# SUPPLEMENTAL DIGITAL CONTENT 1

## COMPLETE METHODS

Studies were performed on male Sprague-Dawley rats, with approval by the local Institutional Animal Care and Use Committee (Philadelphia, PA). General anesthesia was induced and maintained with intraperitoneal pentobarbital (40–60 mg/kg initially, 10-20 mg/kg hourly for maintenance), the trachea intubated (14-gauge catheter; BD, Franklin Lakes, NJ), and the glottis sealed (DAP Products Inc., Baltimore, MD) to prevent gas leakage[1]. Paralysis was obtained with pancuronium bromide (1 mg/kg IV; Abbot Labs, North Chicago, IL) injected through a 24 GA tail vein catheter. Rats also received carotid artery catheterization with a 24 GA catheter for arterial blood pressure monitoring and blood gas measurements. Animals were maintained supine throughout the whole study and were ventilated by a small animal ventilator developed in the authors' laboratory[2]. Positive end expiratory pressure (PEEP) was generated by connecting the expiratory gas line to a graduated water column. Airway pressure was continuously recorded using a fiber-optic sensor (Samba Sensors AB, Sweden), and heart rate and peripheral oxygen saturation ($SpO_2$) levels were monitored by a veterinary pulse-oximeter (Nonin Medical, Inc. Plymouth, MN) attached to the hind foot. All animals received subcutaneous (30 ml/kg) hydration with normal saline after induction of anesthesia; intravenous saline (10 ml/kg) was administered up to a maximum of three times when arterial blood pressure was lower than 40 mmHg. A rectal probe was used to monitor the animal's body temperature, which was carefully maintained at 37 °C by a heated pad (Gaymar Industries, Orchard Park, NY) placed under the body. After the last set of measurements, animals were removed from the scanner and euthanized by lethal pentobarbital injection.

Two groups of rats were studied: rats with acid aspiration ventilated with moderate tidal volume ($V_T$), and normal rats ventilated with high $V_T$.

**Acid Aspiration with moderate $V_T$:** We studied secondary progression of primary lung injury in 20 rats (353±26 g) ventilated after intra-tracheal administration of hydrochlocic acid (HCl, 2.5 ml/kg, pH 1.25). HCl was injected in two aliquots with the animal in the right and left lateral positions and 45° head elevation. Rats were immediately returned to the supine position and allowed to stabilize while ventilated with PEEP 10 cm $H_2O$ and $V_T$ 6 ml/kg for one hour. No effort was made to spread the liquid in the airways: in pilot experiments with this injection technique, we confirmed patchy distribution of HCl solution marked with Evans-blue in the dorsal lungs. After stabilization, rats received ventilation with moderate $V_T$ of 12 ml/kg, PEEP 3 cm$H_2O$, FiO2 1.0, and respiratory rate 53 breaths per minute, continued for up to three hours or until they became irreversibly hypotensive; then rats were euthanized. Because we aimed to investigate secondary progression of *mild* primary lung injury, no randomization was performed and animals were recruited sequentially: only rats that survived long enough to display the propagation were studied. Rats were included in the data analysis it they had baseline $PaO_2/FiO_2$ >300 mm Hg after stabilization and were able to tolerate moderate $V_T$ ventilation for at least two hours. Six additional healthy rats (365 ± 28 g) were ventilated for three hours with these settings, to show the radiological and physiological effects of moderate $V_T$ ventilation in absence of underlying lung injury.

**Normal Animals with Large $V_T$:** To study the evolution of VILI in absence of primary injury, we ventilated 10 healthy rats (480±87 g) with $V_T$ of 30 ml/kg, PEEP 0 cm$H_2O$, $FiO_2$ 0.5, rate 27 breaths per minute for up to 3 hours (Figure 1). Ventilation was continued until peak inspiratory pressure (PIP) increased 50% or more. In this group, we intentionally tolerated a broader range of body weights to introduce variability of lung capacity and strain in absence of primary injury.

**Computed tomography: *CT:*** High resolution whole lung CT scans were acquired at each time point using a commercial microCT scanner (eXplore CT120 system, Gamma Medica, Inc., Northridge, CA). The lungs were easily contained within the default field of view (85 mm transaxial diameter). Settings used for imaging were: 80 kVp, 32 mA, 16 ms exposure time, 220 projections (half-scan), and 200 μm isotropic resolutions [3]. Imaging was ventilator-gated and performed during 500-ms breath-holds; only a single view per breath was acquired during each breath-hold. In rats with acid aspiration injury, both end-inspiratory and end-expiratory images were obtained at the beginning of moderate $V_T$ ventilation (baseline) and repeated hourly until the end of the experiment (without changing $V_T$). Each image acquisition required 11 minutes. In healthy rats, imaging was performed at baseline and was repeated hourly or more often if PIP increased rapidly during high $V_T$ ventilation. In this group, $V_T$ was reset at 12 ml/kg (with rate 60 breaths/min) during each CT acquisition, to allow closer image comparisons with the other group; only inspiratory images were obtained to minimize scan time, totaling about seven minutes.

*Image analysis:* Three independent observers (MC, YX, JZ), blinded to group assignment, semi-quantitatively evaluated 3 coronal (frontal, carinal, dorsal) and 3 axial (apical, mid-lung, subcardiac) slices for each inspiratory image. Observers set image contrast to a fixed intensity window (2000 HU) and level (-1000). Each slice was divided in 4 peripheral and in 4 central sectors and each sector received a score of 0 to 4, estimating the fraction of its surface (in 25% increments) that had abnormally high density (ground glass and dense consolidation) [4,5]. Individual sector scores were summed to obtain a global injury score that was used to estimate the spatial extension of injury in the whole lung. The regional distribution of injury was studied by grouping sectors in one ventral vs. one dorsal area of interest in the three axial images.

For quantitative analysis, three dimensional whole-lung regions of interest (ROI) were segmented, i.e. separated from non- pulmonary tissue, at each time point, using a semi-automated, multi-landmark, registration-based scheme for lung segmentation developed by the authors[6]. Using accepted CT density analysis methodology[7-9], we then quantified lung gas volumes based on the following equation:

$$\text{Gas volume}_{ROI} = \text{Total volume}_{ROI} \times \frac{Mean\ CT_{ROI}}{-1000}$$

Where the Total volume$_{ROI}$ included both gas and tissue volumes, Mean $CT_{ROI}$ denoted the mean value of the lung density in Hounsfield units.

In the acid-injured rats, both inspiratory and expiratory CT images were available at each time point and we obtained end-inspiratory and end-expiratory lung gas volumes (EILV, EELV). $V_T$ was measured as $EILV - EELV$. Baseline lung strain was measured as $\frac{V_T}{EELV}$, [7,10] using the first CT after injury. Dynamic compliance was calculated as $C_{dyn} = V_T / (PIP - PEEP)$.

In the healthy group, only inspiratory images acquired with $V_T$ 12 ml/kg were available and only EILV could be measured directly. Therefore, EELV was calculated by subtracting the dialed $V_T$ ($V_{T12}$) from EILV; strain imposed at baseline by high $V_T$ of 30 ml/kg was then calculated as $\frac{V_{T30}}{EELV}$, where $V_{T30}$ was the dialed $V_T$ value during high stretch ventilation. In pilot data in healthy and injured rats, we confirmed adequate correlations between the dialed values of $V_T$ and the values measured as $EILV - EELV$.

Lung weight was calculated at each time point in both the injured and in the healthy groups using the following equation: $Lung\ weight_{ROI} = \text{Total volume}_{ROI} \times \frac{-1000 - Mean\ CT_{ROI}}{-1000}$ [11,12].

The total weight of the lungs was then partitioned between tissue compartments with different aeration [13,14] (13, 14). These were defined based on density ranges: [a] non-aerated (-100 to +100 HU); [b] poorly aerated (-500 to -101 HU); [c] normally aerated (-900 to -501 HU); and, [d] hyperinflated (-1000 to -901 HU). Each compartment was measured as the percentage of the total lung weight. Tidal recruitment (Table 1) was measured at baseline as the expiratory-inspiratory change of weight of the non-aerated compartment [15].

**Histology:** Lungs were fixed with intratracheal formaline, paraffin embedded, sliced in the coronal plane at multiple levels, and stained with hematoxyline-eosine in 5 animals with acid aspiration and in 3 healthy rats. Slides were reviewed by a collaborating pathologist (CGD) and evaluated for edema, hyaline membranes, perivascular neutrophil infiltration, and alveolar neutrophils.

**Statistical analysis:** The sample size was decided based on pilot studies where we noticed a survival rate of approximately 75% at two hours and of 50% at three hours, which allowed us to study propagation and to test whether baseline characteristics (chosen *a priori*) were predictive of such propagation. Group mean and standard deviation of all the computed quantities were calculated. For semi-quantitative CT analysis, inter-observer agreement for the injury score was calculated using the quadratic weighted kappa statistic [16]. Because there were three raters, kappa was calculated three times for each rat between the three possible pairs of data. The injury score ranged between 0 and 192 and was therefore treated as a continuous variable in subsequent analyses. Correlations between variables were tested by linear regression. Phi coefficient was calculated as a measure of association for binary variables. To study the relations between baseline variables and the propagation of injury in the lung, multiple ANOVA tests were performed separately for non-imaging and imaging markers followed by post hoc t-tests to specifically identify which means were significantly

different from each other. Bonferroni adjustment was performed to control the Type 2 errors. Repeated measurements two-way ANOVA was conducted to examine the main effects of time on CT-derived and physiological variables. Binary variables were tested using Fischer's exact test. $P<0.05$ (for two-tailed hypothesis) was considered statistically significant. Statistical analysis was performed using "R" (R Foundation for Statistical Computing; Vienna Austria, http://www.R-project.org) applications developed in the authors' laboratory.

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