ltd, South Croydon, UK). Our previously described step-wise dynamic PEEP strategy from an initial 8 cmH2O PEEP was performed immediately after birth.(3) PEEP was manually increased by 2 cmH2O every 20s until 14 cmH2O (PMAX), and then decreased step-wise to 8 cmH2O prior to a transient re-recruitment at 14 cmH2O for 20s before ongoing PEEP at 8 cmH2O for remainder of experimental period (total duration approximately 150-180s; Figure 1).

**2) Increasing VT (VTinc):** PPV was delivered as per VTstatic in VTV mode, including the use of an initial dynamic PEEP strategy, except VT was commenced at 3 ml/kg. VT was then increased to 5 ml/kg at 90s (coinciding with PEEP 14 cmH2O), and then increased to 7 ml/kg at 180s (coinciding with final PEEP 8 cmH2O). This exposed the lung to approximately 90 inflations at each of VT 3 ml/kg and 5 ml/kg.

**3) Alternating VT (VTalt):** PPV was delivered as per VTstatic in VTV mode, commencing at VT 7 ml/kg. At 90s (coinciding with PEEP 14 cmH2O) the VT was decreased to 3 ml/kg during the decremental phase of the dynamic PEEP strategy, before being increased again to 7 ml/kg at 3 minutes (coinciding with final PEEP 8 cmH2O) for the remainder of the study.

All lambs were supported with their assigned strategy until 15 minutes when the endotracheal tube was clamped and PPV ceased. In all groups pressure was delivered at a set bias flow of 8-10 L/min. Following 15 minutes of ventilation lambs, the endotracheal tube was clamped and PPV ceased and lambs were then maintained on placental support and external heat for 30 minutes to allow upregulation of injury markers as per the method of Hillman and co-workers.(8, 9) At 45 minutes, a static *in vivo* pressure-volume curve was generated from atmosphere to 35 cmH2O to calibrate the EIT signal and determine static lung mechanics and all lambs received a lethal dose of pentobarbitone.(3-5, 10, 11) Ten foetal lambs received a lethal dose of pentobarbitone upon delivery as an unventilated control (UVC) group for comparison of lung injury data.

***Data Acquisition and Analysis***

Physiological parameters were recorded at 200 Hz (LabChart V8, AD Instruments, Sydney, Australia), analysed at key time points with EIT data and DP, tidal volume (VT) and dynamic respiratory system compliance (Cdyn) calculated.(12) Dynamic tidal mechanical power (MPtidal) was calculated using the simplified equation of Becher and co-workers: MPtidal (J/kg/min) =0.098 x VT x DP; VT was expressed in L/kg.(13) Time-course EIT image data were reconstructed using an anatomically correct custom-built lamb algorithm,(4, 12, 14) filtered to the respiratory domain (IBEX software package, Sentec).(5-7) This algorithm accounts for the specific shape of the lamb chest, including the greater lung size in the right and dorsal hemithoraces.(4) Change in lung volume(∆VL) was calculated from the tidal time-course EIT signal.(3, 11) Global EEV from the pre-aerated state was calibrated from the static pressure-volume curve. Relative ∆VL (aeration) within the gravity dependent and non-dependent regions was determined from the raw data, and weighted to the known pixel (anatomical) contribution of each region to provide a measure of relative aeration states within the lung.(4, 5, 11) The ventral-dorsal centre of ventilation (CoV) was used to define the spatiotemporal distribution of VT.(12, 15)

Physiological and EIT data reported at key time points were determined from 30 s of consecutive artefact-free data for data from 3 to 15 minutes. During the first 3 minutes of respiratory support, data were determined over 10 s intervals to coincide with each PEEP step in the Dynamic PEEP strategies. Consistent with our previous studies of dynamic PEEP the values at PMAX and at PEEP 8 cmH2O on completion of the initial step-wise PEEP decrease were reported as 1 and 2 minutes rather than the actual time points as these are physiologically more meaningful.(3, 9, 14)

Immediately after completing the protocol the lungs were removed *en bloc*. Protein concentration of left lung lavage fluid was determined using bicinchoninic protein assay.(16) The right upper lobe was inflation fixed at 20 cmH2O with 4% paraformaldehyde buffered in PBS solution, hematoxylin and eosin–stained from each of the dependent and non-dependent zones and assessed using our previously reported standardised criteria.(4, 11, 17, 18) All image analysis was performed blinded using FIJI software on 20 fields of view.(19)

***Proteome Analysis***

*Sample acquisition:* Lung tissue samples for proteome analysis from the gravity-dependent and non-dependent zones of the right lower lobe (corresponding to the portion of right lung most included in the field of EIT imaging) were snap frozen and stored at -80 °C until analysis.

*Protein extraction and digestion:* 25 mg of frozen lung tissue was homogenised on ice in 500 µL lysis buffer (8 M Urea, 50 mM HEPES, 1x Halt protease/phosphatase inhibitor (78440; Thermo Fisher Scientific), pH 8) using the Tissue TearorTM fitted with a 5 cm probe (BioSpec, OK, USA). Protein extracts were centrifuged at 13,000 rpm for 20 minutes at 4 °C (FA-45-30-11 rotor, Eppendorf 5430 R) and supernatants transferred to low protein-binding tubes. Protein concentration was determined using the Pierce MicroBCATM protein assay (23235; Thermo Fisher Scientific) according to manufacturer’s instructions. Protein lysates (20 µg/50 µL) were reduced with 10 mM dithiothritol (DTT) (C-1029-5G, Astral Scientific, Australia) at 450 rpm for 1 h at room temperature (RT, 25 °C) followed by alkylation with 20 mM iodoacetic acid (IAA) (I1149-25G, Sigma-Aldrich, Castle Hill, AU) at 450 rpm for 30 minutes at RT (light protected). Samples were immediately quenched with 10 mM DTT before being subjected to singlepot, solid-phase-enhanced sample separation (SP3).(20) Magnetic beads were prepared by mixing SpeedBeads™ magnetic carboxylate modified particles (65152105050250, 45152105050250, Cytiva Life Sciences, USA) at 1:1 (v:v) ratio and washing twice with 200 µL MilliQ.(21) Beads were reconstituted to 100 µg/µL and transferred to protein extracts in a 10:1 total bead to protein ratio, with addition of 50% (v/v) ethanol. Samples were mixed at 1000 rpm for 10 minutes at RT and transferred to a magnetic rack where the supernatant was removed, and protein-bound beads were washed three times with 80% (v/v) ethanol. Samples were reconstituted in 50 mM TEAB pH 8 and digested with MS grade trypsin (enzyme: substrate 1:50, Promega V5113) and Lysyl Endopeptidase (enzyme:substrate 1:100, 125-05061, Wako Pure Chemical Industries) for 18 h, 1000 rpm at 37 ℃. Peptide digests were centrifuged at 20,000 g for 1 minute, supernatants transferred to fresh low-protein binding tubes, snap frozen and lyophilised via vacuum centrifugation (Savant SPD121P, Thermo Fisher Scientific).

*TMT-labelling:* Peptides were reconstituted in 100 mM TEAB pH 8.5, quantified using PierceTM Fluorometric Peptide Assay (#23290, Thermo Fisher Scientific), adjusted to 0.1 µg/µL and TMT-labelled according to manufacturer’s instructions with minor modifications. A pooled internal reference channel was prepared across all sample groups. Samples were labelled using tandem mass tag (TMT) TMT11-plex (A34808/06, Lot VL312003, Thermo Fisher Scientific) (nine samples and two pooled internal reference channels, combined in equal ratio). TMT tags were prepared in anhydrous acetonitrile (ACN) and transferred to 5 µg of peptide digests in a 1:4 peptide: TMT ratio. Samples were incubated at RT for 2 h at 450 rpm, quenched with 1 µL of 5% hydroxylamine at RT for 15 minutes at 450 rpm, and combined in equal ratio. A list of the sample labelling strategy is available in PRIDE proteomeXchange (PXD041917). Combined, labelled peptides were acidified to 3% formic acid (FA), desalted using solid phase extraction manifold (186002321/186001831, Waters Corporation), eluted in 50% ACN and 0.1% formic acid in MS-grade water, and dried by vacuum centrifugation. Samples were reconstituted in 0.07% trifluoroacetic acid (TFA) in MS-grade water, quantified using Pierce™ Quantitative Peptide Assay (#23275, Thermo Fisher Scientific) and normalized to 0.5 µg/µL.

*TMT-based proteomics:* TMT-labelled peptides were analysed on a Dionex 3500RS nanoUHPLC coupled to an Q Exactive HF-X benchtop Orbitrap mass spectrometer equipped with nanospray ion source in positive, data-dependent acquisition mode as previously described.(22) Peptides (0.5 µg) were sequentially loaded (Acclaim PepMap100 C18 3 mm beads with 100 Å pore-size, Thermo Fisher Scientific) and separated (1.9-µm particle size C18, 120 Å pore-size, 0.075 × 200 mm, Nikkyo Technos Co. Ltd) and a gradient of 3–80% (0-3 min, ACN containing 0.1% (v/v) FA over 240 min (2-28% over 224 min, 28-80% to 228 min, 80-2% to 234 min) at 300 nl min-1 at 55°C (butterfly portfolio heater, Phoenix S&T). Peptides were injected into the trap column at an isocratic flow of 5 μL/min of 0.1% (v/v) FA for 5 min, applied before switching in-line with the analytical column.

An MS1 scan was acquired from 350–1,500 m/z (120,000 resolution, isolation window of 0.7 Thomsons) in ‘top speed’ acquisition mode with 3 s cycle time (max IT 250 ms) on the 25 most intense precursor ions; ions with charge states of 2 to 5 were selected. AGC target was set to 3×106 automatic gain control, followed by MS/MS data-dependent acquisition with high-field collision-induced dissociation and detection in the Orbitrap (45,000 resolution, 1×105 AGC, 90-ms injection time, normalized collision energy of 32), with minimum AGC target 4.5×103. Dynamic exclusion was activated for 20 s, with data acquired using Xcalibur v4.5 software.

The mass spectrometry proteomics raw files and data analysis files have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with identifier PXD041917.

Database searching and protein identification: Raw MS data was analysed using MaxQuant (v1.6.14.0, Uniprot Ovis aries (sheep) database; UP000002356, 23111 entries, Feb 2022) using the built-in search engine Andromeda (23) with a contaminants database employed. Cysteine carbamidomethylation was selected as a fixed modification and N-terminal acetylation and methionine oxidations as variable modifications. Data was processed using trypsin/P and LysC as the proteolytic enzymes with up to 2 missed cleavage sites allowed. For TMT-based analyses, reporter ion MS2 (11plex TMT) settings were employed, with trypsin/P and LysC as the proteolytic enzymes set as digestion enzymes allowing a maximum of two missed cleavages. False discovery rate (FDR) for peptide and protein spectrum match was set at 0.01. Peptides were identified with an initial precursor mass deviation of up to 7 ppm and a fragment mass deviation of 20 ppm. Quantification was performed based on intensity of the reporter ion, with a mass tolerance of 0.003 Da and correction for isotopic impurities. Protein group tables were exported as text files for analysis.

*TMT normalisation:* Search results and TMT reporter ion intensities were exported as text files. Within the data set, unreliable protein groups (razor peptides <1, contaminants, peptides only identified by site, reversed) and proteins with reporter ion intensities missing from all samples in a 11-plex run were removed. A Two-step normalisation procedure was employed as per Plubell et al., in RStudio (v4.1.2).(24) The first normalisation was applied within each 11-plex experiment. The grand total reporter ion intensity for each channel was multiplied by global scaling factors to adjust its total intensity to the average total intensity across the 11 channels. This corrects for small sample loading and labelling reaction efficiency differences. Secondly, common, pooled internal standards were used to normalise reporter ion intensities of proteins between different TMT experiments. This allowed preservation of individual intensity-scale measurements and avoided calculation of relative intensity measures such as ratios or percentages within each TMT experiment. To accomplish this, scaling factors which were calculated from the internal standards in each run (minimum two standards/run) and used to adjust the summed reporter ion intensities for each protein in the remaining eight experimental samples in each TMT experiment.

***Statistical Analysis***

From previous preterm lamb studies within our program, we have shown 8-10 lambs/gp sufficient to demonstrate differences in left lung lavage total protein concentration between treatment and control at 15 minutes (80% type 1 error and p=0.05).(25) All non-proteomics data were first tested for normality prior to analysis and investigated with either one-way ANOVA, Kruskal-Wallis test or mixed effects analysis (ventilation strategy and time as variables) and appropriate post-test analysis. Statistical analysis was performed with PRISM 9 (GraphPad Software, San Diego, CA). Proteome analysis to identify differentially expressed proteins (DEPs; identified as having a significant difference in protein abundance in ventilated groups compared with the unventilated control group) was performed using an average reporter intensity from two standardised tissue sites/lamb with the EdgeR software package in RStudio.(26, 27) with preliminary analysis performed as previously described.(22, 28) Significance was confirmed by QL F-testing with multiplicity correction applied using Benjamin-Hochberg method on the p-values to control the false discovery rate (FDR), with significance set FDR<5%.

***Proteome Bioinformatic Analysis***

Multivariate analysis using Principal component analysis (PCA) was performed in ClustVis.(29) Gene set enrichment analysis (GSEA) to identify enriched pathways and biological processes associated with the proteome data sets were identified using WebGestalt, with FDR<0.05 considered significant.(30) Similar to previous studies, (25, 31) to address ovine database limitations in analysis tools, homology of sheep to human proteins was assessed by NCBI Basic Local Alignment Search Tool (BLAST); with 93% of proteins exhibiting homology ≥75%.

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